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Structure and diversity of "pathogenic" and "non-pathogenic" fungal endophyte community of *Fagus sylvatica* in the Mediterranean Basin

by

BARBARA CECCARELLI

Coordinator

Prof. LEONARDO VARVARO

Supervisor

Dr. ANNA MARIA VETTRAINO

Assistant Supervisor

Prof. ANDREA VANNINI

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A tutti quelli che aspettano l’ora della loro vita...

"Dilige, et quod vis fac: sive taceas, dilectione taceas; sive clames, dilectione clames; sive emendes, dilectione emendes; sive parcas, dilectione parcas: radix sit intus dilectionis, non potest de ista radice nisi bonum existere".

Sant'Agostino (In Io. Ep. tr. 7, 8)

Abstract

This thesis has investigated diversity and distribution of "pathogenic" and "non-pathogenic" fungal endophyte communities in twigs, leaves and buds of *Fagus sylvatica* in beech Italian forest into Monte Cimino area. In last decade European beech has been subject to a number of biotic and abiotic stresses included the increasing impact of drought and, as a consequence, the impact of native parasites that attack weakened hosts. Among these, *Biscogniauxia nummularia* (Bull.) Kuntze, associated with severe beech-declines, has been studied for its importance as latent pathogen of healthy beech tissues. Understanding how fungal endophyte communities differ in abundance, diversity and taxonomic composition is key to understanding the ecology and evolutionary context of endophyte-plant associations. Fungal ITS sequences were used for validation of morphological identification and phylogenetic analysis. According to the ITS phylogeny, endophytes studied belong to the main non-parasitic and non-lichenised orders of Pezizomycotina. The effects of native tissue, site exposure, and season on endophyte assemblages were investigated. Native tissue was the major factor shaping endophyte assemblages. Exposure and seasonal differences played a significant but lesser role. Several recently developed molecular methods offer new tools for determining the presence and diversity of fungi in complex microbial communities. Terminal restriction fragment (TRF) pattern analysis was tested as a method for assessing the molecular diversity of endophyte fungal community within asymptomatic beech tissues. ITS ribosomal region of communities DNA isolated from ten beech twigs was amplified and then digested with *Msp*I enzyme. The ITS region showed high degree of specificity in matching observed TRF profiles to those generated from GenBank sequence data for species identification. These data suggest that ITS rDNA TRF pattern analysis has great potential as a rapid and specific method for fungal community analysis and species identification. Results of quantitative Real-time PCR (Rt PCR) assay using DNA of *B. nummularia* isolated from different geographic areas were compared. The assay confirmed higher risk associated with our beech forest in Central Italy, compared to the other equal phyto-coenosis located at most latitudes, in terms of declining damages and loss of biodiversity.

Keywords: *Fagus sylvatica*, endophyte communities, *Biscogniauxia nummularia*, ITS phylogenetic analysis, TRFLP, quantitative Real-time PCR.

Riassunto

Questa tesi ha studiato diversità e distribuzione delle comunità degli endofiti fungini "patogeni" e "non patogeni" in rami, foglie e gemme di *Fagus sylvatica* in una faggeta italiana nella zona del Monte Cimino. Negli ultimi dieci anni il faggio è stato oggetto di una serie di stress biotici ed abiotici inclusi il crescente impatto della siccità e, per conseguenza, l'impatto di parassiti nativi che attaccano ospiti indeboliti. Tra questi, *Biscogniauxia nummularia* (Bull.) Kuntze, associato a gravi deperimenti di faggio, è stato studiato per la sua importanza come agente patogeno latente in tessuti sani faggio. Capire come le comunità fungine endofite differiscono in abbondanza, diversità e composizione tassonomica è la chiave per comprendere l'ecologia e il contesto evolutivo delle associazioni endofita-impianto. Le sequenze ITS sono state utilizzate a conferma dell'identificazione morfologica e per l'analisi filogenetica. In base alla filogenesi ITS, gli endofiti studiati appartengono ai principali ordini non parassiti e non lichenici dei Pezizomycotina. Sono stati studiati gli effetti del tessuto d'origine, dell'esposizione e della stagione sui gruppi endofiti. Il tessuto è stato il principale fattore di formazione dei gruppi endofiti. L'esposizione e le differenze stagionali hanno giocato un ruolo significativo, ma minore. Diversi metodi molecolari recentemente sviluppati offrono nuovi strumenti per determinare la presenza e la diversità dei funghi in comunità microbiche complesse. L'analisi dei frammenti terminali di restrizione (TRF) è stata verificata come metodo per la valutazione della diversità molecolare della comunità endofitica fungina all'interno di tessuti asintomatici di faggio. La regione ITS dell'rDNA delle comunità isolate da dieci rami di faggio, è stata amplificata e quindi digerita con l'enzima *Msp*I. La regione ITS ha mostrato un alto grado di specificità, nella corrispondenza tra profili di TRF osservati e quelli generati da sequenze in banche dati ufficiali, per l'identificazione delle specie. Questi dati suggeriscono che il modello di analisi TRF basato sulle regioni ITS ha un grande potenziale come metodo rapido e specifico per l'analisi delle comunità fungine e l'identificazione delle specie. Sono stati confrontati i risultati dell'analisi quantitativa *Real time* PCR (Rt-PCR) del DNA di *B. nummularia* isolato da diverse aree geografiche. L'analisi ha confermato un più alto rischio associato alla nostra faggeta nel Centro Italia, rispetto ad altre simili fitocenosi a maggiori latitudini, in termini di deperimento e perdita di biodiversità.

***Keywords*:** *Fagus sylvatica*, comunità endofite, *Biscogniauxia nummularia*, analisi filogenetica delle regioni ITS, TRFLP, analisi quantitativa *Real-time* PCR.

Contents

Preface

Concerning forest preserve large economic and environmental importance is recognized to *Fagus sylvatica* in Italy with an important ecological and hydrogeological role. On the Apennines, beech forests represent the most widespread mountain climax association (Scoppola, 1999). Cause of its frequency, ability to grow on sites of wide ecological variability and its longevity, beech is well used to evaluate the growth and climate relationships in different bioclimatological units, and to estimate future prospects and possible ecological risks associated with "Global Climate Change" (Eckstein *et al*., 1984; Gutierrez, 1988; Biondi, 1992; Rozas, 2001; Dittmar *et al*., 2003; Piovesan *et al*., 2005; Lebourgeois *et al*., 2005; Di Filippo *et al*., 2007; Biondi, 2008). Currently, among European regions, the Mediterranean Basin, that is at the southern limit of beech geographical range, is consedered an "hot spot" of both climate change and biodiversity and could be most affected by global warming in term of habitat loss and extinction (Brook *et al.*, 2002). In ecological and evolutionary time the preserving of so sensitive biocenosis as beech forests, is only possible after understanding of interactions complex existing in these ecosistems. In this context, "patogenic" and "non-pathogenic" endophyte communities, which living in healthy beech tissues, could show differential response to physiological changes in trees. In the Mediterranean Basin beech is able to provide a unique opportunity to explore connection between tree physiological condition and long-term drought stress that promotes aggressiveness of weak parasites, among which *Biscogniauxia nummularia*, responsible of beech decline (Luchi *et al.*, 2006).

1 Background

1.1 Global climate is changing

The latest assessment report of the Intergovernmental Panel on Climate Change (IPCC, 2007) on the impacts of human-induced climate change reconfirms that global atmospheric concentrations of greenhouse gases have increased markedly since 1750 as a result of human activities, and cause radiative forcing. The warming of our climate system is unequivocal, as is now evident from observations of increases in global average air and ocean temperatures, widespread melting of snow and ice, and rising global average sea level (Haeberli and Beneston, 1998; Joughin *et al.*, 2004; Rignot, 2006; IPCC, 2007). During the last 100 years the earth has heated up by 0.74°C; with

the linear warming trend over the last 50 years nearly twice as much that for the last 100 years (IPCC, 2007).

The frequency of extreme temperature events has changed: cold days, cold nights and frost have become less frequent, while hot days, hot nights, and heat waves have become more frequent. Changes in precipitation patterns are at least as divergent as in temperature patterns: rainfall increased in eastern parts of North and South America, northern Europe and parts of northern and Central Asia, while drying has been observed in southern Europe, southern Africa and parts of southern Asia. Droughts are linked to higher temperatures, reduced precipitation, changes in sea surface temperatures, wind patterns, and decreased snow pack and snow cover. Meanwhile, the frequency of heavy precipitation events has increased over most land areas, consistent with warming and accompanying increases of atmospheric water vapour.

Projections for the future predict a warming of about 0.2°C per decade, analogous to a temperature change of 1.8°C (1.1-2.9°C; B1 scenario: environmental sustainability) to 4.0°C (2.4-6.4°C; A1FI scenario: fossil intensive, very rapid growth) until the end of the century (IPCC, 2007) (Fig. 1).

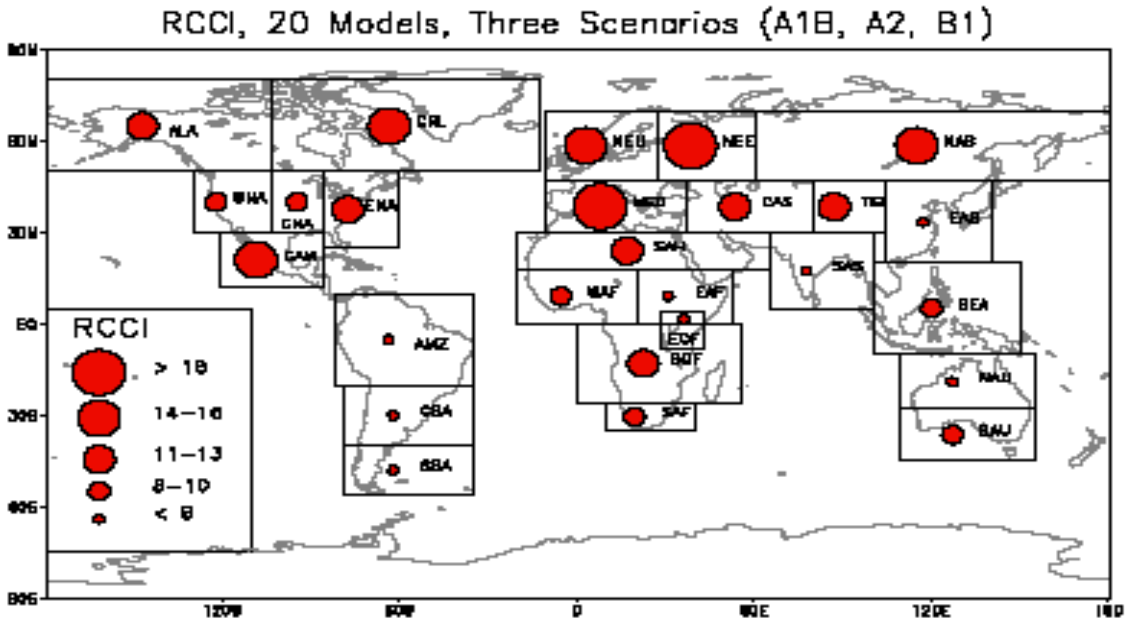


Fig. 1. Regional Climate Change Index (RCCI) over 26 land regions of the World calculated from 20 coupled AOGCMs and 3IPCC emission scenarios (A1B, A2, B1).

Even if all radiative forcing agents are held constant at year 2000 levels, a further warming trend would occur in the next two decades at a rate of about 0.1°C per decade,

mainly due to the slow response of the oceans, and would continue for centuries. Since the world's energy needs and consequently the emission of carbon dioxide will continue to grow for at least the next three decades (IEA, 2005), constancy at 2000 levels is not very likely. Continued greenhouse gas emissions will cause further warming and induce many changes much larger than those observed during the 20th century.

Global warming will influence the global hydrological cycle. Projections indicate decreasing water availability and increasing drought risk in many regions of the world (Gerten *et al.*, 2007), manifest as reductions in river discharge (e.g., Chalecki and Gleick, 1999), ground water resources (e.g., Sandstrom, 1995), or soil moisture (Gregory *et al.*, 1997; Wetherald and Manabe, 2002). Even though, rate and distribution of precipitation strongly depend on a variety of parameters, e.g., topography, vegetation structure, and land use, and therefore strongly differ in their spatial and temporal distribution, hot extremes and summer heat waves like in summer 2003 will continue to become more frequent in particular in central and southern Europe (EEA, 2004, Kundzewicz *et al.*, 2006; Rowell and Jones, 2006).

1.1.1 Shift of species ranges as response to global warming

In the past, migration has been the most common response of plants to Quaternary climate change (Huntley *et al.*, 1991). Palynological data indicate that plant species tracked during Quaternary climate changes favourable conditions with estimated migration rates of 150-500 m year^{-1} (Huntley *et al.*, 1991). Global analyses document significant range shifts of present vegetation toward the poles (or metres per decade upward) of 600 m year^{-1} (Grabherr *et al.*, 1994; Meshinev *et al.*, 2000; Kullman, 2001; Parmesan and Yohe, 2003; Peñuelas and Boada, 2003; Meier and Leuschner, 2008). These maximum rates of migration are one or two orders of magnitude too slow to track the predicted climatic changes in the next century and species may not be able to avoid the severest effects of global warming. Climate-induced ecological change is also expected to outpace the rates at which successional processes could occur in forests. The understanding of the plasticity of plant responses towards changing climatic conditions therefore becomes increasingly important.

1.1.2 Plant responses to elevated carbon dioxide and temperature

The predicted rapid and simultaneous changes in several environmental factors controlling forest ecosystem functions are raising concerns about future terrestrial

ecosystem productivity. Among the most critical of these concerns are the increase of atmospheric concentrations of CO_2 and extreme temperature, and changes in precipitation distribution (Bazzazz, 1990; Mooney *et al.*, 1991). The primary effect of elevated CO_2 in most ecosystems is through a direct positive effect on photosynthetic C-fixation that increases net primary production (NPP) (Lindroth *et al.*, 1993; Hamilton *et al.*, 2002; Norby *et al.*, 2002). However, long-term growth in elevated CO_2 concentrations has caused in forest species like beech reduced sensitivity and acclimation of stomatal conductance to vapour pressure deficit (Heath, 1998), reduced sensitivity to drought (Heath and Kerstiens, 1997), and reduced sensitivity to atmospheric CO_2 concentrations (Santrucek and Sage, 1996; Idso, 1999). Increasing temperatures and vapour pressure deficits additionally increase the rate of evapotranspiration (Norby and Luo, 2004). Photosynthetic acclimatization to elevated CO_2 and temperature reduces the water use efficiency. Thus, actual water use per individual tree may increase, if the stomatal response to CO_2 and temperature is weak. Further, CO_2 and temperature can also influence ecosystem processes via their effects on soil moisture (Hungate *et al.*, 1997; Yang *et al.* 2003). Rising temperature and CO_2 concentration will impair the water supply for plants that will be already diminished due to lower precipitation amounts in summer. Thus, water deficiencies might become more hazarding for ecosystems than altered CO_2 or temperature regimes, indeed, without commensurate increases in precipitation, water stress can result even at constant rainfall levels.

1.1.3 Increasing water stress and loss of biodiversity

In many regions of the world, net primary production is in the first instance limited by available soil water and only secondly by temperature, CO_2, or radiation (Nemani *et al.*, 2002; Xiao and Moody, 2004). The geobiosphere as a whole appears to be currently in a state of water deficiency (Lee and Veizer, 2003). Precipitation is often used as a proxy of soil water availability; therefore declining precipitation can be linked to a decrease in soil moisture. If soil water content falls below some species-specific level, plants experience drought stress that alters both soil-root and leaf-atmosphere interfaces and threatens the integrity of the liquid phase continuum from soil to leaves (Bréda *et al.*, 2006). In this so critical state, drought-induced physiological disorders dramatically can change the distribution of potential forest types increasing tree vulnerability to secondary stresses like frost or another drought, insect damage (Tuomi *et al.*, 1988;

Docherty *et al.*, 1997; Rouault *et al.*, 2006) and pathogens (Dale *et al.*, 2001; Desprez-Loustau *et al.*, 2006). Such cumulated stress factors rapidly may end either with partial recovery of tree growth, or with a final shift into decline and eventual death (Law *et al.*, 2002; Sturrock, 2007) with a final consequence of the dramatic loss of biodiversity.

In spite of this, the effects of climate change in the next few years will hardly be perceptible as measured by changes in tree health and species composition, and conservationists are far from able to assist all species under threat (Myers *et al.*, 2000). One effective way to support the most species threatened with extinction is to identify "biodiversity hotspots" where exceptional concentrations of endemic species are undergoing exceptional loss of habitat. As many as 44% of all species of vascular plants are confined to 25 hotspots comprising only 1.4% of the land surface of the Earth. Concentrating a large proportion of conservation support on these areas would go far to stem the loss of biodiverity that is now underway (Myers *et al.*, 2000). According to Myers *et al.* (2000) one of the hottest under threat area in term of rate of habitat loss is the Mediterranean Basin, where, in recent decades, on several Fagaceae species the impact of forest plant pathogen has clearly increased in connection with exceptionally dry years (Desprez-Loustau *et al.*, 2006). In particular, beech trees under water stress can become affected by *Biscogniauxia nummularia* (Bull.: Fr:) O. Kuntze., which causes necrosis and cankers on branches and stems (Nugent *et al.* 2005). This specific beech pathogen is able to spend most of its life cycle in latent form in symptomless aerial plant organs as endophyte to switch pathogenic face once the host becomes subjected to physiological stresses, in particular water stress (Granata and Whalley, 1994; Paoletti *et al.*, 1996; Capretti *et al.*, 2003; Granata and Sidoti, 2004; Nugent *et al.*, 2005; Luchi *et al.*, 2006).

In conclusion, to better understand the whole complex of forest ecosystem functions is necessary to well know its biodiversity in terms not only of plant species diversity but also of microbial diversity and place each species into a trophic level or into its niche dimension within the host plant. Indeed, recent studies have shown that physiological changes in trees may also lead to differential response of those organisms that normally colonize beech tissues (Hendry *et al.*, 1998), that suggests the importance of role of endophytic fungi associated with woody perennials, which can be complex and labile both in ecological and evolutionary time (Saikkonen, 2007).

1.1.4 Evolutionary risk for European beech forests

Concerning Forest Preserve large economic and environmental importance is recognized to European beech (*Fagus sylvatica* L.) that is a characteristic feature of central and southern Europe with a wide natural distribution range (Fig. 2), and also plays an important ecological and hydrogeological role in the Italian mountain landscapes. Timber quality, landscape value and role in preventing soil erosion justify the economic and environmental importance attached to this tree in the Italian mountain areas. On the Apennines, beech forests represent the most widespread mountain climax association (Scoppola, 1999). Cause of its frequency, ability to grow on sites of wide ecological variability and its longevity, beech is well used to evaluate the growth and climate relationships in different bioclimatological units, and to estimate future prospects and possible ecological risks associated with climate change (Eckstein *et al.*, 1984; Gutierrez, 1988; Biondi, 1992; Rozas, 2001; Dittmar *et al.*, 2003; Piovesan *et al.*, 2005; Lebourgeois *et al.*, 2005; Di Filippo *et al.*, 2007; Biondi, 2008). In this century, in the Mediterranean Basin, that is southern limit of beech geographical range, beech forests are able to work as bio-indicator and provide a unique opportunity to explore connection between tree physiological conditions and long-term drought stress (Piovesan *et al.*, 2003-2008) European beech either will have to respond to rapidly changing climatic conditions or will face local extinction if not sufficiently adapted to altered drought and temperature conditions, that can promotes aggressiveness of weak parasites living inside plant tissues (Schröter *et al.*, 2005). Rapid climate change as predicted requires a better understanding of structure and diversity of "pathogenic" and "non-pathogenic" fungal endophyte community of *Fagus sylvatica* in the Mediterranean Basin.

1.2 The European beech (*Fagus sylvatica* L.)

The European beech (*Fagus sylvatica* L.) belongs to the family Fagaceae, order Fagales, which includes woody plants with flowers pollinated by wind and characterized by a particular structure called “cupule”, partially or entirely enclosing the fruit. Beech is the most frequent deciduous forest tree species in Central Europe, it predominates at all natural or near-natural forest sites that are not extremely dry, wet or acidic (Thomas and Sporns, 2008).

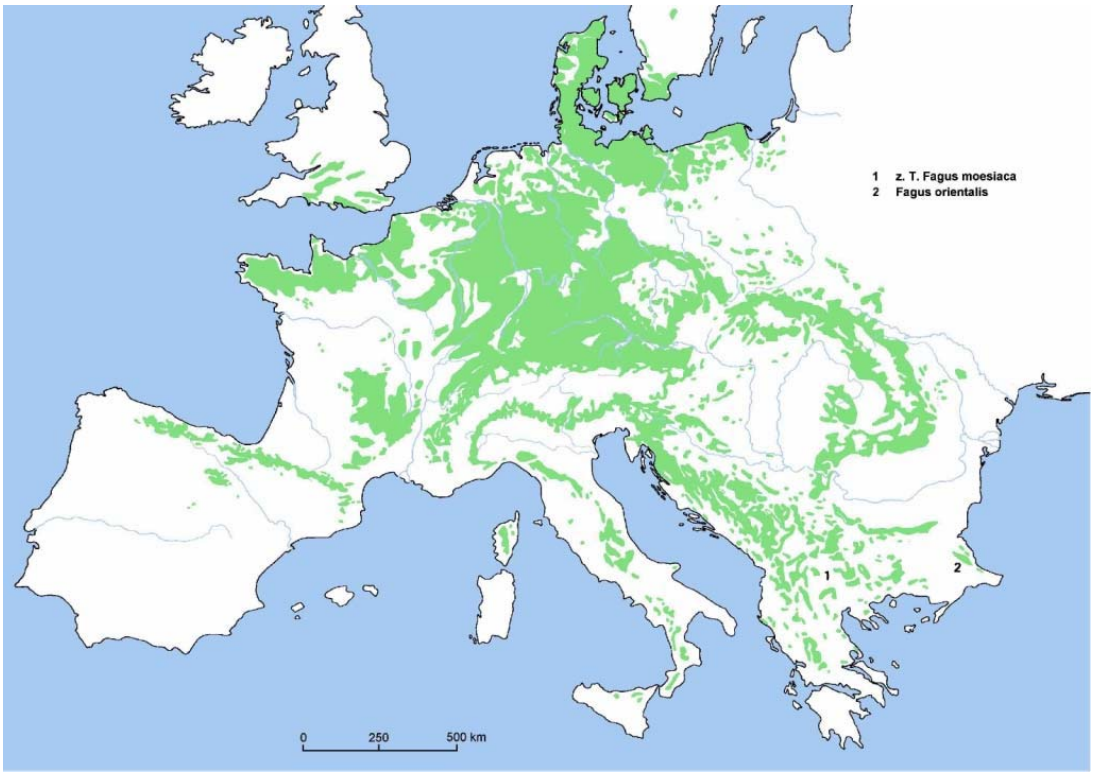


Fig.2. Natural distribution of beech forests in Europe (according to Bohn 1992)

With a slow growth rate *F. sylvatica* is a large, deciduous tree, capable of reaching heights up to 40–50 m, with thin, cylindrical trunk and conical, later wide-branched treetop. Root system is heart-shaped with strong many-sides roots. Bark is smooth, grey to white-grey, rarely fissured. Annual shoots are red-brown, primarily white-hairs, later glabrous. The buds are alternately double-rowed, thin fusiform, 10–25 mm long, cinnamon brown, with scales whitish on the top. Leaves are smooth, shining above, lighter beneath, with short petiole; lamina is elliptic to oval-elliptic, 3–12 cm long, entire to shallow crenate, undulate at the edges, ciliate. Leaf primary vein is whitish hairy, in the axils of the marginal veins are long-haired. Secondary veins are in 5–9 pairs. Petiole is 5–10 mm long, pinnate. Stipules are narrowly lanceolar, light brown, smooth, and soon deciduous. Male flowers are in the axils of the leaves on long pedunculated bundle, female flower are 2–3 in reddish cup with hairy nodulation. The seeds are about 1 cm long, brown, smooth, triangular, at the edges with winged achenes – beechnuts. The beechnuts borne singly or in pairs in soft-spined husks 1.5–2.5 cm long, known as cupules are closed by 3-4 lobels in brown, ligneous, echinate, pedunculated cup which is about 2 cm long and dehiscent by four lappets (Pignatti, 1982). Raw beechnuts can be slightly toxic for some people. You can lower the toxicity

by thermal treatment, for example roasting (Leugnerová, 2007). Beech flowers in late April and May. Seeds ripen in the autumn and coloration of leaves comes from yellow over the reddish until dark brown. European beech has lifespan of 200 to 400 years.

European beech is an oceanic and sub oceanic climate woody species (De Philippis, 1937) with need of 800–1000 mm precipitation. This tree creates multi-storeyed forest, mostly unmixed, because of its huge its shading.European beech prefers freshly wet, well aerated soils, often calcific and rich in humus and minerals. It dislikes waterlogged but also dry and sandy soils. It is sensitive to drought and late frost too. European beech is diagnostic species for *Fagion* alliance (Leugnerová, 2007).

Beech forests characterise the landscape of many mountain areas in Italy, from the Alps down to the southern regions of Campania, Basilicata, Calabria and Sicily in the Mediterranean area, except Sardinia (Fig. 2). According to the National Forest Inventory (INFC, 2005), the total area covered by beech in Italy is 1.042.129 hectares, which corresponds to 9.4% of the country's total forest area. In the Alps, beech generally forms pure stands above 1000 m a.s.l. altitude in areas with relatively low rainfall, while it grows at around 600-700 m a.s.l. in more humid areas. On the Apennine mountains beech usually grows above 900-1000 m a.s.l. In the central Apennines sporadic beech or beech stands can also be found at lower altitudes (< 700-800 m asl) associated with chestnut or mesophilous mixed oak forests; these beech spots are considered relict sites and proof of a much wider diffusion of beech in the area (Montelucci, 1956; Anzalone, 1961; Anzalone, 1980; Scoppola and Caporali, 1997; Scoppola, 1999). Beech forests are more widespread on the northern slopes and where rain and fog maintain moist air conditions. On the sunnier and warmer southern slopes, the lower vegetation limit for beech tends to move higher (Hofmann, 1991;Pignatti, 1998). On the northern slope of the Mount Etna in Sicily beech reaches an altitude of 2000 m (Hofmann, 1960; Del Favero, 2008). In the southern regions, in areas with high air moisture conditions, beech can descend to an altitude of 400-500 m, where it comes into contact with evergreen oak (*Quercus ilex* L.). In some valleys on the Aspromonte mountain range at the southern most tip of Calabria, there is an inversion of the vegetation planes, with beech occurring at lower elevations compared to evergreen oak. In the "Gargano" peninsula (Puglia) beech grows at an altitude of 200-300 m a.s.l. (Hofmann, 1961; Fenaroli, 1966; Pignatti, 1998). Beech forests also host other mountain hardwoods, such as helm, linden, cherry, sycamore and Norway maple

(Pignatti, 1998). Silver fir (*Abies alba* Mill.) is found in beech forests along the Apennine mountains and, especially, in the Alps (Nocentini, 2009).

Beech, with plenty of cultivars, is often setting out as solitaire wood species in larger gardens and parks; it is also usable in hedge (Leugnerová 2007).Wide use has European beech, which are different by its habitat, shape or colour of leaves in gardens.

European beech is one of the most important economic leafy woody species in Europe. It has interspersed porous wood, skin-pink to pink-brown colour, without marked true heartwood. Its wood is hard, heavy and little flexible. However, false heartwood with red-brown colour that occurs in old trees lowers the quality of wood. Beechwood can be used in production of bent-wood furniture (beechwood contains a huge amount of lignin, which softens at high temperatures, so than is wood very shapable), for veneer, plywood, parquet. In the Middle Ages was using in production of wood coal.

1.2.1 Main damages

Usually none diseases cause death of plants provided soil is not compacted and is well-drained. Several fungi cause leaf spots but are generally not serious to warrant chemical control. Among these *Apiognomonia errabunda* (Roberge ex Desm.) Höhn causes leaf anthracnose with discrete, irregular, brown necroses distributed in patches on leaf blade. Infected areas are often found along the veins and midrib of the leaf (Fig. 3).

Fig. 3. *A. errabunda* symptoms on beech leaves *F. sylvatica* (www.arsia.toscana.it).

Fruit honey-brown bodies are formed on the undersides of the leaves. Many studies have reported *A. errabunda* living in symptomless tissues as endophyte in beech trees (Sieber and Hugentobler, 1987; Morelet, 1989; Hämmerli *et al.*, 1992; Toti, 1993; Viret *et al.*, 1993; Kowalski and Kehr, 1996; Danti *et al.*, 2002).

Similar necroses are cuased in the spring by the larva of the beech leaf miner, *Rhynchaenus fagi* L., an *curculionidea* insect, but these can be distinguished by the presence of the mined area which consists of a pale brown patch linked by a narrow tunnel to the midrib of the leaf (Butin, 1995).

Another diseases are: powdery mildew most common late in the season, by fungi belong to Erisyphaceae family, causes a white coating on the leaves; bleeding canker forms small or large patches of dying bark on the stems or branches from which were scattered drops of rusty-red, yellow-brown or almost black, gummy liquid ooze. Crown symptoms include leaves of smaller size and lighter green color than normal. In severe cases the leaves wilt and the branches die. Avoid feeding with high nitrogen fertilizers as it seems to worsen the condition of infected trees. Beech bark disease occurs when the feeding site of woolly beech scale is invaded by a fungus. The fungus kills the bark and in the process favoured the attacks of insects, such as bark beetles, and aphids. There are no satisfactory controls for the fungus. Cankers infect, girdle, and occasionally kill branches. (Michigan State University Extension Ornamental Plants 1999).

Beech root system can be strongly damaged after infection of soil pathogens which cause root rot in host plants. Among these were remembered, for their virulence and geographic distribution and diffusion, differents type of species in the oomycete genus *Phytophthora*. These root rot fungi produce motile, flagellate zoosperes as dispersal units with which move through soil water. The species most virulance at our latitudes are *P. cambivora* (Petri) Buism (Jung *et al*., 2005**)**, *P. citricola* Sawada (Wang *et al.*, 2003) e *P. cactorum* (Lebert & Cohn) J. Schröt., while most spread in UK are *P. kernoviae (*Brasier *et al*. 2005) *e P. ramorum* Werres (Heungens *et al.*, 2006) (Fig. 4) and are responsible of devasting and diffusine dieback of forest tree species.

Fig. 4. Bleeding canker by *P. ramorum* on beech trunk (http://www.ilvo.vlaanderen.be)

Serious threat of *F. sylvatica* is *Heterobasidion annosum* (Fr.) Bref.. This fungus is a wood-decay fungus widespread in the coniferous forests of temperate and boreal regions of the northern Hemisphere. It causes great financial losses due to root and butt rot on several conifers, especially in managed forests (Stambaugh, 1989; La Porta *et al.*, 1997). *H. annosum* kills resinous hosts directly by decaying roots and killing the cambium around the root collar. Trees are often predisposed to bark beetle infestation and usually die standing. Some non-resinous hosts are affected in the same way, but others develop extensive butt decay rather than being killed outright. The most reliable way to identify the disease in the forest involves finding its fruiting bodies, called "conks" (Fig. 5).

Fig. 5. Fruit bodies of *H. annosum* on trunk of *F. sylvatica.*(www.unipd.it)

Advanced decay caused by the fungus is often laminated with pitting and it can also be stringy and moist with white streaks and scattered black flecks (USDA Forest Service Gen. Tech. Rep. PSW-GTR-165. 1998).

Stressed and damage hosts were attacked by weak parasites, among these, some are *xylariaceous* fungi, such as *Xylaria polymorpha* Pers. ex St. Amans, Kretzchmaria deusta Fr. [ex Ustulina deusta] and *Biscogniauxia nummularia* (Prljincevic, 1982; Sinclair *et al.*, 1987; Schwarze *et al.*, 1995; Hendry *et al.*, 1998; Luchi *et al.*, 2006). Over the last 20 years decline of european beech in Sicily and Calabria (Italy) was observed to be associated with the ascomycete *B. nummularia*. This fungus is naturally present in beech stands and to date has not been considered a primary pathogenic agent (Granata and Whalley, 1994; Paoletti *et al*., 1996; Moriondo *et al*., 1999; Capretti *et al*., 2003; Granata and Sidoti, 2004). Many studies reported *B. nummularia* living in asymptomatic tissues and being able to cause strip-canker and wood decay in trees that suffer severe water stress during the growing season (Granata and Whalley, 1994; Hendry *et al.*, 1998; Hendry *et al.*, 2002; Capretti *et al.*, 2003; Nugent *et al.*, 2005; Luchi *et al.*, 2006). According to the redefinition proposed by Petrini (1991), it could thus be considered an endophyte during the latent period.

1.3 Endophytism: an age-old phenomenon

Endophyte ownes its origin to De Barry (1866) who first coined the term. Since then it has deeply embedded in the literature. Within the last decade, different authors have proposed a range of similar, but more complex definitions (e.g., Carroll, 1986; Petrini, 1991; Wilson, 1993). However, “endophyte” does not stand alone as a term whose definition has changed over time, or as a term whose definition biologists might not agree over. At the most basic level, endophyte could simply refer to the location of the organism: *endo* means within and *phyte* means plant. This is contrasted to “epiphyte” which refers to organisms living on the outside of the plant (Kogel *et al*., 2006). The organisms commonly associated with the term endophyte are fungus and bacteria (Fahey *et al.*, 1991) and this restricted definition (Wennström, 1994) has evolved into a definition which describe not only the location, but also the nature of the association between one organism with its host (Kogel *et al*., 2006).

Symbiosis between a fungus and a plant is a widespread phenomenon in nature. Most, if not all, plants studied in natural ecosystems are infested by fungi that live in intercellular space or inside cells of host plant without cause any kind of disease

(Saikkonen *et al*., 1998; Kogel *et al*., 2006) or apparent damage, if by disease we mean the result of an interaction between a host (susceptible), a pathogen (virulent) and an environment (favourable to the pathogen). During the long co-evolution term of endophyte and plant, equilibrium between these organisms have been established (Gao *et al*., 2010). The true endophyte will exist once equilibrium between fungal activity and the plant reaction is achieved and is maintained over time (Giménez *et al*., 2007), therefore a mutualistic interaction does not mean the absence of plant defence but is a finely tuned balance of antagonisms that keeps the host–microbe interaction in a stable state that disadvantages neither partner (Kogel *et al*., 2006) (Fig. 6).

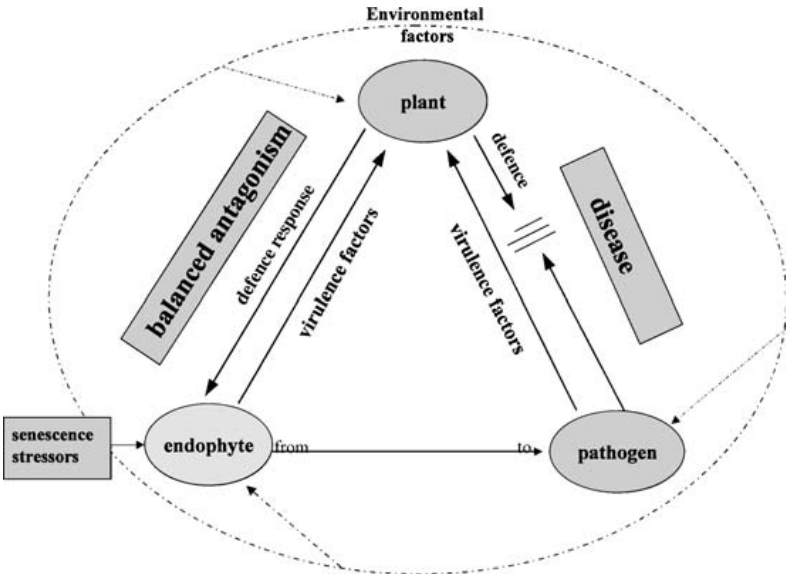


Fig. 6. Hypothesis: a balance of antagonisms between endophytic virulence and plant defence response results in asymptomatic colonisation.

Commensalism and mutualism represent the balanced stages plant–endophyte interactions. Commensalism provides benefit to the endophyte by enabling an undisturbed existence and nutrient supply without affecting the host. Mutualism, by contrast, is defined as an interaction that is beneficial for both partners (Kogel *et al.*, 2006). In addition to the providing benefits for the endophyte, mutualism can frequently result in promoted growth of the host (Dai *et al.*, 2008), improved tolerance to abiotic stress (Redman *et al.*, 2002, Lewis 2004; Malinowski *et al.*, 2004) and increased resistance against pathogens (Colditz *et al.*, 2005; Tanaka *et al.*, 2005; Vega *et al.*, 2008). Direct effect of the potential mechanisms of endophytes inhibition of plant

pathogen is characterized by producing of antifungal and antibacterial secondary metabolites like antibiotic, terpenoids, alkaloids, aromatic compounds and polypeptides (Gunatilaka, 2006), and by releasing of lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellu-lose and DNA (Tripathi *et al*., 2008). When endophytes colonize the plant surface, they produce enzymes to hydrolyze plant cell walls and to start the growth within host tissues. These enzymes also have the function to suppress plant pathogen activities directly by degrading of cell walls of fungi and oomycetes eventually present on the surface of host tissues (Gao *et al*., 2010). However, the direct interactions between fungal endophytes and pathogens are complex and sensitive to species-specific antagonism (Arnold *et al*., 2000).

Under stress conditions, the plant-endophyte interaction can become unbalanced and disease symptoms can appear or the fungus can be excluded by induced host defence reactions (Clay and Schardl, 2002; Schulz and Boyle, 2005). Actually, some fungal endophytes are patogens of plant host and may evolve from plant pathogenic to non-pathogenic state go through an endophytic or latent face of its lifecycle (Carroll, 1988; Freeman and Rodriguez, 1993; Saikkonen *et al*., 1998; Kogel *et al.*, 2006) (Fig. 6). These pathogens are known to be symptomless colonizers of healthy forest trees in an unstable equilibrium between transient mutualism or neutralism and latent pathogenesis. Climatic factors can change the endophytic nature of these fungi turning it into a weak pathogen or an opportunistic invader of senescing trees (Müller and Krauss 2005, Schulz and Boyle 2005, Moricca and Ragazzi 2008, La Porta *et al.* 2008, Gao *et al.* 2010). The host–microbe interactions can range from mutualism through commensalism to parasitism in a continuous manner (Johnson *et al*. 1997, Redman *et al.* 2001) (Fig. 6).

Symbioses of plants with beneficial or neutral endophytes share many common attributes with plant interactions with pathogens. The molecular and biochemical basis for the switch from endophyte to parasite is still to be elucidated, but recent findings of compatible plant–microbe interactions have enhanced our understanding of what factors determine endophytic and parasitic lifestyles(Kogel *et al*., 2006).

1.4 The genus *Biscogniauxia* Kuntze

The genus *Biscogniauxia*, a member of the family *Xylariaceae*, has a worldwide distribution with over 50 *taxa* recognised (Nugent *et al.*, 2005).

Biscogniauxia has long been known as *Nummularia* Tul. & C. Tul., until Miller (1961) placed most of its members in his section *Applanata* of *Hypoxylon.* The current concept of *Biscognauxia* was defined by Pouzar (1979, 1986) and resumed by Ju, Rogers, San Martin and Granmo (1998).

This genus is represented in Europe by ten known species, five of which having applanate stromata and low margins, i.e., *B. anceps*, *B. cinereolilacina*, *B. granmoi*, *B. mediterranea and B. nummularia*, and thus being likely to be confused with *Hypoxylon*. The five other species *B. dennisii*, *B. marginata*, *B. querna*, *B. repanda* and *B. simplicior* differ in having more or less cupulate (concave) stromata with conspicuously raised margins.

Biscogniauxia shares with *Hypoxylon* discoid ascal apical rings and *Nodulisporium*-like anamorphs as defined in Ju and Rogers (1996). It is separated from *Hypoxylon* primarily in having bipartite stromata with an outer stromatal layer which disappears on mature stromata, and in lacking KOH-extractable pigments. Moreover, the stromatal carbonization is important in *Biscogniauxia* while it is weak in *Hypoxylon* section *Annulata* and usually absent in *Hypoxylon* section *Hypoxylon* (Ju and Rogers, 1996; Ju *et al.*, 1998). The outer dehiscent stromatal layer is often overlooked as it is only present on young stromata, remnants of this layer may be found at margins of mature stromata, but are usually inconspicuous. It is noteworthy that in *Biscogniauxia*, the natural anamorph develops on young stromata either between the outer layer and the stromatal surface, before the outer layer dehisces (Ju *et al*., 1998), or on the upper side of the dehiscing layer and on stromatal margins.

In the field, applanate stromata of *Biscogniauxia* also are easily confused with those of *Diatrype* Fr. (*Diatrypaceae*) and *Graphostroma* Piroz. (Graphostromataceae). In *Diatrype*, stromata differ in lacking carbonaceous tissue and in having a well-developed interperithecial tissue (entostroma) of usually white granules; moreover, asci are typically long-stipitate, ascospores are yellowish and allantoid, and conidia are scolecosporous (anamorph *Libertella* Desm.). *Graphostroma* is somewhat intermediate between the Xylariaceae and the Diatrypaceae through a *Nodulisporium*-like anamorph recalling the Xylariaceae and light-coloured suballantoid ascospores recalling the Diatrypaceae.

Biscogniauxia species develop in bark of trees and shrubs, especially on dead or dying branches, more rarely on trunks, and are suspected to be at least weak pathogens (Ju *et al.*, 1998). Therefore they are to be searched for on dead branches still attached to

the trunk, and specimens of *Biscogniauxia* found on dead branches lying on the ground are usually old and their perithecia are empty, unless the branches have broken off recently. It is also probable that many of these species occur "unseen" in healthy host tissue but only become apparent when stromata develop following senescence or death of the host material in which they occur. Whether they are classed as endophytes or are seen as "sneaky" fungi sensu Rogers (2000) is open to debate.

Identification of *Biscogniauxia* mainly relies on stromatal characters and ascospore size and morphology. Identification of host is a decisive feature in several species which are apparently host-specific, i.e., *B. cinereolilacina* specific to *Tilia sp.*, *B. granmoi* specific to *Prunus sp.*, *B. nummularia* specific to *F. sylvatica* and *B. simplicior* specific to *Rhamnus catharticus.*

The stromatal surface may be plane to slightly convex and then with low margins to more or less concave or cupulate, and then with thick raised margins, (*B. marginata*). The stromata are considered *carbonaceous* when, sectionned with a razor blade, they turn out to be broken rather than properly cut. The dehiscing outer layer is only seen on very young stromata, and therefore is rarely observed. It can be thin and torn up, operculum-like to crust-like.

Ostioles may be umbilicate or discoid when opening lower than stromatal surface, to papillate or coarsely papillate when opening higher than stromatal surface.

Perithecia may be obovoid to tubular, but in some species this character may be variable within a same collection.

Ascospores may be one-celled to two-celled, ellipsoid to subglobose, with a germ slit that can be straight, sigmoid or bilateral.

The germ slit morphology is frequently a diagnostic character and its observation is made easier by the use of mounting media such as lactic acid or lactophenol which improve the clearness. Permanent mounts of ascospores in Polyvinylic alcohol (Rhodoviol®: Rhodoviol 50g, water 150g, lactic acid 80g, phenol 40g.) dissolved in lactophenol (Van Brummelen, 1967) make the germ slits particularly conspicuous. Bilateral germ slits are best observed on ascospores that are not exactly in front view, when focusing alternately on upper and lower side.

1.4.1 *Biscogniauxia nummularia* (Bull.:Fr.) O. Kuntze

B. nummularia is known to cause the Beech Tarcrust. The Latin "nummus" meaning a coin, is applied at the encrustations that are often rounded and coin-like (Phillips,

1981). This fungus causes strip-cankers and wood decay in consequence of severe dry spell (Hendry *et al*., 1998) These striking lesions, often many metres long, typically spiralled around the trunks of affected trees, terminating at a dead branch or root. Yet despite their great size, such strip-cankers were formed in a single growing season (Lonsdale, 1983; Rayner, 1986; Hendry *et al*., 1998). The disease symptoms also include yellowing and leaf drop (Granata and Sidoti, 2004). The necrotic bark on the faces of cankers bore fungal fruiting structures (Hendry *et al*., 1998). The fruiting body forms a thick and shiny black crust, on beech bark and is found at all times of the year (Hendry *et al*., 1998). It is not edible (Phillips, 1981). Young specimens are covered by a light brown outer layer. The spores are black to dark brown.

Taxonomic classification of this fungus following:

Kingdom*:* Fungi

Division*:* Ascomycota

Class*:* Ascomycetes

Order*:* Xylariales

Family*:* Xylariaceae

Genus*: Biscogniauxia* Kuntze

Species: *Biscogniauxia nummularia* (Bull.) Kuntze

B. nummularia is distinctive among other members of genus with applanate stromata in the combination of black stromatal surface with slightly papillate ostioles, blackish brown ellipsoid ascospores 10-13 x 7.5-8.5 µm and specificity for *F. sylvatica.*

The closest taxon is *B. granmoi*, thoroughly described by Læssøe *et al.* (1999). They pointed out the main differences between these two species: in *B. granmoi*, ostioles are pitted and not papillate, stromatal margin is slightly raised, ascospores are medium brown, narrower (x 5-6.5 µm) and all records were made on *Prunus padus* (in Europe) and *P. padus* var. *pubescens* (in Far Eastern Russia).

Morphological characters are used in description and for identification of this type species:

Stromata applanate, discoid 5-20 mm diam to irregularly ellipsoid 15-50(-120) mm long x 10-22(-60) mm broad x 0.6-0.8 mm thick; surface black, carbonaceous(Fig. 7a-c), when young covered by a dehiscing light brown outer layer; tissue beneath perithecia inconspicuous (Fig. 7b).

Perithecia subglobose to obovoid, 0.4-0.6 mm diam x 0.5-0.7 mm high.

Ostioles discoid, slightly papillate to nearly at the same level as the stromatal surface.

Asci short-stipitate, with a discoid amyloid apical ring.

Ascospores dark brown to blackish, ellipsoid, frequently with narrowly rounded ends, 11.5 x 13.5 µm, with straight, inconspicuous germ slit spore-length (Fig. 7d).

Fig. 7. a-c: Stroma(ta) at maturity . b: immature stroma(ta). d: Ascospore of *B. nummularia*. Herbarium: JF99047-JF00114 JF99047

1.4.2 *B. nummularia* a latent invaders with higher damage potential due to climate change in Mediterranean Basin

In Italy a serious decline of *F. sylvatica* was reported by the Forestry Commission of Messina (Sicily) in the lower region of a beech wood on the Nebrodi mountain range in 1990, and a few years later, in old beech coppice in the Ferdinandea forest, on the jonic side of the Calabrian Serre. Furthermore was observed that *B. nummularia* was capable of rapid growth at high temperatures, and that it was able to colonize large volumes of wood within host under water stress (Granata and Whalley 1994, Paoletti *et al*. 1996, Moriondo *et al*. 1999).

Currently, the presence of *B. nummularia* as endophyte was been also reported in Italian beech forests at major latitude (Capretti *et al*. 2003, Luchi *et al*. 2006). Given that, how was already suggested for other pathogens like *B. mediterranea* on mediterranean oaks (Vannini and Valentini 1994; Vannini *et al*. 1996; Collado *et al*. 2001, Anselmi *et al*. 2000, 2004, Vannini *et al*. 2009), *Sphaeropsis sapinea* on *Pinus resinosa* (Stanosz *et al*. 2001) and *Sclerotinia pseudotuberosa* on chestnut (Vettraino *et al.* 2005), the potential ecological risk predicts a spread of damages caused by *B.*

nummularia infection more and more along north, just as result of drought increasing due to Global warming. Thus, the potential spread of disease is not due to the movement of pathogen but to "climate shift" linked to global climate change in progress. On the basis of this possibility is necessary well full prevention of these critical evens, that can be possible just after full knowledge of composition and dynamics of fungal endophytic community associated with *F. sylvatica*.

2. Aims

Fungal endophytes that colonize forest trees are widespread phenomenon, but relations between fungal endophytes and forest trees have begun to be the subject of survey more recently. The few studies on endophytes in trees mainly concern the tropical areas and the most northern latitudes, whereas similar investigations in the Mediterranean region have so far been scarce and sparce. Actually endophytes are studied mostly in economically important forests suffering from diseases such as oak forests, but now, the discovery of old-growth beech forests is very interesting to undrestanding the long-term ecosystem dynamics in relation to natural disturbances. Within forest ecosystem dynamics new informantions about the endophytic distribution patterns, in term of possible effects of location, geographical and seasonal factors can provides an opportunity to develop future guess concerning beech decline in relation to climate changes.

This present study was undertaken in order to :

1. Evaluate the composition of fungal endophyte community of *Fagus sylvatica* in Center Italy (twigs, leaves and buds);
2. Analyze the endophytic distribution patterns within tissues, and the dynamics of symbiotic relationship over time and in reference to the effects of station (twigs and leaves);
3. Compare two approaches used to address description of fungal endophyte community: traditional isolation techniques and DNA-based, cultivation-independent techniques (TRFLP, *Real-time* PCR);
4. Assess the incidence of *B. nummularia* in asymptomatic beech tissues from two different climatic stations (in collaboration with Prof. Capretti, University of Florence).

3. Materials and methods: objectives 1

To evaluate how fungal endophyte communities differ in taxonomic composition and abundance is key to understanding the ecology and evolutionary context of endophyte–plant associations.

In this study were examined endophytes associated with healthy beech leaves, twigs and buds in different stations.

3.1 Study sites

The sampling site is an old-growth beech forest called "La Faggeta" characterized by minimal human disturbance during the last 50 years located in Central Italy (Fig. 8) inland Soriano town on Monte Cimino (Viterbo Province, Latium), between 950 and 1050 m a.s.l. (Fig. 9A).

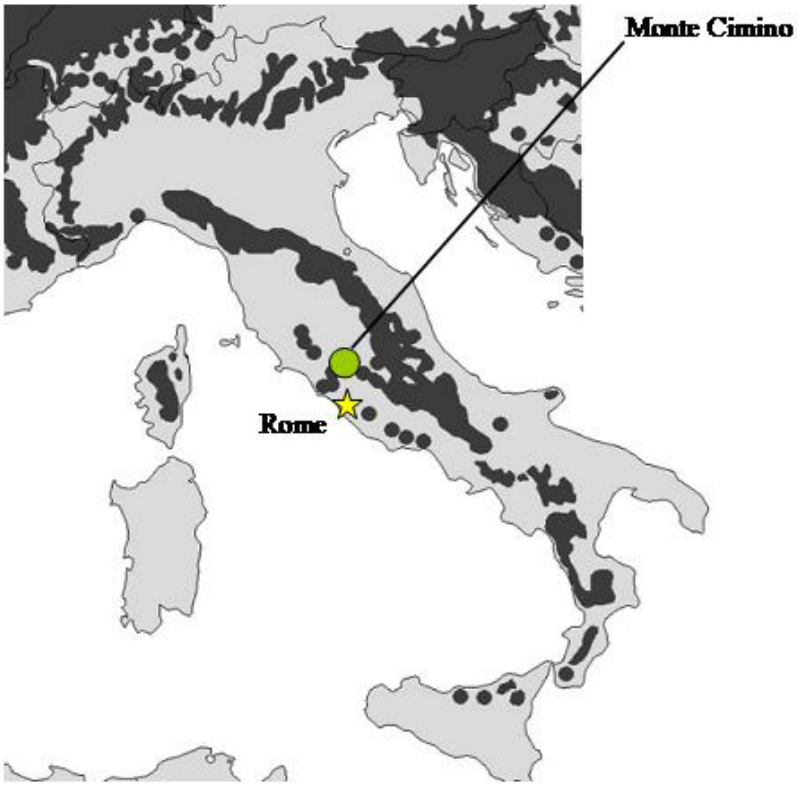


Fig. 8. Geographic distribution of *F. sylvatica* in Italy (dark area; Von Wuehlisch 2006), together with the position of study sites in Monte Cimino area.

"La Faggeta" is an oldgrowth secondary forest (58 ha) in the demographic transition stage (Frelich 2002), passing from an impressive single-layer canopy, where trees can reach >40m in height, to an old, yet multiaged, structure (Piovesan, 1998; Di Filippo *et*

al., 2005) (Fig. 9B). It is dominated by a widespread even-aged cohort (120–150 years old) that has suffered mortality in consequence of windthrows and glaze storms (Piovesan, 1998; Di Filippo *et al*., 2005); the last timber logging occurred in 1947–1949.

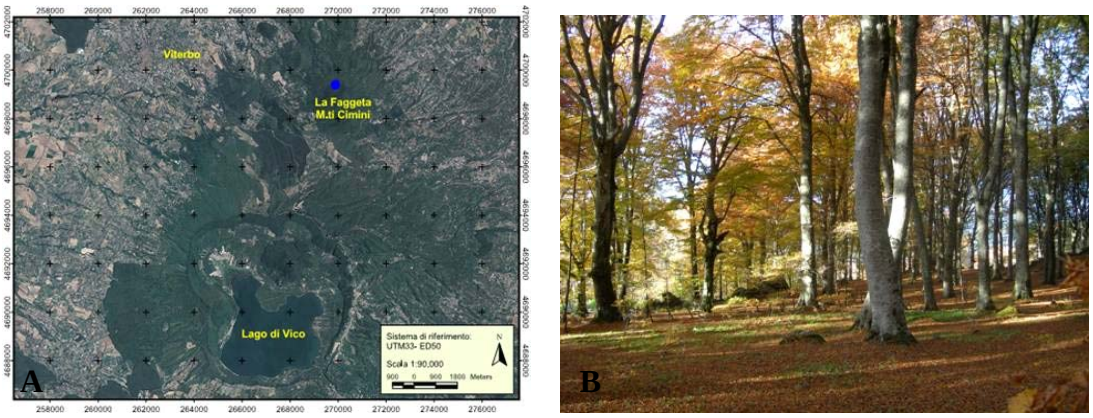


Fig. 9. A) Study site tracking on Monte Cimino area (referent system UTM33-ED50). It's possible see Viterbo city on the East and Vico lake on the South side. B) "La Faggeta" beech stand.

"La Faggeta" are characterized by the occurrence of European Beech (*F. sylvatica*) and Sweet Chestnut (*Castanea sativa* Mill.), at lower vegetation limit of beech stand, as main tree species with a group of least species as Sycamore Maple (*Acer pseudoplatanus* L.) and European Hornbeam (*Carpinus betulus* L.) and more rarely Wild Cherry (*Prunus avium* L.), Turkey Oak (*Quercus cerris* L.) and Downy Oak (*Quercus pubescens* Wild.). According to the phytosociological analysis "La Faggeta" belongs to the *Aquifolio-Fagetum* unit, where the limestone rocks are dominant (Pignatti, 1998) and has been managed following high-forest silvicultural systems so that trees older than a century are not uncommon over the landscape.

The geographic and environmental characteristics of sampling area are summarized in table 1.

Table 1 Geographic and structural features of sampled beech forest.

Site	Latitude (°)	Longitude (°)	Elevation (m a.s.l.)	Slope (%)	N (n ha -1)	H (m)	Phytoclimatic unit	Annual mean temperature (C°)	Annual mean precipitation (mm)	Xerothermic index
Monte Cimino	42.2429 N	12.1608 E	1042 (950–1050)	0–35	132	41	*Aquifolio-Fagetum*	10.03	1239	38.50

Soil has developed from a volcanic bedrock, are generally deep (41 m), and was classified as Vitrandic Hapludalf (Lorenzoni *et al*., 1995) following the Soil Taxonomy of the US Soil Survey Staff (1992). Site topography is gently sloping.

The pluviometry and mean temperature values of the stand were obtained from meteorological station located in Caprarola (Viterbo Province) about 10 km from trial site, at 42°19' of latitude, 12°10' of longitude (reference system UTM33-ED50) and 650m of altitude, the station has been activated since 1992/10/1 and climatic data were provided by the UCEA service (www.ucea.it).

Sampling was carried out in two different forest sites, called A and B, with North and South facing aspect respectively (Fig. 10).

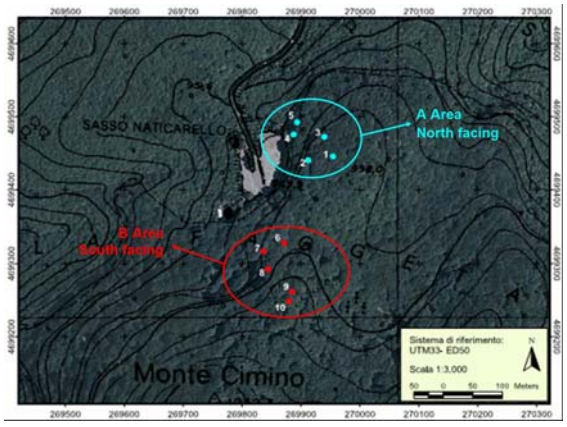


Fig. 10. Sampling sites into "La Faggeta" beech stand with different exposure. For each area five symptomless plants are randomly chosen.

3.2. Plant sampling

In beech forest stand five plants from each site with different exposure are randomly chosen for a total of ten apparently healthy beech trees. Each tree was marked with coloured paint and identified with a progressive number from 1 to 10 (Fig. 11A). Sampling collection was carried out in five different periods: November (2007),

February (2008), June (2008), September (2008) and January (2009). During which stems were always picked up, whereas leaves were presented only three times (Febuary, June and September 2008). From the basal and outer portion of the crown 3 twigs, 30 cm long, and 15 leaves per plant were collected (Fig. 11B).

Fig. 11. A) Plant n. 5 marked with coloured paint. B) Plant materials picked up from beech plants.

During last sampling in January 2009 were also collected buds of beech trees. From each tree 3 branches from the basal and outer portion of the crown carrying at least 30-40 buds were cut, labeled and taken to the laboratory. Five buds with bud-carrying twig segments were picked from each branch (Fig. 12).

Fig. 12. Fifteen buds per plant were picked during the last sapling period (January 2009).

Once collected tissues samples were taken to laboratory and were stored immediately at 4°C into plastic shoppers to avoid umidity loss until further processing within 24 h.

Shoot portions 30 mm longs, leaves and buds were surface-sterilized with 75% ethanol (1 min), 3% NaClO (3 min) and 75% ethanol (30 s) according to Luchi *et al*. (2006) and rinsed three times with sterile water. Each shoot, leaf and bud was split longitudinally into two parts: one was used for conventional isolation on PDA medium (PDA - MERCK KGaA, Darmstadt, Germany) added with Streptomycin (0,06 g/l) (PDS medium) and the second for DNA extraction.

The portion of shoot and leaf used for fungal isolation was cut into 5-6 small fragments (5 mm each), placed in Petri dishes on PDS and incubated in darkness at 20°C for 10 days (Fig. 13).

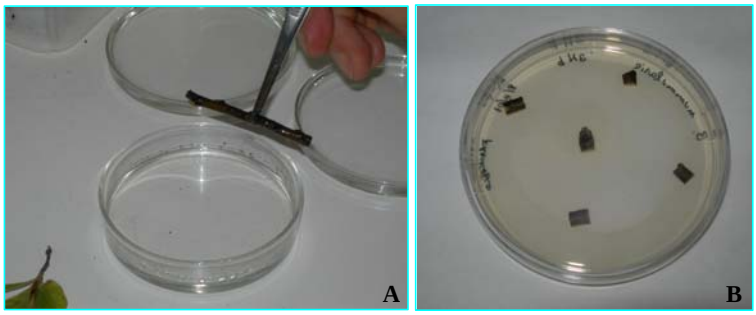


Fig. 13. A) Sterilization of plant materials. B) 5-6 small fragments were cut for each tissue and placed in Petri dishes on PDS medium.

Each bud was divided into its different component tissues: scales, rolled up leaves and current-twigs. Contiguous twig tissues were separated into two pieces: twig pieces directly underneath the buds were called contiguous, as opposed to the distal ones.
Bud scales, rolled up and twig pieces were placed in serial order in PDS medium and incubated in darkness at 20°C for 10 days.

3.3 Preparation of culture media.

The following components were added for every liter of PDS medium to be prepared: 39g of Potato Dextrose Agar (PDA - MERCK KGaA, Darmstadt, Germany) powder in 1000 ml of distilled water. Solution was autoclaved at 115°C for 15 minutes. After cooling to 55°C Steptomycin was added to solution (0,06 g/l) to suppress bacterial growth and the medium was dispensed to the Petri plates.

3.4 Isolation, cultivation and identification

The mycelia growing out of the fragments were individually subcultured on potato-dextrose agar (PDA). Pure cultures were identified according to their morphological and molecular features.

Cultures were grouped into morphotypes according to cultural and morphological characteristics (e.g. growth rate, habit and colour of colonies) and colonies were identified morphologically at the genus or species level under light microscopes (Zeiss Axioscope). Taxonomy followed dicotomic keys in standard mycological manuals (Watanabe, 2002) and descriptions of "Index Fungorum" (http://www.indexfungorum.org).

3.5 DNA extraction, amplification and sequencing

As control, at least one isolate was taken from each morphotype for sequence analysis.

Mycelial cultures were frozen and lyophilized. Genomic DNA was extracted with DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Dry mycelium was transferred into steril ceramic mortar and pulverized with steril ceramic pestle. After, a portion (20 mg of dry weight) was trasferred to 2-ml microfuge tubes with 0,4-ml lysis buffer AP1 and 0,004 ml of RNase (Dneasy Plant Minikit; Qiagen) and ground with a Vortex (2 min at max rpm) to homogenize mixture. After incubation at 65°C for 10 min, 0,13 ml of buffer AP2 was added to the lysis mixture and incubated for 5 min on ice to precipitate detergents, proteins and polysaccharides. After centrifugation for 5 min at 14.000 rpm (Beckman Cuolter ™ Microfuge® 22R Centrifuge), the supernatant was loaded into a QIAshredder™ (Qiagen, Hilden, Germany) spin column and centrifuged for 2 min at 14.000 rpm to remove all precipitates and cell debris. The flow-through fraction was then mixed with

buffer AP3, which contained ethanol, to create conditions for optimal binding to the DNeasy mini-spin column. An aliquot (0,65 ml) of the mixture was applied to the Dneasy mini-spin column and centrifuged at 8.000 rpm for 1 min. After one washing step with buffer AW to remove potential PCR inhibitors, DNA was eluted in 0,1-ml of distilled water and was then ready for PCR processing. To ensure a successful extraction, DNA was separated by gel electrophoresis on 1.5% agarose gels (Agarose D-1 low eeo, Eppendorf Italy) with ethidium bromide (1.2%) in TBE buffer (45mM Tris-borate, 1mM EDTA, pH 8.0) for 25 minutes (70 V). Metagenomic DNA was detected spectrophotometrically under UV lightwave.

The ITS1, 5.8s, and ITS2 regions of rDNA were amplified using the primers ITS1 5′-TCC GTA GGT GAA CCT GCG G-3′ and ITS4 5′-TCC TCC GCT TAT TGA TAT GC-3′ (White *et al*., 1990). Polymerase chain reactions (PCR) were performed using a PCR- Beads^{TM} (Amersham Pharmacia Biotech) that included:

- 1,5 U/ml Taq Polymerase;
- 10 mM Tris- HCl;
- 50 mM KCl;
- 1,5 mM $MgCl_2$;
- 200 mM for each dNTP;

to this misture was added 1µl of each primer ITS1 and ITS4 [10 µM], 1µl DNA template [20ng/µl] in 25 µl total reaction volume. Cycling reactions were run on DNA Thermal Cycler (Mastercycler personal, Eppendorf) with the following protocol: 95°C for 2 min; 35 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 1 min, with a final elongation step at 72°C for 7 min after cycling. The PCR amplicons were initially scored for successful amplification on 1.5% agarose gels and were purified using Ultra Clean-Up DNA Purification Kit (MoBio Laboratories, Inc., Solano Beach, CA) according to manufacturer protocols. Sequencing PCRs were produced by Macrogen DNA sequencing service under Big Dyes™ terminator cycling conditions. Consensus sequences were computed from forward and reverse sequences using BioEdit software (BioEdit version 7.0.0) and the identity of the amplicon sequence was determined with the standard nucleotide–nucleotide BLAST (blast n) of the NCBI GeneBanck (http://www.ncbi.nlm.nih.gov/).

3.6 Alignment and phylogenetic analyses

For each identified species one sequence generated for this study and two sequence available from official GeneBank was retained for phylogenetic analysis. Sequences were aligned with the program Clustal X (Thompson *et al*., 1997). Distance analysis was performed using Neighbour-joining method based on K2P distances. A consensus tree was calculated from 1000-fold bootstrapped analyses. Phylogenetic analyses were performed in PHYLIP 3.6 (Felsenstein, 1993).

3.7 Statistical analysis of data

Frequency of species occurrence was calculated as a percentage of the number of samples with the species to the total number of samples tested. The percentage of twig, leaf and bud pieces with single and multiple infections was computed in Microsoft Excel. The colonization frequency (CF) of an individual endophytic taxon was calculated with the formula

CF=Ni/Nt*100

where Ni= number of tissue units from which the fungus were isolated and Nt= total number of units examined. The CF divergences between different plant tissues (twigs, leaves and buds) and between plant samples from different stations (A with North facing and B with South facing) were evaluated by analysis of variance (ANOVA) of percentages.

When the frequency of a species on any tissue type was significantly ($P<0.05$) higher than zero by Fisher's exact probability test, the species was regarded as frequent. The chi-square test was used when comparing the frequency of a species among different bud component tissues: rolled-up leaves, bud scales, and twigs.

A statistical interpretation and characterization of the isolated fungal populations in different bud tissues such as diversity and distribution were analyzed for ecological indices (Maria and Sridhar, 2002) computed with software PAST v. 2.04. in particular were considered: Margalef index (D_{Mg}); Shannon's Diversity Index (H') and Shannon's equitability or evenness index (J).

The differences among tissues were evaluated by analysis of variance (ANOVA) and Tukey test ($p<0.05$) for number taxa and ecological indices and were performed using the GraphPad Prism 4 Istat ® 3.5 (GraphPad Software inc., San Diego, California, USA).

4. Results: objective 1

4.1 Taxon richness at species and order level

3668 isolates from 150 twigs, 450 leaves and 150 buds collected from 10 symptomless beech trees in 5 successive samplings were used for the assessment of species richness. According to molecular analysis morphotype grouping was evaluated by BLAST searches using sequence data from GeneBanck NCBI(Tab. 2).

Tab. 2. Taxa list of beech tissue (twigs, leaves and buds) endophytes in alphabetical order and references of avalaible ITS sequences in NCBI GeneBank using to complete identification of taxon (BLAST searches and ITS phylogeny).

	Taxa	Reference	Code
1	*Alternaria alternata*	This study	FF32F5
2	*Alternaria alternata*	This study	FF3F5
3	*Alternaria alternata*	Wicklow and Poling, 2009	GQ221851*
4	*Alternaria alternata*	Ghosta, 2002	AY154682*
5	*Alternaria* sp.	This study	FF3F9
6	*Apiognomonia errabunda*	This study	RF5G1
7	*Apiognomonia errabunda*	This study	FF3F12
8	*Apiognomonia errabunda*	Sogonov (unpublished)	DQ313530*
9	*Apiognomonia errabunda*	Bahnweg (unpublished)	AJ888477*
10	*Arthrobotrys* sp.	This study	R1R9
11	*Arthrobotrys* sp.	Hagedorn and Scholler, 1999	AF106536*
12	*Arthrobotrys* sp.	Arhipova (unpublished)	GU062287*
13	*Ascochyta fagi*	This study	F2R2
14	*Ascochyta fagi*	Hashizume (unpublished)	AB472195*
15	*Ascochyta fagi*	Hashizume (unpublished)	AB472196*
16	*Aspergillus niger*	This study	F2FF
17	*Aspergillus niger*	Kausar (unpublished)	HM438947*
18	*Aspergillus niger*	Espino del Castello (unpublished)	EU833208*
19	*Aspergillus terreus*	This study	F4F16
20	*Aspergillus terreus*	Peterson, 2008	EF669580*
21	*Aspergillus terreus*	Chang, 2009	FJ842764*
22	*Aspergillus versicolor*	This study	F4F26
23	*Aspergillus versicolor*	Li (unpublished)	EU497952*
24	*Aspergillus versicolor*	Espino del Castello (unpublished)	EU833210*
25	*Aureobasidium pullulans*	This study	F5G1
26	*Aureobasidium pullulans*	Manitchotpisit, 2009	EU719541*
27	*Aureobasidium pullulans*	Manitchotpisit, 2009	EU719545*
28	*Biscogniauxia mediterranea*	This study	RF3R6
29	*Biscogniauxia mediterranea*	This study	RF3R10
30	*Biscogniauxia mediterranea*	Sanchez Marquez (unpublished)	FN394711*
31	*Biscogniauxia mediterranea*	Collado *et al.* , 2001	AF326482*
32	*Biscogniauxia nummularia*	This study	FF3F4
33	*Biscogniauxia nummularia*	Volkenant (unpublished)	EF155488*
34	*Biscogniauxia nummularia*	Pinto-Sherer, 1999	AF201706*
35	*Chaetomium globosum*	This study	RF3R18
36	*Chaetomium globosum*	Zhou (unpublished)	EU301639*
37	*Chaetomium globosum*	Tian, 2009	AB470915*

(*)= Accession number of GeneBank source sequence

Tab. 2. (continued)

	Taxa	Reference	Code
38	*Cladosporium cladosporioides*	This study	RF3R17
39	*Cladosporium cladosporioides*	Braun *et al.* , 2003	AY251074*
40	*Cladosporium cladosporioides*	Arteau, 2010	GQ458030*
41	*Cylindrocarpon faginatum*	This study	R41
42	*Cylindrocarpon faginatum*	Hallen *et al.* , 2004	AY677277*
43	*Diaporthe phaseolorum*	This study	FF3F15
44	*Diaporthe phaseolorum*	Restrepo (unpublished)	EU436686*
45	*Diaporthe phaseolorum*	Lopera (unpublished)	EU821481*
46	*Diaporthe viticola*	This study	FF3F14
47	*Diaporthe viticola*	This study	FF3F13
48	*Diaporthe viticola*	Sanchez Marquez (unpublished)	FN386282*
49	*Diaporthe viticola*	Bakys *et al.* , 2009	FJ228188*
50	*Epicoccum nigrum*	Zhou (unpublished)	HM776421*
51	*Epicoccum nigrum*	This study	R7
52	*Epicoccum nigrum*	Gorfer (unpublished)	HQ115657*
53	*Fusarium lateritium*	Wu (unpublished)	HM061323*
54	*Fusarium lateritium*	This study	M1RF
55	*Fusarium lateritium*	Santori *et al.* , 2010	FN547420*
56	*Fusarium oxysporum*	Zheng (unpublished)	EU364842*
57	*Fusarium oxysporum*	This study	RF5R3
58	*Fusarium oxysporum*	Soca-Chafre (unpublished)	EU715659*
59	*Lecythophora hoffmannii*	Vasiliauskas *et al.* , 2005	AY781227*
60	*Lecythophora hoffmannii*	This study	R14R
61	*Lecythophora hoffmannii*	Menkis *et al.* , 2004	AY805566*
62	*Massarina corticola*	This study	RF3R3
63	*Massarina corticola*	Liew *et al.* , 2002	AF383957*
64	*Neonectria* sp. 1	This study	RF3R5
65	*Neonectria* sp. 2	This study	RF3R4
66	*Penicillium commune*	This study	F4R5
67	*Penicillium commune*	Huang (unpublished)	GU183158*
68	*Penicillium commune*	Huang (unpublished)	GU183165*
69	*Phoma cava*	This study	FF3 2F8
70	*Phoma glomerata*	This study	F5GF1
71	*Phoma glomerata*	Lindqvist-Kreuze (unpublished)	AJ428533*
72	*Phoma* sp.	Bitter (unpublisher)	HQ130716*
73	*Phomopsis* sp.	This study	FF3F13
74	*Phomopsis* sp.	Kacergius (unpublished)	EU571102*
75	*Phomopsis* sp.	Qi *et al.* , 2009	FJ176469*
76	*Pleospora herbarum*	This study	FF3F3
77	*Pleospora herbarum*	Saito, 1999	AB026164*
78	*Pleospora herbarum*	Saito, 1999	AB026165*
79	*Pyrenochaeta cava*	Merhautova (unpublished)	FJ379832*
80	*Pyrenochaeta cava*	Gorfer (unpublished)	HQ115698*
81	*Sordaria fimicola*	This study	R1R19
82	*Sordaria fimicola*	Rotella, 2010	FN868475*
83	*Sordariomycetes* sp.	This study	RF3R14
84	*Sordariomycetes* sp.	Hoffman and Arnold, 2010	GQ153239*
85	*Sordariomycetes* sp.	Arnold (unpublished)	GQ153034*
86	*Verticillium* sp.	This study	R2R2
87	*Verticillium* sp.	Li (unpublished)	HM346536*
88	*Verticillium* sp.	Hoffman and Arnold, 2010	GU062214*

(*)= Accession number of GeneBank source sequence

All taxa were identified by a combination of morphological and DNA-based analysis at least at order level (Tab. 3).

Tab. 3. List of total taxa identified in alphabetical order and taxonomic setting.

	Endophyte taxon	Family	Order	Class	Phylum
1	*Alternaria alternata*	Pleosporaceae	Pleosporales	Dothideomycetes I	Ascomycota
2	*Alternaria* sp.	Pleosporaceae	Pleosporales	Dothideomycetes I	Ascomycota
3	*Apiognomonia errabunda*	Gnomoniaceae	Diaporthales	Sordariomycetes	Ascomycota
4	*Arthrobotrys* sp.	*Incertae sedis*	*Incertae sedis*	*Incertae sedis*	Deuteromycota
5	*Ascochyta fagi*	Pleosporaceae	Pleosporales	Dothideomycetes I	Ascomycota
6	*Aspergillus niger*	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota
7	*Aspergillus terreus*	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota
8	*Aspergillus versicolor*	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota
9	*Aureobasidium pullulans*	Dothioraceae	Dothideales	Dothideomycetes II	Ascomycota
10	*Biscogniauxia mediterranea*	Xylariaceae	Xylariales	Sordariomycetes	Ascomycota
11	*Biscogniauxia nummularia*	Xylariaceae	Xylariales	Sordariomycetes	Ascomycota
12	*Chaetomium globosum*	Chaetomiaceae	Sordariales	Sordariomycetes	Ascomycota
13	*Cladosporium cladosporioides*	Davidiellaceae	Capnodiales	Dothideomycetes II	Ascomycota
14	*Cylindrocarpon faginatum*	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota
15	*Diaporthe phaseolorum*	Diaporthaceae	Diaporthales	Sordariomycetes	Ascomycota
16	*Diaporthe viticola*	Diaporthaceae	Diaporthales	Sordariomycetes	Ascomycota
17	*Epicoccum nigrum*	Pleosporaceae	Pleosporales	Dothideomycetes I	Ascomycota
18	*Fusarium lateritium*	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota
19	*Fusarium oxysporum*	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota
20	*Lecythophora hoffmannii*	Sordariaceae	Sordariales	Sordariomycetes	Ascomycota
21	*Massarina corticola*	Massarinaceae	Pleosporales	Dothideomycetes I	Ascomycota
22	*Neonectria* sp. 1	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota
23	*Neonectria* sp. 2	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota
24	*Penicillium commune*	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota
25	*Phoma cava*	Pleosporaceae	Pleosporales	Dothideomycetes I	Ascomycota
26	*Phoma glomerata*	Pleosporaceae	Pleosporales	Dothideomycetes I	Ascomycota
27	*Phomopsis* sp.	Diaporthaceae	Diaporthales	Sordariomycetes	Ascomycota
28	*Pleospora herbarum*	Pleosporaceae	Pleosporales	Dothideomycetes I	Ascomycota
29	*Sordaria fimicola*	Sordariaceae	Sordariales	Sordariomycetes	Ascomycota
30	*Sordariomycetes* sp.	Sordariaceae	Sordariales	Sordariomycetes	Ascomycota
31	*Verticillium* sp.	*Incertae sedis*	Hypocreales	Sordariomycetes	Ascomycota

In total 31 taxa from 6 orders were recognised with high total isolation frequency of 93,7%. The list reported in table 4 shows number of total isolates collected from twigs, leaves and buds for each taxon identified. The taxa list includes 6 doubletons and 7 singletons (Tab. 4).

Preliminary results showed that the most abundant endophytes in twigs of *F. sylvatica* collected in 5 samplings were *Biscogniauxia mediterranea* (37.4%) and *B. nummularia* (26.1%), both from Xylariales order, whereas in leaves, sampling in 3 periods, were *Apiognomonia errabunda* (31.9%) (Diaporthales) and again *B. mediterranea* (26.6%). The most frequency endophte in buds, only picked during last

sapling, was again *B. mediterranea* (27.4%) followed by *Fusarium oxysporum* (21.8%) (Hypocreales), *A. errabunda* (14.2%) and *Alternaria* sp. (13.4%) (Pleosporales).

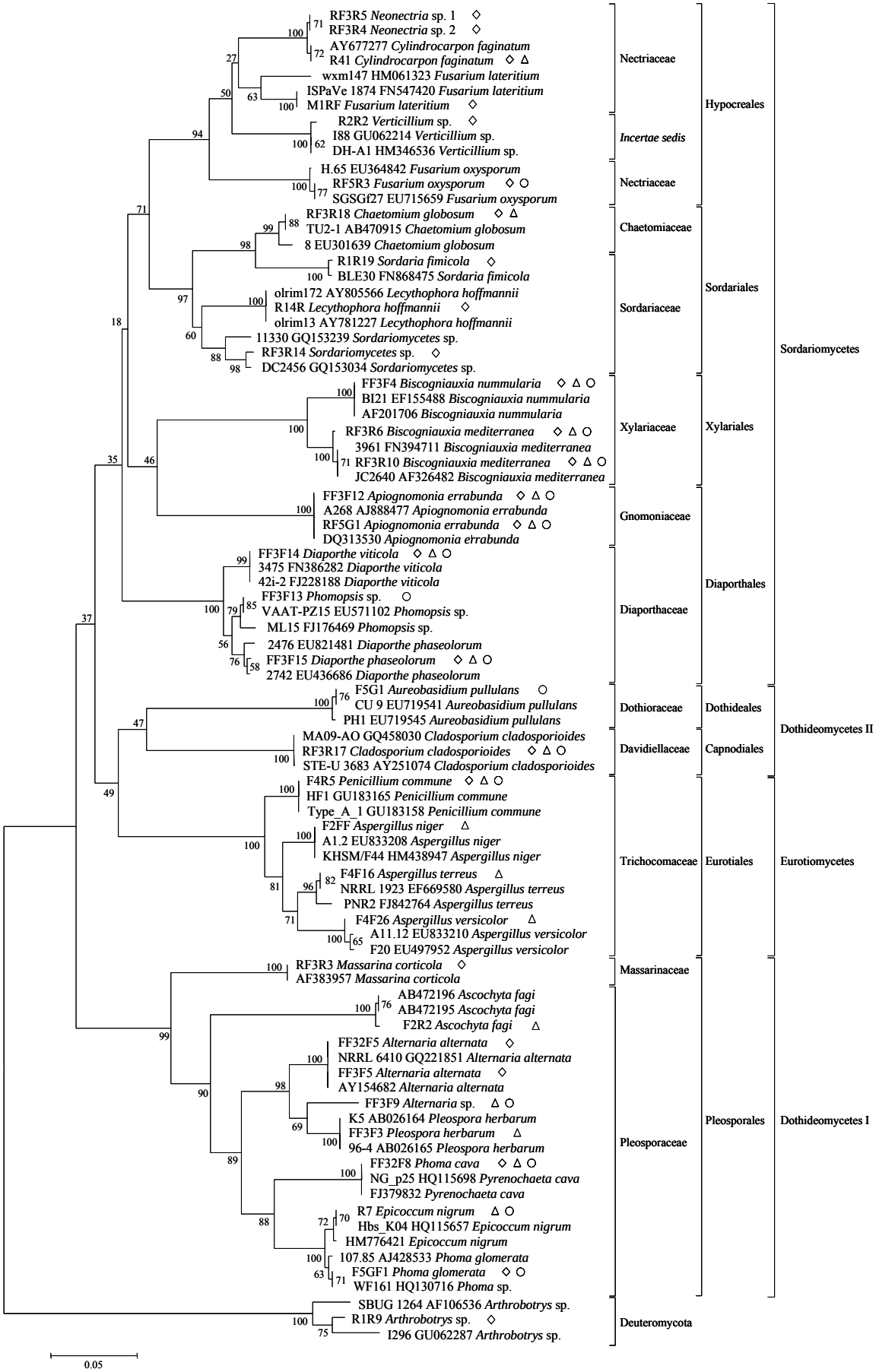
Tab. 4. Species abundance information of different tissue pieces isolated including doubletons and singletons.

	Endophyte taxon	N. twigs isolates	N. leaves isolates	N. buds isolates
1	*Alternaria alternata*	10	0	0
2	*Alternaria* sp.	0	143	164
3	*Apiognomonia errabunda*	10	546	174
4	*Arthrobotrys* sp.	2	0	0
5	*Ascochyta fagi*	0	24	0
6	*Aspergillus niger*	0	2	0
7	*Aspergillus terreus*	0	2	0
8	*Aspergillus versicolor*	0	1	0
9	*Aureobasidium pullulans*	0	0	16
10	*Biscogniauxia mediterranea*	272	456	336
11	*Biscogniauxia nummularia*	190	139	44
12	*Chaetomium globosum*	6	1	0
13	*Cladosporium cladosporioides*	41	85	2
14	*Cylindrocarpon faginatum*	5	6	0
15	*Diaporthe phaseolorum*	72	173	68
16	*Diaporthe viticola*	12	33	64
17	*Epicoccum nigrum*	0	29	58
18	*Fusarium lateritium*	36	0	0
19	*Fusarium oxysporum*	1	0	268
20	*Massarina corticola*	2	0	0
21	*Neonectria* sp1	1	0	0
22	*Neonectria* sp2	1	0	0
23	*Penicillium commune*	2	8	4
24	*Lecythophora hoffmannii*	3	0	0
25	*Phoma cava*	13	14	6
26	*Phoma glomerata*	1	0	4
27	*Phomopsis* sp.	0	0	20
28	*Pleospora herbarum*	0	50	0
29	*Sordaria fimicola*	42	0	0
30	*Sordariomycetes* sp.	1	0	0
31	*Verticillium* sp.	5	0	0
	Total isolates	728	1712	1228

Identification of taxa was completed by ITS phylogeny, pairwise distances of ITS1 and ITS2 (Tab. 2). The phylogenetic relationship of foliar beech endophytes is shown in Fig 14 as consensus tree of the Bayesian analysis. The corresponding trees for maximum likelihood and maximum parsimony resulted in congruent topologies and are therefore not shown, but, statistical branch support is shown (Fig 14). Results of phylogenetic analyses of taxa sequences showed representative endophytes from each host tissue and representative Ascomycota until for each class level. Many endophytes recovered in this study were reconstructed as part of larger clades of endophytic fungi from diverse beech tissues: Diaporthales, Eurotiales, Hypocreales, Pleosporales,

Sordariales are shown as diverse endophytic lineages, whereas Xylariales, Dothideales and Capnodiales are represented as comparatively species-poor clades.

Fig 14 (Next page). Phylogenetic relationships of beech endophytes rooted with the endophytic Deuteromycota. With the exception of the deuteromycete endophyte, all taxa belong to the Pezizomycotina (Ascomycota). The phylogeny is based on 90% ITS rDNA sequence similarity. Numbers above branches indicate branch support. Simbols after endophyte taxa name indicate native plant tissue: rhomb indicates woody tissue, triangle indicates foliar tissue and circle indicates bud tissue. All named taxa, endophytic fungi from other studies and GenBank accession numbers are shown in table 2.


RF3R5 Neonectria sp. 1
RF3R4 Neonectria sp. 2
AY677277 Cylindrocarpon faginatum
R41 Cylindrocarpon faginatum
wxm147 HM061323 Fusarium lateritium
ISPaVe 1874 FN547420 Fusarium lateritium
M1RF Fusarium lateritium
R2R2 Verticillium sp.
I88 GU062214 Verticillium sp.
DH-A1 HM346536 Verticillium sp.
H.65 EU364842 Fusarium oxysporum
RF5R3 Fusarium oxysporum
SGSGf27 EU715659 Fusarium oxysporum
RF3R18 Chaetomium globosum
TU2-1 AB470915 Chaetomium globosum
8 EU301639 Chaetomium globosum
R1R19 Sordaria fimicola
BLE30 FN868475 Sordaria fimicola
olrim172 AY805566 Lecythophora hoffmannii
R14R Lecythophora hoffmannii
olrim13 AY781227 Lecythophora hoffmannii
11330 GQ153239 Sordariomycetes sp.
RF3R14 Sordariomycetes sp.
DC2456 GQ153034 Sordariomycetes sp.
FF3F4 Biscogniauxia nummularia
BI21 EF155488 Biscogniauxia nummularia
AF201706 Biscogniauxia nummularia
RF3R6 Biscogniauxia mediterranea
3961 FN394711 Biscogniauxia mediterranea
RF3R10 Biscogniauxia mediterranea
JC2640 AF326482 Biscogniauxia mediterranea
FF3F12 Apiognomonia errabunda
A268 AJ888477 Apiognomonia errabunda
RF5G1 Apiognomonia errabunda
DQ313530 Apiognomonia errabunda
FF3F14 Diaporthe viticola
3475 FN386282 Diaporthe viticola
42i-2 FJ228188 Diaporthe viticola
FF3F13 Phomopsis sp.
VAAT-PZ15 EU571102 Phomopsis sp.
ML15 FJ176469 Phomopsis sp.
2476 EU821481 Diaporthe phaseolorum
FF3F15 Diaporthe phaseolorum
2742 EU436686 Diaporthe phaseolorum
F5G1 Aureobasidium pullulans
CU 9 EU719541 Aureobasidium pullulans
PH1 EU719545 Aureobasidium pullulans
MA09-AO GQ458030 Cladosporium cladosporioides
RF3R17 Cladosporium cladosporioides
STE-U 3683 AY251074 Cladosporium cladosporioides
F4R5 Penicillium commune
HF1 GU183165 Penicillium commune
Type_A_1 GU183158 Penicillium commune
F2FF Aspergillus niger
A1.2 EU833208 Aspergillus niger
KHSM/F44 HM438947 Aspergillus niger
F4F16 Aspergillus terreus
NRRL 1923 EF669580 Aspergillus terreus
PNR2 FJ842764 Aspergillus terreus
F4F26 Aspergillus versicolor
A11.12 EU833210 Aspergillus versicolor
F20 EU497952 Aspergillus versicolor
RF3R3 Massarina corticola
AF383957 Massarina corticola
AB472196 Ascochyta fagi
AB472195 Ascochyta fagi
F2R2 Ascochyta fagi
FF32F5 Alternaria alternata
NRRL 6410 GQ221851 Alternaria alternata
FF3F5 Alternaria alternata
AY154682 Alternaria alternata
FF3F9 Alternaria sp.
K5 AB026164 Pleospora herbarum
FF3F3 Pleospora herbarum
96-4 AB026165 Pleospora herbarum
FF32F8 Phoma cava
NG_p25 HQ115698 Pyrenochaeta cava
FJ379832 Pyrenochaeta cava
R7 Epicoccum nigrum
Hbs_K04 HQ115657 Epicoccum nigrum
HM776421 Epicoccum nigrum
107.85 AJ428533 Phoma glomerata
F5GF1 Phoma glomerata
WF161 HQ130716 Phoma sp.
SBUG 1264 AF106536 Arthrobotrys sp.
R1R9 Arthrobotrys sp.
I296 GU062287 Arthrobotrys sp.
0.05
Nectriaceae
Incertae sedis
Nectriaceae
Chaetomiaceae
Sordariaceae
Xylariaceae
Gnomoniaceae
Diaporthaceae
Dothioraceae
Davidiellaceae
Trichocomaceae
Massarinaceae
Pleosporaceae
Deuteromycota
Hypocreales
Sordariales
Xylariales
Diaporthales
Dothideales
Capnodiales
Eurotiales
Pleosporales
Sordariomycetes
Dothideomycetes II
Eurotiomycetes
Dothideomycetes I

4.2 Isolation frequencies of endophytic fungi from terminal buds

613 isolates with 85% of isolation frequency were detected from 721 bud fragments (scales, rolled-up leaves, contiguous and distal twig pieces) in one sampling (January 2009) and were used for the assessment of species richness. In total 14 taxa were recognised. All taxa were identified by a combination of morphological and DNA-based analysis (Tab. 5.).

Tab. 5. Number of endophyte taxa isolated from different component tissues of beech buds.

Endophyte taxa	Bud scales	Contiguous twig pieces	Distal twig pieces	Rolled-up leaves
1 *Alternaria* sp.	80	36	34	14
2 *Apiognomonia errabunda*	54	46	66	8
3 *Aureobasidium pullulans*	2	2	10	2
4 *Biscogniauxia mediterranea*	66	114	108	48
5 *Biscogniauxia nummularia*	6	20	18	0
6 *Cladosporium cladosporioides*	0	0	2	0
7 *Diaporthe phaseolorum*	22	26	18	2
8 *Diaporthe viticola*	18	23	23	5
9 *Epicoccum nigrum*	18	14	20	6
10 *Fusarium oxysporum*	76	48	70	74
11 *Penicillium communis*	2	0	0	2
12 *Phoma cava*	6	0	0	0
13 *Phoma glomerata*	0	4	0	0
14 *Phomopsis* sp.	9	7	4	0

Endophytes were recovered at higher frequencies from bud scales and contiguous twig pieces to the buds (Table 5). *B. mediterranea* (27.4%), *F. oxysporum* (21.8%), *A. errabunda* (14.2%) and *Alternaria* sp. (13.4%) were detected with higer frequencies and on any parts of the overwintered terminal buds before bud burst. Even though with low frequency (3.6%) *B. nummularia* was also detected in beech bud tissues except for rolled leaves tissues.

Ecological indices showed no significant differences in richness and specific distribution between component bud tissues, however confirmed higher number of taxa in bud scales and twigs compared to rolled leaves (Fisher's exact test) $P<0,001$, and differences in species diversity (Fisher's exact test) $P<0,05$ (Fig. 15).

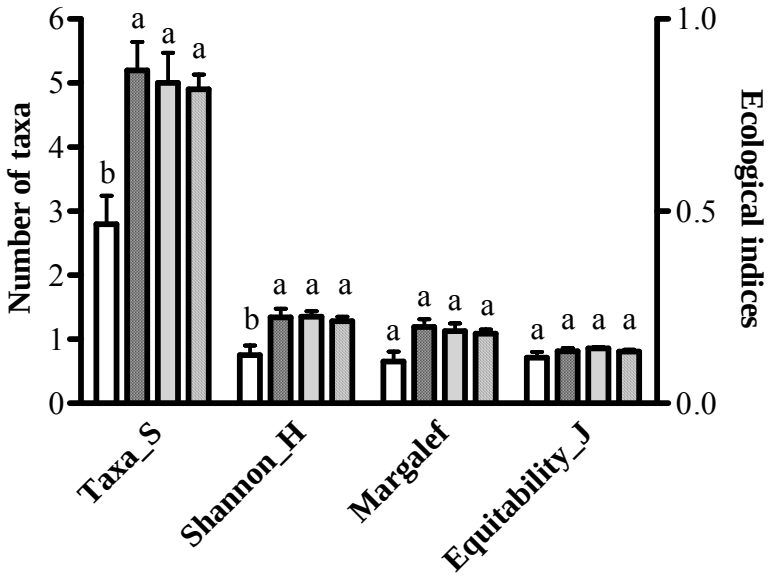


Fig. 15. Taxa number and ecological indices of endophyte assemblages with regard to different beech bud component tissues. =rolled-up leaves; =scales; =distal twigs; =contiguos twigs.

5. Discussion: objective 1

5.1 Fungal endophyte community composition

Endophytic fungi are an important group of symbionts in the tissues of many plant species with which they interact in many different ways. They can be dormant saprophytes, latent pathogens or mutualistic symbionts, and their relationship with the host may change during their life cycle (Stone *et al.*, 2000; Wilson, 2000; Sieber, 2002). Endophytes can produce toxins against insects, growth substances of use to the plant and can occupy the microhabitat of some pathogenic fungi (Carroll, 1986; Miller, 1986; Petrini, 1986; Clay, 1989; 1991). Some fungi that are potentially pathogenic can spend part of their life cycle as neutral endophytes, giving rise to symptoms only when ecological and physiological conditions of the host are modified, as for example in response to environmental stresses. The continuum colonisation patterns of endophytes within their host plants spans and includes fungi that are specialized to occupy every niche within the host.

This study has been produced a monitoring of *F. sylvatica* fungal endophyte community composition in area included into the Mediterranean Basin. To my knowledge, there have been only few studies about specific assessment of cultivated fungal endophyte biodiversity in healthy European beech forests (Sieber and Hugentobler, 1987; Toti *et al.*, 1993; Danti *et al.*, 2002; Unterseher and Schnittler 2009).

Overall, in this study, 31 different taxa of fungal endophytes were isolated in five samplings carried out on beech twigs, leaves and buds from a site in the Central Apennines (Viterbo Province). Danti *et al.* (2002) and Sieber and Hugentobler (1987), using the same method of sterilization, isolated from beech twigs 44 different species from a site in the Tuscan-Emilian Apennines (Pistoia Province), and 20 different species from a site in the Jura mountains (Switzerland, alt. 675 m) respectively. Toti *et al.* (1993) obtained 26 species by examining bud and the bud-carrying twig segments from various sites in the region of Zurich and Ticino (Switzerland). Unterseher and Schnittler (2009) obtained 35 total taxa using two different cultivation techniques of foliar samples collected near Greifswald (Germany).

It seems clear, that geographic origin, tissue age and size, in addition to a different method of surface sterilization and culturing, may have contributed to the observed differences between this study and the other ones. Kowalski and Kehr (1996), studying

endophyte communities of several European tree species, examined the basal parts of beech branches with very high stem runoff and moisture and were able to isolate 67 different endophyte species. In the present study, twigs were mainly collected from the periphery of the crowns where the microclimatic conditions are drier than in the centre and, thus, less suitable for spore germination and infection of many fungal species. Further, the use of other incubation methods such as drying regimes (Chapela and Boddy, 1989; Bissegger and Sieber, 1994) or of various selective media (Bills, 1996) might have revealed additional species.

However, the observed species composition with total isolation frequency about 94%, are well consistent with the data obtained by Danti *et al.* (2002), with 98% of isolation frequency.

Among the dominant fungi, *Biscogniauxia mediterranea*, well known as the causative agent of charcoal canker in cork oak (Anselmi *et al.*, 2000; 2004; Mazzaglia *et al.*, 2001; Desprez-Loustau *et al.*, 2005; Vannini *et al.*, 2009), in this study was reported for the first time on *F. sylvatica*, broadly presence in twigs (37%), leaves (27%) and also buds (27%). Another xylariaceous fungus very abundant in all tissue samples was *B nummularia*. In this study this fungus was reported for the first time on bud tissues of *F. sylvatica*. This species is considered a weak pathogen highly associated with stem material (Granata and Whalley, 1994; Hendry *et al.*, 2002; Capretti *et al.*, 2003; Nugent *et al.*, 2005; Luchi *et al.*, 2006) and switch to pathogenic phase, causing strip-canker and wood decay, in trees that suffer severe water stress during the growing season (Hendry *et al.*, 1998). Studies about the role of xylariaceous fungi in endophytic symbioses speculate that this species are simply waiting for their host to senescence, at which time they can begin decomposition of cell wall materials (Petrini *et al.*, 1995; Whalley, 1996). Until now, in studied beech forest, result presence data of this species, seem to confirm its mutualistic behaviour. Indeed, how outlined by Carroll (1988), ubiquity and geographic spread, absence of any minimal disease symptoms, vertical and efficient horizontal transmission, growth throughout host tissues, or with high proportion into a particular organ are all characteristics that can prove current endophytic state of this species in my forest.

Further, *Apiognomonia errabunda* (anamorph *Discula umbrinella*), which dominate in the leaves of many deciduous, broadleaved trees, was among the five most abundant fungi collected mainly in beech leaves and buds but also in twigs in this study. It is perhaps the most intensively studied beech endophyte (Danti *et al.*, 2002). Its

morphological, ecological and genetic features as well as its infection and colonization mechanisms of *F. sylvatica* were analysed in various studies (Sieber and Hugentobler, 1987; Morelet, 1989; Hämmerli *et al.*, 1992; Toti, 1993; Viret, Scheidegger and Petrini, 1993; Kowalski and Kehr, 1996; Danti *et al.*, 2002). This endophyte can exhibits two lifestyles: mutualism under some circumstances and parasitism under others. The endophytic thalli of these endophyte resumes growth in response to an external stimulus, e.g. oviposition of gall-forming or leaf-mining insects or infection by a pathogenic fungus. Necroses develop in the leaf areas where infection or oviposition occurred, eliminating the food base of the pathogen or herbivore insect and hereby reducing the population of the antagonist. For example, oviposition of the gallmidge *Mikiola fagi* close to endophytic thalli of *A. errabunda* in beech leaves elicits such a reaction (Pehl and Butin, 1994). In other cases, the endophytic thalli resumes growth for, as yet, unknown reasons and develops the diseases known as anthracnose. However, in general, anthracnose of this fungus often occurs on the leaves of one branch but is absent on other branches of the same tree. Perhaps, *A. errabunda* is genetically diverse and forms a complex of morphologically identical cryptic species (Fisher *et al.*, 2002; Grünig *et al.*, 2007) some of which may be pathogenic and others non-pathogenic. Indeed, up to four different genotypes were detected in the same beech leaf by Hämmerli *et al.* (1992). Thus this study confirms the role of *A. errabunda* as an endophyte stably associated with healthy beech trees.

In contrast with early studies, *Alternaria alternata* and *Cladosporium cladosporioides* were collected with low relative abundances. These fungi are considered on *Fagus* genus 'common primary saprophytes' (Osono and Takeda, 1999). According to Danti *et al.* (2002), it is likely that the endophytic habitus provides them with an advantage in saprotrophic colonization of twigs after their death. Compared with purely saprotrophic fungi, litter fungi with an endophytic phase may have an advantage because they are inside the host tissues where they usually do not have to compete with other microorganisms and from where they can start to decompose the hosts tissues without delay as soon as the tissue age and die.

It is important to point out high frequency of *Diaphorte* sp. (anamorpf *Phomopsis*), potential pathogen causing canker lesions, that was frequent reported as common endophytes of branches of forest trees (Chapela and Boddy, 1988; Kowalski and Kehr, 1996; Danti *et al.*, 2002; Kaneko and Kaneko, 2004; Murali *et al.*, 2007).

Otherwise, *Neonectria* sp. and his anamorpf *Cylndrocarpon* sp., which are endemic pathogens with high virulence, causing cankers on tree species of Fagales, were rarely and alternatively detected as endophytes from *F. sylvatica* in this study, consistently with other surveys in Europe (Danti *et al.*, 2002; Dorworth *et al.*, 1996; Sieber *et al.*, 1991a). Thus, a high frequency of colonization of the internal of healthy plant tissues is a clear indication of low virulence of a fungus (Sieber, 2007).

Likewise, *Ascochyta fagi*, dominant in other studies and known as a yellow leaf-spot pathogen of different Fagaceae like Japanese beech (*Fagus crenata*) or *Quercus crispula* (Wei and Harada, 1998; Kaneko and Kaneko, 2004), in this study was sporadically detected. Thus, this isolates might represent a latent pathogen.

Subtle physiological and biochemical host-specific mechanisms are known to be involved in recognition and establishment of endophytic symbioses (Chapela *et al.*, 1991; 1993; Toti *et al.*, 1992; Toti, 1993; Viret *et al.*, 1993). Endophytic infections may be limited to few or even a single cell (Stone, 1987), but it is difficult to determine the factors involved that keep fungi, among them potential parasites, at bay. Preformed or elicited phenolic compounds were considered good candidates as protectants against fungal attacks (Bahnweg *et al*., 2005)

As reported by many authors (Chapela and Boddy, 1988; Kowalski and Kehr, 1996; Danti *et al*., 2002; Kaneko and Kaneko, 2004; Murali *et al.*, 2007), some other common fungal endophytes characterize community composition of my beech forest. Even if seem to be less dominant compared to early studies, were found *Aspergillus* spp., *Epicoccum nigrum*, *Phoma cava* and *Pennicillium commune*. In the present study, the diversity of endophytes increased marginally during the wet season. Indeed, wet conditions are favourable for sporulation and infection and causes this slight increase due to the appearance of some fungi commonly isolated as endophytes and no truly unique of *F. sylvatica* (Murali *et al*., 2007).

5.2 Detection of fungal endophytes in buds

Analysis of endophyte community composition and structure reveled a great amount of fungal endophyte isolates from foliar tissues of sampling carried out in February 2008, with number of isolates much higher than other sampling periods. Thus, inspection of climatic conditions and factors affecting the months befor winter sampling (data not showed), prove that precipitation and temperature levels determined composition and frequency of endophyte assemblages. Indeed, according to

meteorological data of weather station of Caprarola (Viterbo Province), was recorded no rainfall (0mm) and average of maximum monthly temperatures above seasonal average (10°/12°C), during Dicember 2007 and January 2008. Probably, these weather conditions determined the physiological drop of trees in studied forest with subsequent increasing of endophytic infections. In this conditions, beech buds can able to work as a possible way of new infections.

Proceeding from this hypothesis, was checked the systemic growth of weak pathogen *B. nummularia* into the leaf tissues and current-year twigs from dormant beech buds, and was assessed the existence of infection by airborne inocula, how was already suggested for *A. errabunda* on *F. sylvatica* (Toti *et al*., 1993) and for *A. fagi* on *F. crenata* (Kaneko and Kaneko, 2004).

In contrast with Toti *et al.* (1993), this study has demostrated for the first time the systemic growth into immature leaves inside the buds of *B. nummularia* over many other endophytic fungi, among which *A. errabunda*. Further, the contiguous part of the twigs is colonized more than the distal one. This suggests that *B. nummularia* and other latent invasors are able to colonize not only leaves but also young twig by mycelia present latently in the buds. It's important point out that vertical and horizontal transmission of *B. nummularia* by airborne spores is effective (Davis *et al.*, 2003), but Moriondo *et al.* (1999) have proposed infection of aerial plant tissues by jewel beetle *Agrilus italicus* as possible colonization mechanism. This finding is consistent with the results of Bayman *et al.* (1998), who reported horizontal transmission was more important than vertical transmission of xylariaceous endophytes of trees.

Further, note well in this study, the high presence in beech buds of *Fusarium oxysporum.* Toti *et al.* (1993) has reported low frequency of *Fusarium* spp.. Whereas is known non-pathogenic endophytic *F. oxysporum* as biocontrol agent of soilborne pathogens, and its effect on concentration increasing of phenolic metabolites, which are considered to be involved in the plant defense reaction (Schulz *et al.*, 1999), until now, as far as I know, remain undefinined the endophyte-host interaction mechanisms existing between this fungus and *F. sylvatica* trees.

5.3 Phylogenetic diversity of beech endophytes

An attempt was made to define taxa at about the level of species, but that was no possible for 7 taxa only identified at level of genus. The initial morpho-taxa groupings were involved on the basis of their morphological traits. Subsequently morpho-taxa

groupings tested using ITS sequences. In most cases, the original morpho-taxa were well supported, but initial groupings were sometimes modified on the basis of the sequencing results. A few of the original morpho-taxa were combined.

According to the present ITS phylogenetic analysis assessed here, the community of cultivable endophytes on beech is composed of the main nonparasitic and non-lichenised Pezizomycotina lineages (Fig 14, all taxa except for the outgroup). The cladogram reflects current knowledge of systematic and taxonomic relationships among the Ascomycota (Blackwell *et al.*, 2006; Hibbett *et al.*, 2007). Orders are clearly resolved as are the classes Sordariomycetes and Eurotiomycetes. The sordariomycete subclasse Hypocreomycetidae also received high branch support. The general weak backbone support is due to the small amount of sequence information of the conserved 5.8S rDNA. The multi-copy ITS region of nuclear ribosomal DNA is mostly used to infer phylogenetic relationships among closely related taxa of various organism groups (Unterseher and Schnittler, 2010). Based on the present study, ITS sequence analyses of Ascomycota seem to have sufficient resolution to accurately evaluate fungal diversity to the family level.

6. Conclusion: objective 1

Differences regarding species composition of the endophyte assemblages and frequencies of species exist between this study and the other ones carried out on endophytes of beech from Central Europe. Early studies reported that other local factors may greatly influence the degree of colonization by endophytes, such as edaphic characteristics (Sieber-Canavesi and Dorworth, 1990; Espinosa-Garcia, Rollinger and Langenheim, 1996; Sieber, 2007), diversity of plant community and amount of fungal inoculum (Kowalski and Kehr, 1996), or still, anthropogenic influences like acid rain or gaseous emissions of air pollutants (Helander *et al.*, 1993, Barengo *et al.*, 2000, Danti *et al.*, 2002).

The broad phylogenetic diversity of *F. sylvatica* endophytes suggests different independent origins of endophytism in different tissues of trees. Some studies have shown how taxonomic affinities of endophyte assemblages reflect relationships with host (Carroll and Carroll, 1978; Petrini and Carroll, 1981). This study confirm the phylogenetic similarity of endophyte assemblages that colonize the same tissue. Endophytic lineages of *Apiognomonia* and *Diaporthe* (Diaporthales), *Cladosporium* (Capnodiales), *Fusarium* and *Neonectria* (Hypocreales) or *Biscogniauxia* (Xylariaceae) may have switched from plant pathogenic life style to a type of symbiosis that can be described as induced mutualism (Carroll, 1995). Upon an external stimulus such as water stress or folivore attacks, these species may become plant-parasitic or -pathogenic for a short period of time leading to localised strong hyphal growth and/or necrosis of host tissues (Bayman, 2006). As a consequence, folivorous, gall-forming and mining insects might be affected (Wilson, 1995; Wilson and Carroll, 1997) and plant infections with microbial pathogens might be limited (Andrews, 2006). Further, fungal species with remarkably adaptive life strategies, e.g., the phylloplane yeasts *Aureobasidium pullulans*, sometimes forms two barriers against pathogen attack: as epiphyllous colonizer with demonstrable biocontrol efficacy (Allen *et al.*, 2004) and as endophyte, that destroys or reduces those microbial pathogens that broke the first barrier (Andrews, 2006). Yet another strategy could explain the presence of ubiquitous microfungi as endophytes in different tissues over time: the fungi entered the wrong host or substratum, in which they could not proliferate and went to dormancy until released upon the appropriate substratum, within the appropriate micro-habitat (Carroll, 2006) or the appropriate isolation method. This could be the case for wood-inhabiting taxa of the

Sordariales and Xylariales clades. Finally, many fungi, including forest trees endophytic taxa are known to be pleomorphic if environmental conditions change (Seifert and Samuels, 2000). The resulting enormous ecological plasticity, was surely needed to invade plant tissues in Mediterrenean basin forests and to establish successfully in the corresponding habitat. On the other hand, it also gave the species have the potential to switch between habitats without the need of evolving too tight substratum preferences. One example is again *A. pullulans*. The species is able to grow as a yeast (e.g., in wet environments) and as a filamentous fungus on various substrata (Andrews, 2006). The evolution of a variety of adaptive ecologies that some authors termed "endophytic continuum" (Saikkonen *et al.*, 1998; Schulz and Boyle, 2005) has led to a diverse and often opportunistically behaving ecological group, that may be as common and important among forest plants as are for exemple the mycorrhizas (Carroll, 1995).

Microbiological and mycological studies largely benefit from advances in molecular biology (Harrington and Wingfield, 1995; Vainio and Hantula, 2000; Adair *et al.*, 2002; Parfitt *et al.*, 2005; Arnold *et al.*, 2007; Hibbett *et al.*, 2007; Guglielmo *et al.*, 2008). Cultivation-independent biodiversity studies are promising to yield a "biodiversity fingerprint" of a sampling site, to detect uncultivable organisms and new clades in the fungal tree of life, or to unveil phylogenetic relations (Johannesson and Stenlid, 1999; Mazzaglia *et al.*, 2001; Pandey *et al.*, 2003; Hunt *et al.*, 2004; Parfitt *et al.*, 2005; 2007; Franzen *et al*., 2006; Arnold *et al.*, 2007; Promputtha *et al.*, 2007; Guglielmo *et al.*, 2008; Buée *et al.*, 2009; Jumpponen and Jones, 2009; Öpik *et al.*, 2009; Unterseherand and Schnittler, 2010) Some molecular approaches in endophytology, however, need improvement. Clearly there is need for caution in assessing the biodiversity of these cryptic organisms based upon single isolation techniques. According to Arnold *et al.* (2007), my own results highlight the complementary of cultivation-based and cultivation-independent isolation techniques. Allen *et al.* (2003) and Arnold *et al.* (2007) compared the effect of cultivation and environmental PCR on the species richness of rhizosphere and phyllosphere endophytes, respectively. Thus, as far as I know, befor this study, there weren't specif works about comparison of cultivation and environmental PCR on the species richness of woody and bud tissue endophytes.

An incorrect assessment of endophytic communities by cultivated-independing methods may be caused by inappropriate protocols of surface sterilisation of tissues, that did not remove all arbitrarily adhering DNA. Biases may have also occurred during environmental PCR due to preferential amplification of specific templates.

Whereas microfungi including endophytes contribute substantially to global biodiversity, the data needed to derive an accurate estimate of microfungal diversity are still insufficient, incomplete and contradictory (Hyde *et al.*, 2007). To increase the number of meaningful comprehensive studies on microfungal diversity, preferably of extreme habitats, and because the separation of cultivable and noncultivable fungi is methodological and not biological, cuttingedge biodiversity studies are urgently needed. They should combine high-throughput cultivation methods (Collado *et al.*, 2007; Unterseher and Schnittler, 2009), high-throughput molecular workflows (Buée *et al.*, 2009; Jumpponen and Jones, 2009; Öpik *et al.*, 2009), barcoding (Seifert, 2008), improved statistical analysis, such as mathematical species richness estimations that consider also the rare species (Colwell and Coddington, 1994; Hughes *et al.*, 2001; Chao *et al.*, 2006; Unterseher *et al.*, 2008, Coddington *et al.*, 2009), and robust phylogenies derived from a sufficient amount of genetic information (Arnold *et al.*, 2007).

7. Materials and methods: objectives 2

7.1 Endophyte fungi in twigs and leaves

As described previously (¶ 3.7), frequency of species occurrence was calculated as a percentage of the number of samples with the species to the total number of samples tested. The percentage of twig and leaf pieces with single and multiple infections was computed in Microsoft Excel. The colonization frequency (CF) of an individual endophytic taxon from twigs and leaves was used for calculating species-accumulation and rarefaction curves. According to the suggestions of Gotelli and Colwell (2001), the taxon richness was plotted as a function of the accumulated number of fungal isolates, not accumulated number of samples, thus the plot of the cumulative number of species collected against a measure of the sampling effort is termed the species accumulation curve. Expected species accumulation curves based on the Mao Tau estimator were computed in PAST v. 2.04 and plotted for individual tree species in each plant tissues separately using Microsoft Excel.

In addiction, a rank- abundance plot was also constructed to represent the structure of twig and leaf endophyte communities. Multivariate techniques were applied using the PAST v. 2.04 and then exponential decay was achieved with GraphPad Prism 4 Istat® 3.5 (GraphPad Software inc., San Diego, California, USA). A rank/abundance curve (or Whittaker plot) can be used to visualize species abundance distributions. In this plot, the number of individuals of each species are sorted in descending order (the most abundant species is given rank 1, the second most abundant is 2 and so on), and the proportion of the total number of individuals for each species is then plotted against the species rank.

The colonization frequency (CF) divergences of an individual endophytic taxon between different plant tissues (twigs and leaves) and between plant samples from different stations (A with North facing and B with South facing) and sampling periods (1th=November 2007, 2nd=February 2008, 3rd=June 2008, 4th=September 2008 and 5th=January 2009) were evaluated by analysis of variance (ANOVA) of percentages.

When the frequency of a species on any tissue type was significantly ($P<0.05$) higher than zero by Fisher's exact probability test, the species was regarded as frequent. Fisher's exact probability test was used when comparing the frequency of a species between twigs and leaves. To evaluate isolation boundary of fungal endophytes in different seasons, isolation data analysis for twig and leaf endophytes in different seasons separately was performed in order to presence/absence of a fungus in a unit and

was coded binarily (1/0) independently of the number of colonized tissues pieces using Microsoft Excel.

A statistical interpretation and characterization of the isolated fungal populations in different tissues (twigs and leaves) such as diversity and distribution were analyzed for ecological indices (Maria and Sridhar, 2002) computed with software PAST v. 2.04. in particular were considered: Margalef index (D_{Mg}); Shannon's Diversity Index (H′) and Shannon's equitability or evenness index (J).

Margalef index (D_{Mg}) was computed to evaluate species richness

$D_{Mg}=(S-1)/\ln N$

Where S= number of different species or groups; N = total numbers of individuals.

Shannon-Weaver index (H′)

$H'= -\sum (p_i \ln p_i)$

Where $p_i = n_i/N$, is proportion of total sample belonging to i^{th} species; n_i= number of individuals belonging the i^{th} species or group; N= total numbers of individuals.

Evenness index (J) can defined as the ratio between the observed species diversity (H′) and the maximum species diversity (Hmax) and was expressed by:

$J= H'/Hmax= H'/\ln S$

J=equitability (range 0–1, max. value when individuals evenly distributed among the species or groups); H′= observed species diversity; Hmax = species diversity under conditions of maximal evenness.

In this study were used non-parametric approaches of data analysis because the endophyte assemblage data were skewed and contained many zero counts, making parametric analysis unsuitable (Anderson, 2001).

Frequency data were square-root transformed to reduce the influence of the most abundant species (Clarke and Warwick, 2001) and Bray–Curtis similarity indices were calculated (Bray and Curtis, 1957). Patterns from the resulting similarity matrix were examined using Nonmetric Multidimensional Scaling (NMDS) ordination in PAST v. 2.04. An analysis of similarities (ANOSIM) was carried out using PAST v. 2.04 to assess the statistical significance of differences between and within hosts and sites, using the same similarity matrix calculated for NMDS. The R value in ANOSIM gives an absolute measure of how separated the groups are, on a scale of 0 (indistinguishable) to 1 (all similarities within groups are less than any similarities between groups) (Clarke and Gorley, 2001). Permutation tests established the statistical significance (P) of the R values.

Variability in the endophyte assemblages at different spatial scales, i.e. among trees, tissues within trees, sites within tissues was measured explicitly using a semi-parametric permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001; McArdle and Anderson, 2001). This approach allows a direct additive partitioning of variation among individual terms using the multivariate analogue of the ANOVA component estimators (e.g. Searle *et al*., 1992). For each season a three factor nested design was applied: trees (ten trees in two sampling sites) tissues (three twigs and fifteen leaves per tree nested within trees) and sampling period (five sampling for collected twigs indicated with 1th-2nd-3rd-4th-5th; and three for leaves indicated with 2nd-3rd-4th). The mean squares derived from PERMANOVA were used to calculate components of multivariate variation following the methods in Anderson *et al*. (2005).
The differences among tissues were evaluated by analysis of variance (ANOVA) and Tukey test ($P<0.05$) for number taxa and ecological indices. Increments of these data during one control year were compared using a two-way ANOVA and Fisher's exact probability test and were performed using the GraphPad Prism 4 Istat ® 3.5 (GraphPad Software inc., San Diego, California, USA).

7. Results: objective 2

7.1 Endophyte fungi in twigs and leaves

To assess the sampling strength, befor to analyse structure of fungal endophyte community from twigs and leaves, species accumulation curve plotted the expected number of species collected against a gradual increase of sampling effort. How showed in figure 16 while species accumulation curve for foliar samples (the green one) reached an asymptote, that showed sampling effort has been sufficient to collect most of the species present and the asymptotic value was a measure of the total species complement; curve for woody samples (the brown one) wasn't smoothed but approximated a linear growth, thus incresing sampling effort could recorded the rarest or occasional species (singletons).

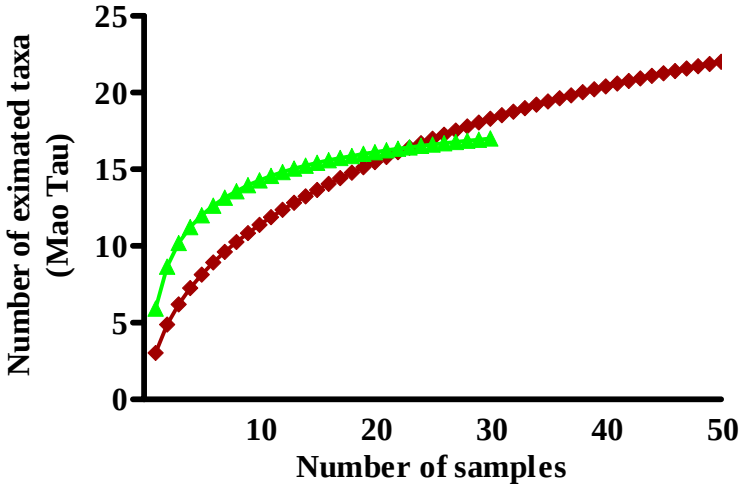


Fig. 16. Expected endophyte species accumulation curves for each native tissue samples based on the Mao Tau estimator calculated using PAST v. 2.04. = twigs; = leaves.

Subsequently, taxa richness analysis with rank-abundance curve showed that method sampling also is capable of catching most abundance species in twig tissues (Fig. 17). Although the abundance curve for woody samples demonstrated relatively lower slope than foliar samples ones, the plot suggesting that our sampling was effective in capturing the species richness of these endophyte communities.

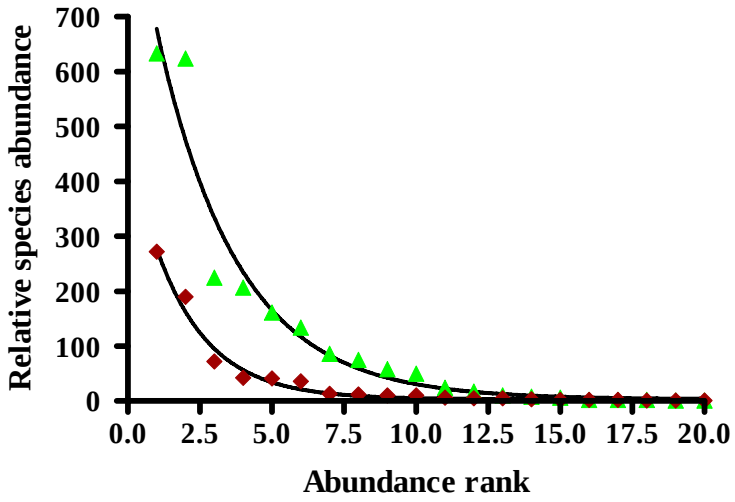


Fig. 17. Rank-abundance curve for each plant tissues separately. —◆— = twigs; —▲— = leaves. Multivariate techniques were applied using PAST v.2.04 and then exponential decay was achieved with GraphPad Prism 4 Istat® 3.5 (GraphPad Software inc., San Diego, California, USA).

Total isolations from leaves and twigs of European beech trees revealed four dominant species: *A. errabunda* (Gnomoniaceae), *B. mediterranea*, *B. nummularia* (Xylariaceae) and *Diaporthe phaseolorum* (Diaporthaceae) and another two taxa occurred frequently: *Alternaria* sp. (Pleosporaceae) and *Cladosporium cladosporioides* (Davidiellaceae). All these taxa were detected in both sampling tissues with different frequencies and isolation results showed in table 6 how Xylariaceae was relatively dominant family both in foliar (34.8%) and woody fragments (63.5%). Remained family occurred with lower frequencies or occasionally.

Tab. 6. Isolation frequencies (%) of family of fungal endophytes detected from foliar and woody beech samples. Family of Xilariaceae was dominant in each tissue.

Family	Foliar islation frequencies (%)	Woody islation frequencies (%)
Chaetomiaceae	0,1	0,8
Davidiellaceae	5,0	5,6
Diaporthaceae	12,0	11,5
Gnomoniaceae	31,9	1,4
Incertae sedis	0,0	1,0
Massarinaceae	0,0	0,3
Nectriaceae	0,4	6,0
Pleosporaceae	13,8	3,3
Pleosporaceae	1,4	0,0
Sordariaceae	0,0	6,3
Trichocomaceae	0,3	0,0
Trichomaceae	0,5	0,3
Xylariaceae	34,8	63,5

Within the limits of the indoor experimental conditions, the obtained results suggested different amount and variable composition of endophyte fungal communities from leaves and twigs (Tab.4). Most of different tissue fragments showed multiple colonization but some endophyte taxa isolated were found to be the main or sole colonizers of specific plant tissue. Plot in figure 18 shows relative abundance of major taxa isolated in twigs and leaves respectively. The polt underlines the different behaviours of fungal endophytes against specific plant tissue: i.e. *B. nummularia* (0.26 in twigs against 0.08 in leaves) and *B. mediterranea* (0.37 in twigs against 0.27 in leaves) were found to be the main colonizers of woody tissues, *S. fimicola* (0.06) and *F. lateritium* (0.05) just were found in twigs, whereas on foliar fragments same dealing showed *A. errabunda* (0.32 in leaves against 0.01 in twigs) and *Alternaria* sp. (0.08) respectively and others like *Diaporthe phaseolorum* or *D. viticola* colonised both tissues indifferently.

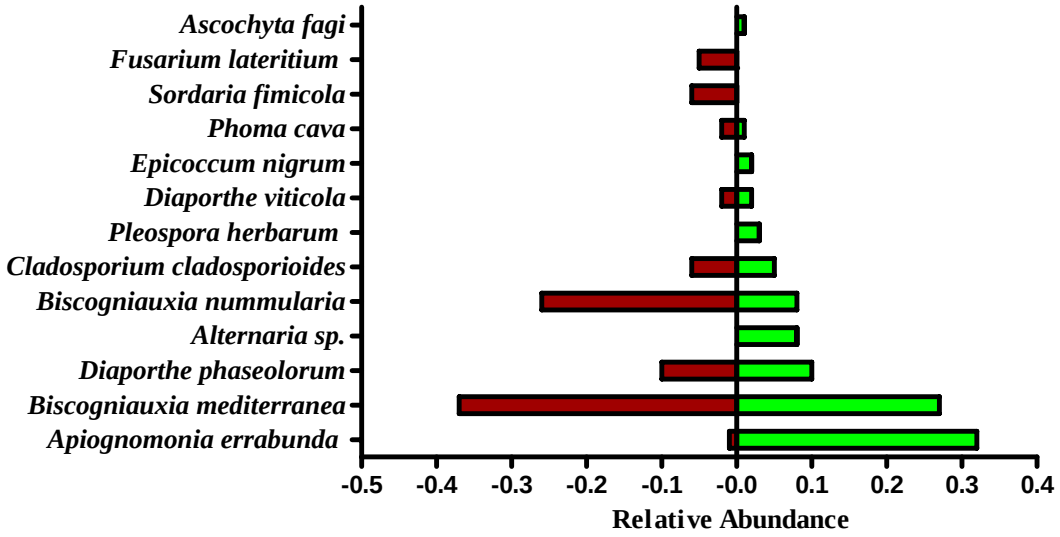


Fig. 18. Relative abundance of major endophytic fungi from woody and foliar beech samples showed different behaviours of fungal endophytes against specific plant tissue. = woody samples; = foliar samples.

7.2 Differences between native tissues

The assessment of isolation boundary of fungal endophytes by analysis of similarity groups showed that endophyte assemblages of the sampling beech forest are strongly shaped by the native tissue (twigs and leaves). Total endophyte assemblages from the same plant tissue clustering together in the NMDS graph (Fig 19) and were highly separated between different tissue groups. Stress level of 0.14 indicate that the NMDS plot provide a satisfactory representation of the real distribution of data (Podani, 1994). The statistical significance of assemblage differences between native tissues was confirmed by ANOSIM (Global R=0.97, P=0.0001) (Tab. 7).

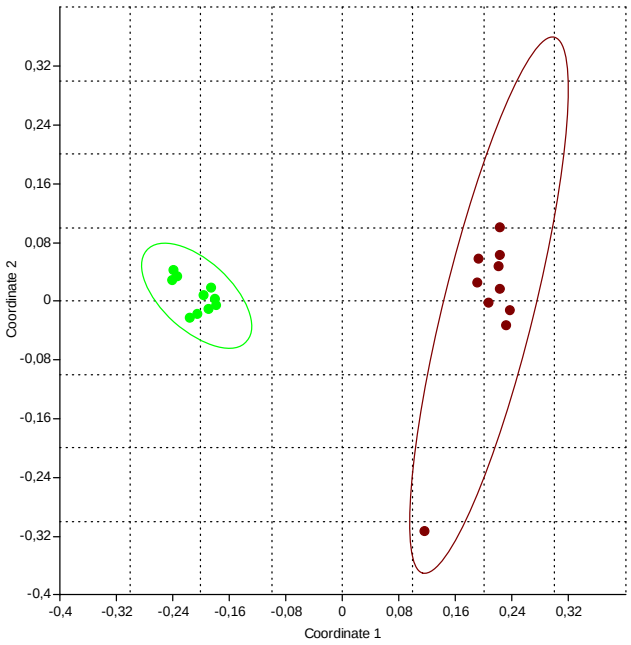


Fig. 19. Nonmetric Multidimensional Scaling plot based on Bray–Curtis similarities, comparing endophyte assemblages across plant tissues. Each circle represents a single tree with different colours for each tissue data point. ●= twigs; ●= leave.

7.3 Differences between exposures

Endophyte assemblages in each native tissue also differed significantly for different exposures. Endophyte assemblages of sites A with North facing clustered together in NMDS plot and were separated from those of site B with South facing in each tissue group (Fig 20). Stress level of 0.13 indicate that the NMDS plot provide a satisfactory representation of the real distribution of data (Podani, 1994). Highly significant differences in endophytes isolated from different tissues and assembled for similar exposure were detected using ANOSIM (Global R=0.8, P=0.0001). Two sites differed significantly in all pair-wise comparisons, however, all similarities within exposure separation level of endophyte groups isolated from the same plant tissue are less than any similarities between exposure groups from different tissues (Tab. 7).

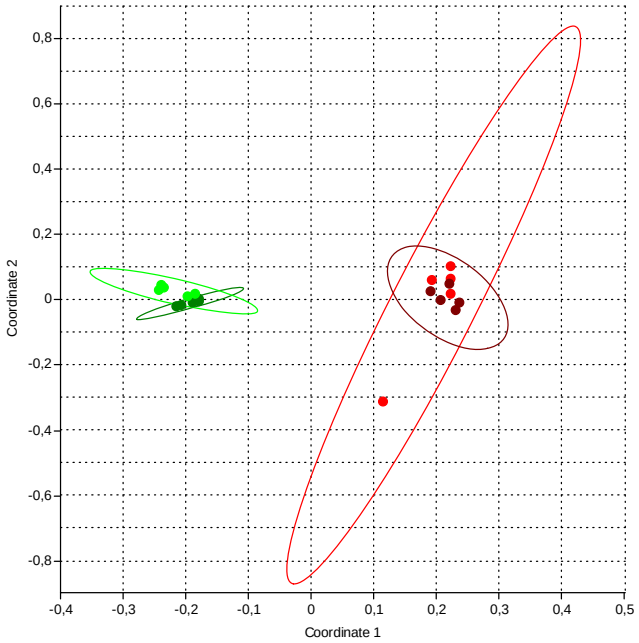


Fig. 20. Nonmetric Multidimensional Scaling plot based on Bray–Curtis similarities, comparing endophyte assemblages across exposures in each plant tissue. Each circle represents a single tree with different colours for each exposure data point within tissue. ●= north-twigs; ●= south-twigs; ●= north-leaves; ●= south-leaves.

Table 7. – Analysis of similarity (ANOSIM) pair-wise comparisons of endophyte assemblages between exposures in each tissue. Global R value=0.8 (P=0.0001). The R value gives an absolute measure of how separated the groups are, on a scale of 0 (indistinguishable) to 1 (all similarities within groups are less than any similarities between groups) (Clarke and Gorley, 2001)

Pair wise comparisons	R	P
Leaves -Twigs	0.97	0.0001
Leaves North-Twigs South	1	0.007
Leaves North-Twigs North	0.95	0.007
Leaves South-Twigs South	1	0.008
Leaves South-Twigs North	1	0.007
Twigs North-Twigs South	0.42	0.01
Leaves North-Leaves South	0.60	0.008

7.4 Differences between seasons

Results of total isolations from leaves and current-year twigs in different times revealed seasonal effects on composition of beech fungal endophytes. Considering five dominant species both in foliar, *A. errabunda* (32%), *B. mediterranea* (27%), *D. phaseolorum* (10%), *Alternaria* sp. (0.8%) and *B. nummularia* (0.8%), and woody samples, *B. mediterranea* (37%), *B. nummularia* (26%), *D. phaseolorum* (10%), *S. fimicola* (6%) and *C. cladosporioides* (6%), plots in figure 21 shows how in leaves *B. mediterranea*, *Alternaria* sp. and *D. phaseolorum* were not detected in June while number of isolates was highest in February, whereas in twigs *B. nummularia* was not recovered in November while *C. cladosporioides* was detected only in February and September.

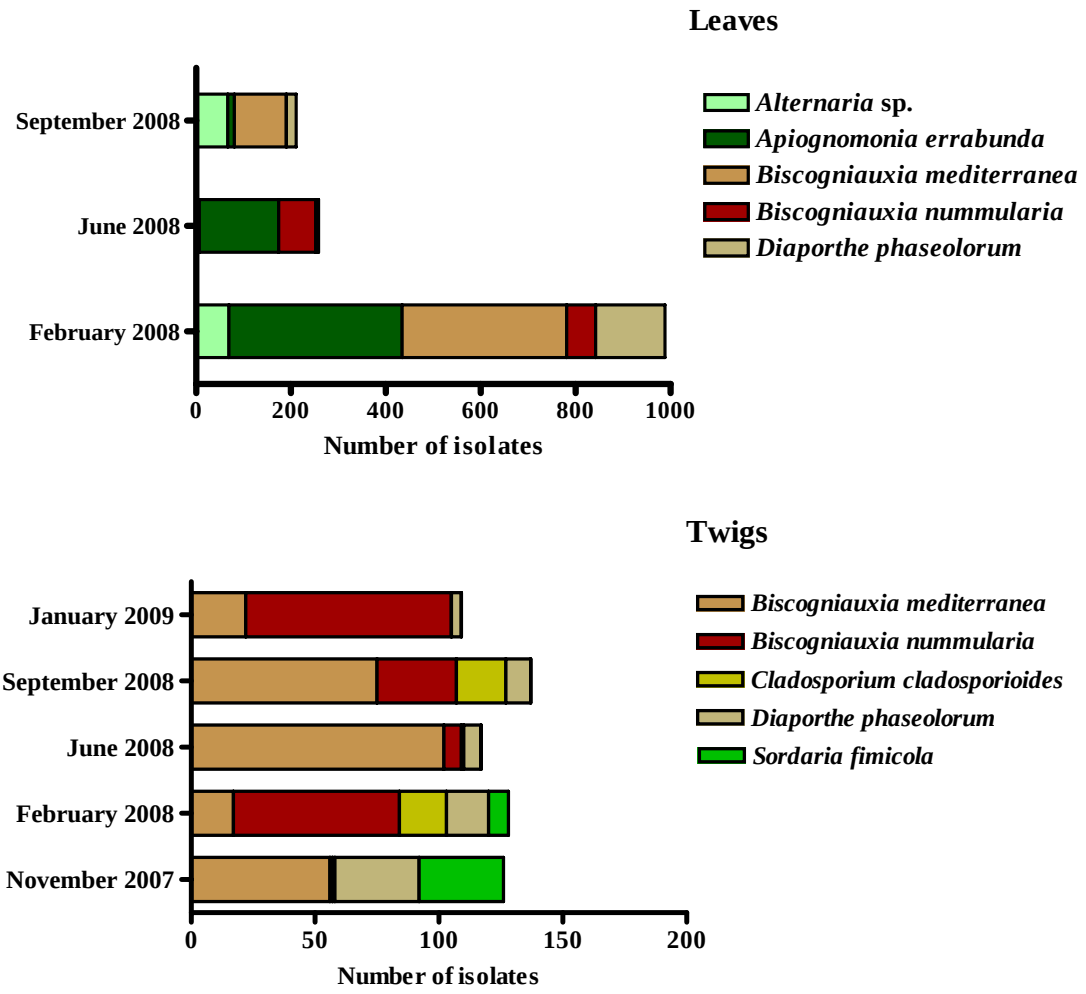


Fig. 21. Seasonal effect on number of isolates of five major endophytic taxa from beech leaves and twigs respectively. Equal color indicates the same taxa.

Isolation boundary of fungal endophytes in different seasons was checked by performing of data in order to presence/absence of any taxa in each sampling period for different leaf and twig tissues (Fig. 22). Plots show how some endophyte were isolated only in specific periods.

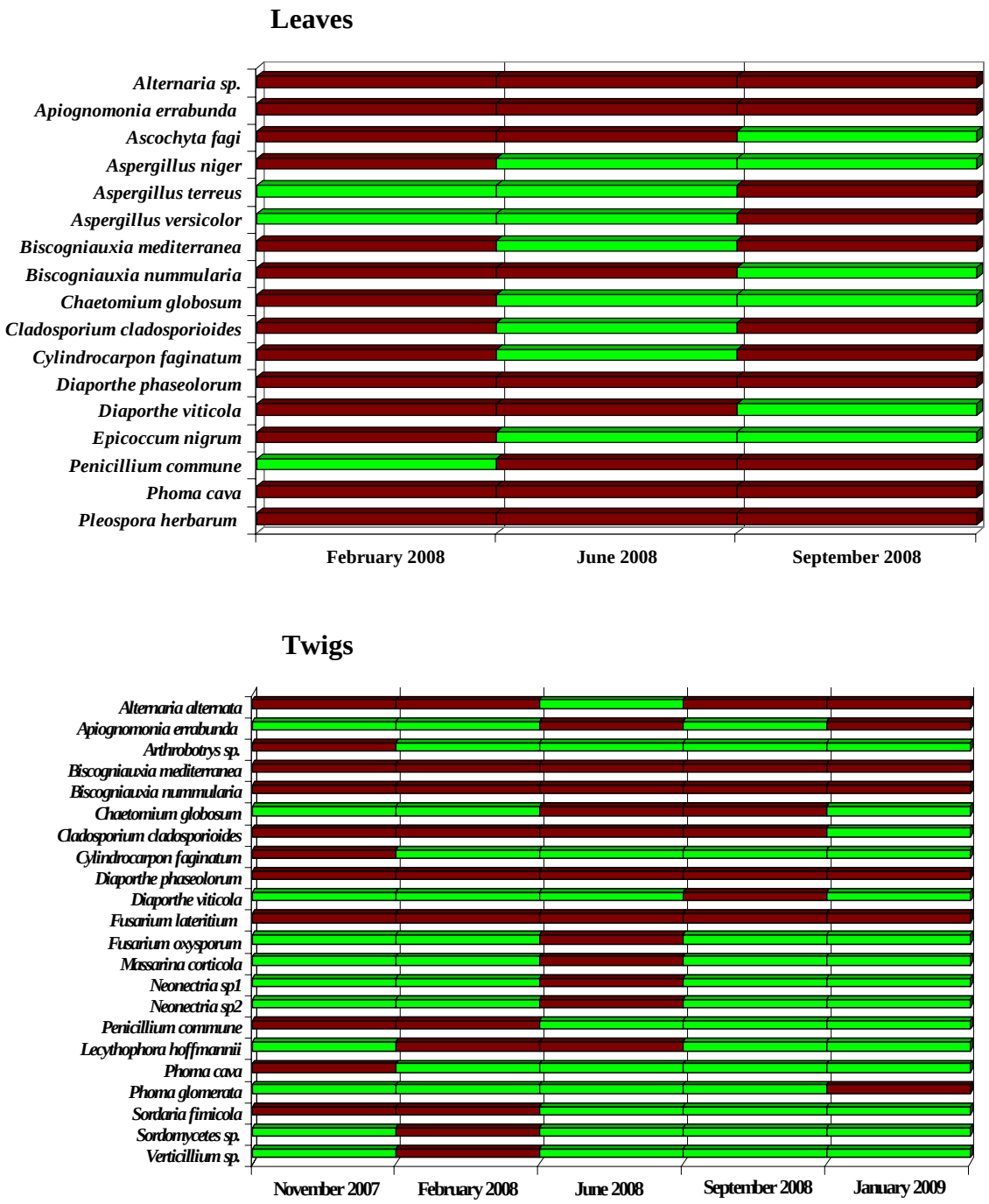


Fig. 22. Seasonal turn-over of fungal endophyte communities in leaves and twigs respectively. In each sampling periods ▬ = presence; ▬ = absence.

7.5 Differences between seasons

Endophyte assemblages in each native tissue also differed significantly for different seasons and endophyte assemblages for the same sampling period (five sampling for collected twigs indicated with 1^{th}-2^{nd}-3^{rd}-4^{th}-5^{th}; and three for leaves indicated with 2^{nd}-3^{rd}-4^{th}) clustered together in NMDS plot and were separated from the others (Fig 23). Stress level of 0.23 indicate that the NMDS plot provide a satisfactory representation of the real distribution of data (Podani, 1994). Highly significant differences in endophytes isolated from different tissues and assembled for similar season were detected using ANOSIM (Global R=0.7, P=0.0001) with significant differeces in all pair-wise comparisons. However, all seasonal similarities of endophyte groups of the same tissue are less than any similarities between seasonal groups from different tissues with a lower global R value for seasonal similarity groups of twigs (Global R=0.43, P=0.0001) compared with leaves (Global R=0.82, P=0.0001).

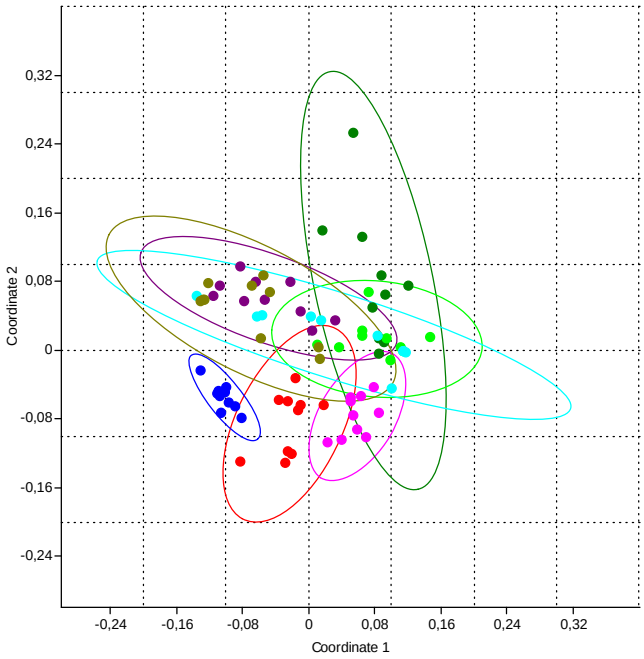


Fig. 23. Nonmetric Multidimensional Scaling plot based on Bray–Curtis similarities, comparing endophyte assemblages across seasonal samplings in each plant tissue. Each cycle represents a single tree with different colours for each exposure data point within different tissues. ●=2^{nd} sampling-leaves; ●=3^{rd} sampling-leaves; ●=4^{th} sampling-leaves. ●=1^{th} sampling-twigs; ●=2^{nd} sampling-twigs; ●=3^{rd} sampling-twigs; ●=4^{th} sampling-twigs; ●=5^{th} sampling-twigs.

7.6 Differences between host trees

The results of a PERMANOVA analysis based on a Bray–Curtis dissimilarity measuring differences in endophyte assemblages between host plant/tissue/season combinations showed significant differences at most spatial levels for tissues. The size of the component of multivariate variation between endophyte assemblages was lower for woody tissues within ten host plants (F=8.05, P=0.0001) compared with foliar tissue data (F=20.36, P=0.0001), with 10.000 permutations number. (Bonferroni pairwise comparisos).

Ecological indices' value of endophite assemblages from the same tissue sampling in different seasons confirmed that diversity in foliar samples was greater than woody ones (Tab.8).

Tab. 8. Ecological indices of endophyte assemblages with regard to differnt tissues and seasonal samplings computed with software PAST v. 2.04

Source	Season	Taxa_S	Individuals	Shannon_H	Evenness_e^H/S	Margalef	Equitability_J	Fisher_alpha
Leaves	Feb. 08	14	1114	1.77	0.42	1,85	0.67	2.26
	Jun. 08	9	290	1.24	0.38	1.41	0.56	1.76
	Sep. 08	11	308	1.76	0.53	1.74	0.73	2.23
Twigs	Nov. 07	11	149	1.59	0.45	2.00	0.66	2.74
	Feb. 08	11	144	1.70	0.50	2.01	0.71	2.77
	Jun. 08	12	133	1.04	0.23	2.25	0.42	3.20
	Sep. 08	8	153	1.44	0.53	1.39	0.69	1.80
	Jan. 09	7	149	1.27	0.51	1.20	0.65	1.52

8. Discussion: objective 2

8.1 Effectiveness of sampling effort

The total diversity of endophytes in the tree species studied was not completely captured for twigs in the present study, as morphotypes continued to accumulate with each additional sampling unit. However, performing of rank-abundance plot, has cleary showed that sampling method used has been effective for detecting of most abundant fungal endophyte taxa, which surely characterize composition and diversity of this forest. This pattern agrees with that observed for fungal endophytes in tropical trees (Arnold *et al.*, 2001) and suggests that although additional sampling would have yielded more morphotypes, these would have primarily comprised 'rarer' taxa. However, was observed a low percentage of singleton morphotypes (0.7% from woody samples and 0.1% from foliar ones).

Observed fungal species composition in studies using culturing methods to assess endophyte diversity can be affected by the size of the tissue segments sampled, as multiple infections per segment may bias species composition in favour of more competitive or fast growing strains (Carroll, 1995; Gamboa *et al.*, 2002). Sampling strategies for studies on endophyte diversity must take into account natural patchiness in the distribution of fungal species across the landscape. The hierarchical sampling structure used in this study allows the proportion of the variances between trees, tissues, sites, and season to be estimated

8.2 Ecological diversity of beech endophytes

The assessment of isolation boundary of fungal endophytes by analysis of similarity groups showed that endophyte assemblages of the sampling beech forest are strongly shaped by the native tissue (twigs and leaves). This is indicated by the clear separation of groups corresponding to foliar and woody sample taxa in NMDS plots (Fig 19), and by significant differences in global (Global R=0.97, P=0.0001) and pair-wise comparisons of endophyte assemblages in different plant tissues (Tab. 7).Our results agree with those of other studies, which also indicated a degree of host tissue preference among endophyte assemblages (e.g. Petrini, 1991). However, was above observed that between two and five endophyte taxa were detected as dominant in different beech tissue and many of these taxa were also isolated in high frequencies from any tissue

(Fig.18). The balanced endophyte-host plant associassions probably reflect the current healthy state of forest studied.

While tissue-related factors were the strongest determinant, geographic separation and seasonal effects also modulated fungal endophytic assemblages, albeit to a lesser extent. Overall, species assemblages differed significantly between the sites studied (Table 7). However, absence of barriers to dispersal and relative proximity between two sites indicate that the observed differences might be linked to climate or history of forest studied, which inside co-evolution of host-endophyte associations have striked a balance as such any endophyte occupy its own spatial and temporal niche.

Endophyte assemblages were also influenced by season. Seasonal differences, although statistically significant, were less pronounced than those observed for host tissues and sites exposure across the total endophyte assemblages (Global R=0.7, P=0.0001). Seasonal patterns differed for individual taxa with a few species being present only either in summer or winter, e.g. *Biscogniauxia nummularia*, *B. mediterranea* , *Diaporthe phaseolorum* from twigs and *Apiognomonia errabunda* and again *D. phaseolorum* from leaves (Fig. 22) More commonly, there were differences in isolation frequency across seasons (Fig 23). The variation observed suggests that there might be a high and ongoing turnover of endophyte populations within a single tissue for effect of ecological pressures (Fig. 22).

In particular, differences between *B. mediterranea* and *B. nummularia* in meaning of colonization spread at different levels tissue/exposure/season, could be explained by the ecological behaviour of two xylariaceous specie, indeed, *B. mediterranea* being more common than *B. nummularia* at least in Italian peninsula (Capretti *et al*., 2003; Granata and Sidoti, 2004). In particular, *B. mediterranea* was mainly recovered from South site in all sampling on leaves, whereas on twigs, gives one's seat to *B nummularia*. Colonization frequency of this fungus, slightly higher in samples from site with South facing (50.4%) compared to ones from North exposure, suggests weak role of this pathogen. Similar behaviour showed *A. errabunda*. Butin (1983) speculates that warm wet spring weather is favourable for an epidemic of *A. errabunda* on beech. In this study higher frequency of this fungus in leaves from site with South facing (54%) compared to ones from North exposure suggests truth of this possibility. Likewise, *Ascochyta fagi* was consider a latent pathogen, because in this study was sporadically detected in winter from leaves of south site.

In addiction, common *Fagus* saprophytes like *Alternaria alternata* and *Cladosporium cladosporioides* (Osono and Takeda, 1999) showed different distribution behaviours. In particular, *A. alternata* was present only on twigs collected from South site. Whereas *C. cladosporioides* was only or mainly isolated from leaves and twigs of South site, but was totally absent during the sampling carried out in summer (June 2008). The isolation frequencies of these common and ubiquitous species were more frequent in winter or autumn (Guo *et al.*, 2008), but also showed high tolerance to sun and desiccation (Park, 1982; Butler and Day, 1998; Osono and Mori, 2003). The vicariation could be due to competition for resources and space, that become too hard during summer.

Probably, the capacity of beech forest ecosystem of the Mediterranea Basin to withstand natural distrurbances is still effective, but more and more an extended drought period, in a scenario of global climatic change characterized by increases in temperature and extreme events, has radical changed the communities of organisms living inside these fragile ecosystems. All that has promoted the presence of no host-specific xylariaceuous fungus, like *B. mediterranea*, but without increase and preservation of biodiversity, the evolutionary trajectory of plant–endophyte relationships is channelled in the direction of decline by *B nummularia*.

9. Conclusion: objective 2

In this study was found that the abundance, diversity and species composition of endophyte communities differed as a function of tissue, exposure and season sampling. Most part of this variation could be explained by natural factors such as climatic and microclimatic environmental conditions affecting the composition of endophytic fungi, which are subjected to different selection pressures in different seasons (Osono and Mori, 2003; Guo *et al.*, 2008). In summary, in studied forest, some fungal endophytes show a great degree of tissue recurrence or specificity, and their colonization and isolation rates are strongly affected by seasonal factor, but the composition of endophytic assemblages is not conspicuously influenced.

Diversity of endophytes in these beech hosts thus appears to be sufficient high, with distinctively structured communities in each tissue and locality and high degree of geographic turnover. How such endophytes might benefit or negatively influence their host plant, particularly in times of severe stress, has not been determined. One of the factors that can be affected the amount of community composition is low precipitation. Wet conditions are favourable for fungal sporulation causing the increase of endophyte infections during growing season (Wilson, 2000). In the present study, the diversity of endophytes increased marginally during the wet season as revealed by rarefaction analysis. This marginal increase was due to the appearance of some fungi such as *Aspergillus* spp., that are commonly isolated as endophytes and not truly unique fungi (Murali *et al.*, 2007).

To assess the diversity and taxon composition of fungal endophytes among different tissues and geographical distinct sites over time, was used a culture-based approach, paired with sequencing of both a fast-evolving locus and a phylogenetically informative locus (ITS rDNA). Well-supported phylogenies are key to diagnosing the taxonomic affinity of unknown endophytes and are necessary for adequately addressing ecological questions. Future studies will benefit from multiple, concurrent analysis methods, especially with regard to linking traditional morphological species concepts to extensive molecular datasets.

10. Materials and methods: objective 3

A careful morphotyping of endophytic isolates results in taxonomic groups could be verifiable with ribosomal DNA sequence analysis. Thus the numerous previous results relying on morphotyping present a good basis for further research with new molecular data. Numerous DNA-based methods have been developed for the characterization of both fungal communities and pure fungal cultures, among these Terminal Restriction Fragment Length Polymorphism (TRFLP or sometimes T-RFLP) has gained notable popularity as a tool to describe the identity and diversity of fungi from biological samples and is one of several molecular tools aimed to generate a fingerprint of an unknown microbial community.

10.1 TRFLP analysis technic

Terminal restriction fragment Length Polymorphism (TRFLP) analysis is a semi-quantitative method based on polymorphism of restriction site of nucleotide sequences. This method was used to assess the genetic diversity of individual present in specific unit (Dickie and FitzJohn, 2007). In this study TRFLP analysis was developed for the rapid comparison of different fungal community structure in beech plant tissues. This method based on differences in the ribosomal DNA sequence of assorted organisms producing amplicons by the PCR reaction of assorted sizes with unique restriction cut sites. Therefore, digestion resulted in numerous labeled terminal DNA fragments (TRF) of various lengths and capillary gel electrophoresis was used to analyze the lengths of the TRFs resulting in a distinctive terminal restriction fragment pattern (TRFP) for each sample (Fig. 24). TRF patterns varied depending on the enzyme used in the restriction. TRFs were then compared to each other to observe shifts in diversity and fungal community members. Overall, the TRFPs proved to be a useful tool in assessing the diversity of fungal species in beech forest studied and with further development, this technique shows promise in analyzing fungal diversity in the environment which standard laboratory plating techniques cannot provide.

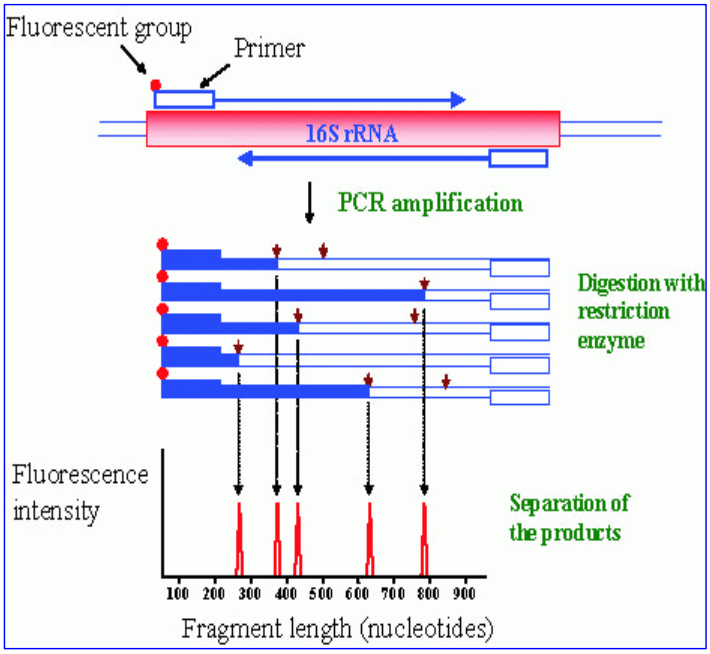


Fig. 24. Flowchart of steps required for a TRFLP assay.

10.2 DNA extraction, amplification and quantification

To begin the analysis, PCR was carried out using DNA of plant tissue samples and primers homologous to regions in the fungal ITS ribosomal gene. Total plant material DNA was extracted from frozen and lyophilized portions of beech twigs, leaves and buds with DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions as described previously (¶ 3.5).

The choice of primers is critical in any molecular study and the ITS regions of fungal rDNA, present in genomic DNA of plant tissues, were amplified using the primers ITS1F 5′-CTT GGT CAT TTA GAG GAA GTA A-3′ and ITS4 5′-TCC TCC GCT TAT TGA TAT GC-3′ (White *et al.*, 1990) widely used for recognition of ascomycetes fungal communities (Haskins and Gehring 2004; Trowbridge and Jumpponen 2004; Dickie and Reich 2005). The forward primer, ITS1F, were 5′-labeled with D4-PA, a fluorescent dye for analyzing samples using the CEQ 8000XL DNA analysis system (Beckman- Coulter, Fullerton, CA, USA). Polymerase chain reactions (PCR) were performed using a PCR- Beads ™ (Amersham Pharmacia Biotech) as described previously (¶ 3.5). The PCR amplicons were purified using Ultra Clean-Up DNA Purification Kit (MoBio Laboratories, Inc., Solano Beach, CA) according to manufacturer protocols. Purified DNA was separated by gel electrophoresis on 1.5%

agarose gels (Agarose D-1 low eeo, Eppendorf Italy) with ethidium bromide (1.2%) in TBE buffer (45mM Tris-borate, 1mM EDTA, pH 8.0) at 100V for 20 minutes. DNA in each purified samples was quantificated spectrophotometrically under UV lightwave by comparison with known quantities of the molecular mass marker 100bp Ladder.

10.3 Choice of restriction enzymes

The choice of restriction enzymes may affect the ability of TRFLP to distinguish species indeed would have recognition sites in a high proportion of fungi (Edwards and Turco, 2005).

The purified PCR products were diluted with water in order to add 100 ng of DNA to the enzyme digest. The enzymes used in the digest were three common 4-base cutters: *Msp*I (C'CG_G), *Hae*III (GG'CC), *Alu*I (AG'CT) and also *Eco*RI (G'AATT_C). The restriction reactions had a total volume of 20µl and contained 100ng of DNA, 6U of restriction enzymeand, 1µl of specific Buffer10X and distilled water. Four separate digests were performed on each sample. The samples were digested at 37ºC for 6 hours and then heated to 80ºC for 20 minutes to deactivate the enzymes.

10.4 Ethanol Precipitation of Digest

To each digest, 50µl of cold 95% ethanol, 1µl 3M sodium acetate (pH 5.2) and 1µl glycerol (20mg/ml) were added and mixed. The mixture was centrifuged for 15 minutes at 15.000 rpm at 4°C to pellet the DNA. The ethanol was removed and the pellet was washed with 100µl of cold 70% ethanol, centrifuging again for 15 minutes at 15.000 rpm at 4°C. The ethanol was removed and the pellet of DNA was dried by vacuum centrifugation.

10.5 Capillary Electrophoresis

The DNA pellet was resuspended in 20µl of Sample Loading Solution (SLS, Beckman Coulter) and was mixed with 0.25µl of Size Standards-600 (Beckman Coulter) and loaded onto a capillary electrophoresis sequencer CEQ^{TM} 8000XL (Beckman Coulter). Analyses were run with a modified version of Frag4 method, including a denaturetion at 90ºC for 2 minutes, an injection at 2000V during 15 seconds and a separetion at 4200V during 65 min. Capillary electrophoresis was performed in laboratiries of Department of Animal Sciences, Unversity of Tuscia, Viterbo. After electrophoresis, the legth and the signal intensity of the fluorescently labelled TRFs

were automatically calculated by comparison with the size standard, using the CEQ 8000XL DNA analysis system software version 4.2.0. Fragments between 60 and 640 bp corresponding to the size range of the standard were considered. For each digest samples were produced at least two replicates. The result was that the labeled terminal restriction fragments presented in each sample were seen on the electropherogram as an individual peak, where the height of the peak represented the relative amount of that fragment in the digested PCR product.

10.6 Developing a TRFLP database

TRFLP database was developed for phylogenetic association of individual TRFs obteined. ITS sequences dataset of isolated fungal endophytes from different beech plant tissues, previously obtained with primer ITS1F and ITS4, were completed with ten published ITS sequences for each taxa. This completed GeneBanck dataset was joined with a subset database of published ITS sequences of all potential fungal edophyte species of *F. sylvatica*, reported by references, in number of ten sequences for each potential fungal endophyte. For each sequence, was simulated restriction digest with chosen restriction enzyme (*Msp*I) using BioEdit software system of Restriction Map (BioEdit version 7.0.0). The result was a complete TRFs database for each beech fungal endophyte taxa. The viewing coparison of 10 sample's TRFLP profile with this TRFs database could determine the association of individual peak with a specific fungal taxa.

10.7 Diversity of fungi in TRFLP profiles

TRF profiles from all 10 samples were analyzed by counting the number of TRF peaks as an index of richness (number of taxa). Analysis of TRFLP profiles also was performed in meaning of sum of peaks area for each detected taxa on total peak area of ten profiles, to evaluated isolation frenquency of each taxa in total 10 woody samples analysed. The Shannon-Weaver diversity index was used to estimate fungal diversity based on the size and number of TRFs using following equation by Brodie *et al*. (2003):

Shannon-Weaver index (H′)=

$H' = -\sum (p_i \ln p_i)$

Where $p_i = n_i/N$, is proportion of total sample belonging to i^{th} TRF where n_i= height of TRF *i* and N= sum of peak heights in a given TRFLP profile.

Dominance within communities was estimated using Equitability index based on the size and number of TRFs present using eq. 5 following equation (Brodie *et al*., 2003):

Equitability (J)= H′/Hmax= H′/lnS

Where S= number of different peak heights in a given TRFLP profile.

J=equitability (range 0–1, with max. value when individuals evenly distributed among the TRFs in a profile); H′= observed species diversity; Hmax = species diversity under conditions of maximal evenness.

10.8 Comparison between TRFLP and isolation methods

In order to compare species frequency of detection culture methods *vs* detection using TRFLP molecular analysis, for each fungus of 10 woody samples a two-way ANOVA and Fisher's exact probability test were performed using the GraphPad Prism 4 Istat ® 3.5 (GraphPad Software inc., San Diego, California, USA).

11. Results: objective 3

11.1 Database predictions

Amplification with ITS1F and ITS4 primers was predicted to yield fragments averaging 650 bp in size. A total of 840 fungal ITS ribosomal DNA sequences were downloaded from NCBI GenBank and analyzed for predicted TRF lengths from the DNA sequences. In Table 9 predicted TRFs for any ITS rDNA sequence of previously endophyte taxa traditional isolated on PDA medium from beech tissues, using 4 chosen restriction enzymes, were reported. According to Lord *et al.* (2002), table 9 showed how *Msp*I using the ITS region for TRF analysis predicted result with great richness and specificity.

Tab. 9. Restriction table of predicted TRFs for any ITS sequences of previously endophytes isolated on PDA medium from beech tissues performing by BioEdit software system of Restriction Map (BioEdit version 7.0.0).

Endophyte taxa	TRF length			
	Msp I	*Alu* I	*Hae* III	*Eco* RI
Alternaria alternata	468	402	471	311
Alternaria sp.	439	373	442	282
Apiognomonia errabunda	186	421	148	329
Arthrobotrys sp.	448	169	174	339
Ascochyta fagi	542	340	542	249
Aspergillus niger	136	nc	72	315
Aspergillus terreus	108	173	42	286
Aspergillus versicolor	87	nc	438	304
Aureobasidium pullulans	181	382	440	291
Biscogniauxia mediterranea	525	479	528	327
Biscogniauxia nummularia	214	195	218	361
Chaetomium globosum	134	nc	131	321
Cladosporium cladosporioides	79	nc	590	302
Cylindrocarpon faginatum	399	nc	498	304
Diapothe phaseolorum	139	475	138	323
Diapothe viticola	82	484	187	332
Epicoccum nigrum	445	380	583	nc
Fusarium lateritium	133	431	132	340
Fusarium oxysporum	135	390	151	292
Lecythophora hoffmannii	230	387	579	280
Massarina corticola	579	222	114	nc
Neonectria sp.	118	nc	80	nc
Penicillium commune	152	489	124	383
Phoma cava	442	378	579	285
Phoma glomerata	441	376	578	285
Phomopsis sp.	139	470	142	320
Pleospora herbarum	618	115	nc	321
Sordaria fimicola	195	191	506	nc
Sordariomycetes sp.	175	nc	133	314
Verticillium sp.	65	422	48	193

nc = no cut. Resulting fragment equaled whole sequence and exceeded the size range of the standard (640 bp).

11.2 Identification of fungi using TRF patterns

TRFLP analysis, performed on DNA isolated from plant samples collected, produced a greater number of TRFs for any restriction with *Msp*I enzyme, confirming predicted results (Tab. 9), lower for *Alu*I, and community members were resolved into smaller and distinct peaks (Fig. 25). To better facilitate this kind of analysis, observed TRF profiles from DNA of 10 woody beech samples collected during November 2007 were viewing compared against those predicted fragment lengths. All peaks produced in these ten profiles by TRFLP analysis were associated with a specific endophite taxa.

Exemple given in figure 25 shows some taxa names identified by association with TRF peak of community profile .

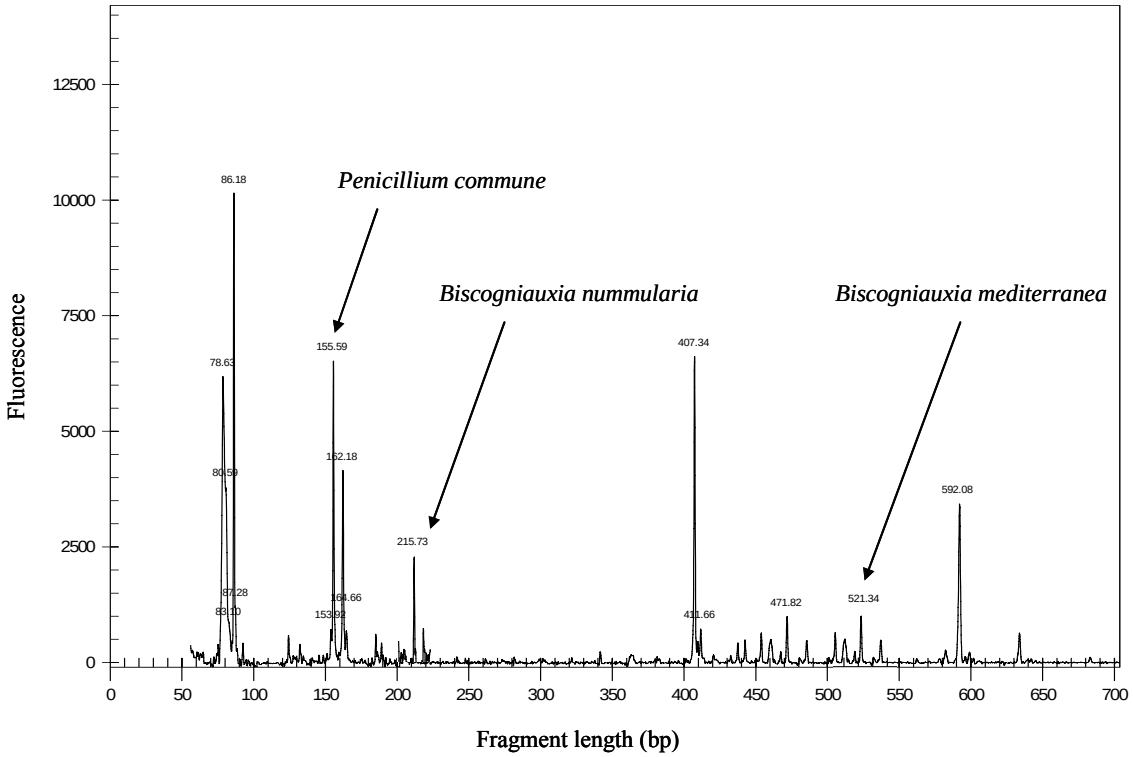


Fig. 25. TRFLP profile of fungal communities generated from ITS rDNA amplified from beech twig DNA using fluorescently labelled primer ITS1F-5′ and primer ITS4, and digested with *Msp*I. The highlighted peaks show an exemple of corresponding of specific fragments to specific fungal endophytes.

11.3 Comparison between TRFLP and isolation methods

In total 23 taxa from TRFLP analysis of DNA from 10 woody beech samples were recognised, with maximum of 14 in sample number seven and minimum of 8 in sample nine, against just 6 total taxa detected by traditional isolation technic, with maximum of 2 in samples 2-3-4-5-6-8-10 and minimum of 1 in the other ones. In table 10 and table 11 were reported list of endophyte taxa detected with different methods and comparation of results between two technics respectively. Results reported in table 11 also showed higher ability of TRFLP to detected specific presence of *B. nummularia* compared to traditional isolation performed on same ten woody samples.

Tab. 10. List of total taxa identified by TRFLP, from DNA of 10 woody beech samples, in alphabetical order. Also were reported for each taxon both value of predicted and real TRF fragment. Letter *i* refers to isolation technic.

Taxon	Predicted TRF peak (bp)	Real TRF peak (bp)
1 *Alternaria alternata*	468	469
2 *Apiognomonia errabunda*	186	184
3 *Biscogniauxia mediterranea* [i]	525	523
4 *Biscogniauxia nummularia* [i]	214	216
5 *Chrysosporium* sp.	83	83
6 *Cladosporium cladosporioides*	79	79
7 *Coniophora puteana*	501	501
8 *Cylindrocarpon faginatum*	399	401
9 *Diaporthe eres*	163	163
10 *Diaporthe phaseolorum* [i]	139	141
11 *Diaporthe viticola*	82	80
12 *Fusarium lateritium* [i]	133	133
13 *Hypoxylon fragiforme*	206	204
14 *Lecythophora hoffmannii*	230	232
15 *Massarina corticola*	579	580
16 *Penicillium commune*	152	153
17 *Phoma cava* [i]	442	440
18 *Pyrenochaeta* sp.	420	422
19 *Rhodotorula* sp.	355	355
20 *Sordaria fimicola* [i]	195	192
21 *Sordariomycetes* sp.	175	174
22 *Verticillum* sp.	66	65
23 *Xylaria* sp.	164	164

i = taxa also detected by traditional isolation technic

Tab. 11. Comparation between traditional isolation and TRFLP approaches to assessing richness of fungal endophyte community performing on the same 10 beech woody samples.

	TRFLP		**Isolation**	
	Total	**Max-min**	**Total**	**Max-min**
Number of Taxa	**23**	**14 - 8**	**6**	**2 - 1**
Number positive samples to *B. nummularia*	**4**		**1**	

Comparisons between number of taxa and ecological indices showed highly significant differences in taxonomic detection and species diversity between two different approaches (Fisher's exact test, $P<0.001$). In contrast, no significant differences were found in order of specific distribution (Fig. 26).

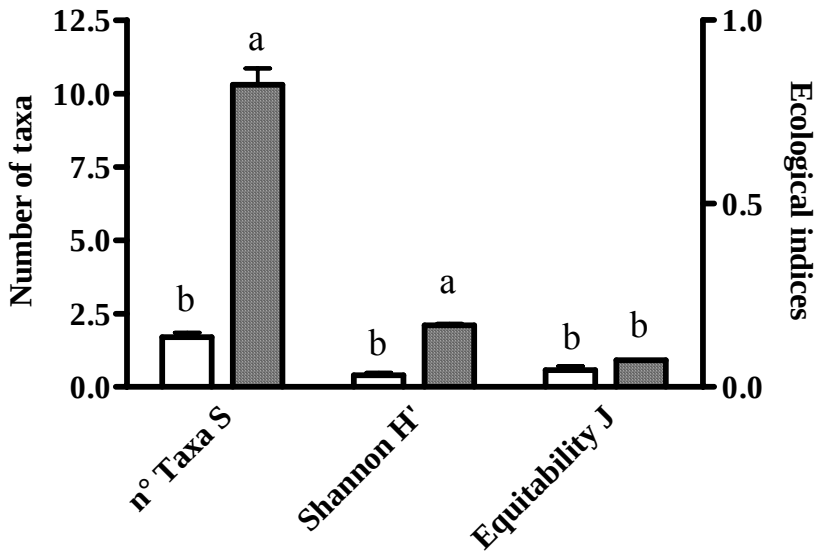


Fig 26. Ecological indices of endophyte assembleges detected using traditional and molecular approaches from the same 10 beech woody samples. ▭ =isolation; ▬= TRFLP.

12. Discussion: objective 3

This study developed a sensitive approach to monitor *F. sylvatica* fungal endophyte community structure based on plant tissue nucleic acid extraction and TRFLP analysis, which, to our knowledge, is the first report using this approach in comparative analyses of fungal endophyte community of European beech forests.

The value of a TRFLP approach is that it may be interpreted on a number of levels. Firstly, the number of TRFs or ribotypes, found in each tissue DNA sample, gives information regarding fungal diversity at a quantitative level (numbers of ribotypes present). Secondly, fragment lengths can potentially be compared with known sequences in previously made database (identification of ribotypes); while fragment peak heights (fluorescence) provide information about the relative abundance of a ribotype within a particular plant sample. In principle, combining these parameters builds a view of fungal community structure.

The purpose of TRFLP analysis in this work was to discriminate between communities in 10 different woody beech samples. Preliminary analyses showed the enzyme *Msp*I was most successful in this regard (Tab. 9). This work showed the ability of TRFLP analysis, agaist traditional isolation technic, to detecting low presence of beech fungal endophyte very ecologically importan (Tab. 10). For istance, *Coniophora puteana* (0.1%) and *Hypoxylon fragiforme* (0.6%), are known to be weak pathogen of *F. sylvatica* and colonize woody tissues with high frequencies (Hendry *et al.*, 2002; Baum *et al.*, 2003), further, this two fungi were reported by Chapela and Boddy (1988) as fungal species strongly isolated from stained zone, continuous with dead bark, in declining beech trees. Also was detected *Pyrenochaeta* sp. (0.1%), that together with *H. fragiforme* are known to be among beech fungal endophytes, isolated with low frequencies by Danti *et al.* (2002) from beech bark samples. In addition, together with *C. puteana*, *Rhodotorula* sp. is another basidiomycetous detected by TRFLP (0.3%) but not by the isolation methods. Recently this least fungus was reported as abundant beech tissue endophyte in Germany (Unterseher and Schnittler, 2009). In addiction, fungal endophyte belonging to genus Rhodotorula (*Rhodotorula minuta*) was studied in bud of Scots pine to able to produce adenine derivatives that may be used as precursors in cytokinin biosynthesis that affect morphology and mitigate browning of callus cultures (Pirttilä *et al.*, 2003), improving plant resistance responses.

As always, with PCR-based community analyses some caution must be exercised in the interpretation of such data, particularly quantitative aspects, due to differing PCR amplification efficiencies of templates within heterogeneous community DNA extracts. However, this study confirm the ability of TRFLP to discriminate a number of total taxa (number of peaks) nearly four time greater than those detected by isolation for the same 10 samples (Tab. 11). Thus, TRFLP assess better than traditional methods, richness and diversity of endophyte communities ($P<0.001$) of environmental samples. Further, low value of standard deviation obteined by TRFLP analysis confirmed the great repeatability of this molecular assay (Clement *et al*., 1998). Lower species distribution value (Equitability *J*) confirmed quanlitative feature of TRFLP; the higher peak is, the more taxa abound.

TRFLP technique was also assessed for the ability to detect specific presence of *B. nummularia* as endophyte in healthy beech tissues. In this study molecular approach showed a specific sensibility four time higher than isolation do (Tab. 11).

The technique has both high sensitivity and throughput making it ideal for comparative analyses (Marsh, 1999). The technique has the significant advantage of low cost and relative simplicity, permitting sufficient replication to address important ecological questions and permits high throughput of samples (Dickie and FitzJohn, 2007).

According to Lord *et al.* (2002), these study suggest that the ITS TRF method has the potential to be a useful tool for assessing differences in fungal community structure, and that also primer region and sequence are a key elements of TRF analysis. The upstream primer used to amplify the ITS region, ITS1F, is known to be fungal-specific (Edwards *et al*., 2004). It was paired with an universal eukaryotic ITS primer, ITS4, assuming that the fungal-specific primer would limit PCR amplification to primarily fungal sequences.

In this study primers to ITS region were identified and successfully used to generate TRF patterns from DNA of beech tissue community samples. Further, according to Lord *et al.* (2002) this work suggested that ITS region provides an accurate and extensive assessment of community richness and species identity. The utility of the ITS region was specifically demonstrated by the great number (i.e. richness) of predicted and observed ITS TRF peaks and the low redundancy when matching observed TRF profiles to predicted TRF ones (Tab. 9). Indeed, in this study was observed only one case of different taxa with the same TRF peak by restriction with *Msp*I enzyme.

Diapothe phaseolorum and *Phomopsis* sp. showed the same terminal fragment 139 bp long. The other analyses previously performed for the same twig samples in this study (morphological survey and molecular identification by ITS sequencing) have allowed overtake this technical limits of TRFLP analysis, but actually real identification of that TRF peak remains uncertain. However, the ITS region was also provided high species-specific indication of community richness and diversity index (Fig. 26). According to Edwards and Turco (2005), this finding was confirmed by experimental observations for *Msp*I and *Hae*III (Table 9). For all enzymes examined in this study, except *Alu*I, sequence database predictions suggested that the ITS region would produce a great number of different TRF lengths per genus (Table 9). The reason that *Alu*I gave such a low number of ITS TRF fragments could be due to a highly conserved cut site. This limit can be overtake using three or four enzyme at the same time (Tab. 9).

13. Conclusion: objective 3

In this study was successfully proven the possibility of identification of specific taxa to the genus and/or species level by matching observed TRF profiles from either ribosomal region with predicted ones. In this study all abteined peaks were identified.

However, just a limited number of samples were tested with one enzyme in this study. Really, matching observed TRF profiles from community samples to individuals in a database isn't so easy. First istance, because identifications could be not specific, in reason that a single TRF may represent a number of species. The reason that a specific enzyme could give a very low number of ITS TRF fragments could be due to a highly conserved cut site. This limit could be improved through the use of more than one restriction enzyme at the same time for the analyses, or through identification of TRFs within clone libraries generated from rDNA sequences predigestion. Second, because identification of fungal community members from TRF profiles will depend largely on the fidelity and completeness of the available fungal rDNA sequence information and database. Sometimes, low return of sequences could depend both by high number of ambiguous and unidentified sequences and by low number of sequence entries long enough to contain the required sequence information (e.g. both primer sites). It is also worthwhile to note that sequences available in the public databases represent only a small portion of the fungi that are known to exist in nature. Thus, improving of current databases with additional and more complete ITS rDNA sequences, and with sofware for rapid maching of peaks patterns, will help pave the way for easier identification of fungi from TRF pattern data.

In conclusion, TRF analysis shows great promise for the study of fungi in complex microbial communities, in particular when primers that amplify the ITS region are used. The T-RFLP technic is rapid and sensitive as originally described. It has been used for comparative community analysis and to derive estimates of the diversity of a phylogenetic group within a community, but also for single strain identification. Because of the high throughput capacity and the supporting sequence databases, the technique will prove most valuable in comparative community analysis. Increasing levels of community dissection can be attained with the systematic use of phylogenetic specific primers in the T-RFLP protocol. The T-RFLP data, when well maching with complementary data on the diversity and distribution of fundamental physiological markers as well as physico-chemical data describing the particular ecosystem, will

provide a level of insight into the demographic structure and function of microbial communities as yet unrealized.

14. Material and methods: objective 4

Quantitative Real time polymerase chain reaction is quantitative method used to amplify and simultaneously quantify a targeted DNA molecule. This type of assay allows the continuous monitoring of amplicon synthesis during thermocycling, and requires no post-PCR sample handling for target quantification (Orlando *et al.*, 1998). Recently, TaqManTM chemistry (Holland *et al.*, 1991; Livak *et al.*, 1995) has been used to develop a quantitative real-time PCR (Rt PCR) assay for the early detection of xylariaceous fungus *B. nummularia* in symptomless beech tissues. This fungal pathogen causes strip-canker and wood decay on European beech but has been widely reported like endophyte, in a latent fase, into plants of different Italian beech forests (Luchi *et al.*, 2006). In this study, in order to detect *B. nummularia* in symptomless tissues of beech, Real-time PCR was proposed as an important tool for epidemiological and ecological studies on the interaction between this fungal species and beech trees under environmental conditions.

14.1 Detection of *B. nummularia* by Real-time PCR

The molecular assay, carried on at laboratory of Florence's University, was based on TaqMan^{TM} chemistry using species-specific primers and a fluorogenic probe designed on the ITS1 sequence of rRNA gene clusters using the methods described by Luchi *et al.* (2005). This study was carried out in collaboration with Prof. Capretti (University of Study, Florence, Italy) and the specificity of the oligonucleotides and the probe was tested using the DNA of *B. nummularia* isolates from two different geographic and climatic Italian beech forest:

Site 1. "La Spianessa", Cutigliano, Pistoia (994-m a.s.l.) (44°04′08″N; 10°48′42″E).

Site 2."La Faggeta", Soriano nel Cimino,Viterbo (1042-m a.s.l.) (42°24′29″N; 12°16′08″E).

In particular forest area of Viterbo (Site 2), although is at major laltitude, is more subject to water stress than forest of Pistoia (Site 1).

14.2 Genomic DNA extraction

A portion of genomic DNA extracted from woody samples collected in "La Faggeta" of Viterbo during four different sampling periods, November 2007, February, June and September 2008, as described previously (¶ 3.5), was sent to Dr. Nicola Luchi

(Dipartimento di Biotecnologie Agrarie, Sezione di Patologia vegetale, Universita` degli Studi di Firenze) for the early detection of *B. nummularia* on symptomless European beech using quantitative Real-time PCR (pg of *B.nummularia* DNA /µg of total DNA extracted) (Luchi *et al.*, 2006).

14.3 Coparison of Isolation with Real-time detection

In order to compare positive frequency of beech shoot samples to specific precence of fungal pathogen *B. nummularia* using detection culture methods *vs* quantitative detection by real time PCR a two-way ANOVA and Fisher's exact test were performed using GraphPad Prism 4 Istat ® 3.5 (GraphPad Software inc., San Diego, California, USA). Assessing of fungus precence was performed by nuber of twigs positive to presence of *B. nummularia* against twigs total number for each sampling period into forest site of Viterbo.

14.4 Coparison of two different forest sites

In order to compare presence/absence data of *B. nummularia* detected by real time PCR in 2 Italian forest sites with different water stress conditions, as number of positive twig samples collected in 4 periods, chi-square value was calculated using GraphPad Prism 4 Istat ® 3.5 software (GraphPad Software inc., San Diego, California, USA).

15. Results: objective 4

15.1 Coparison of Isolation with Real-time detection

Iteresting results were obteined by comparaison between traditional isolation on fungal culture media and real-time PCR approaches to assessing incidence of *B. nummularia* into 30 symptomless beech shoot samples collected from 10 plants during 4 different sampling periods in Viterbo. How suggests plot in Figure 27, results showed highly significant differences between two methods in detection of the pathogen in all sampling periods (Fisher's exact test, P<0.001). Isolation from symptomless shoots on PDA was poor, with higher frequency in February (53%), whereas the percentage of detection of *B. nummularia* from symptomless tissues using quantitative Rt PCR was always very high with an incidence equal or slightly less of 100%.

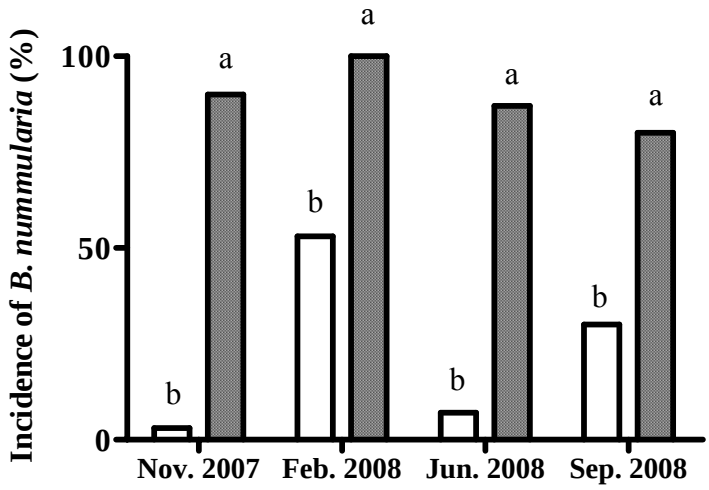


Fig. 27. Incidence of *B. nummularia* comparing detection data by isolation on agar medium and quantitative real-time PCR from European beech shoots, sampling into Viterbo forest area during four periods. = isolation; = realtime PCR.

15.2 Coparison of two different forest sites

Results show that were always obteined high positive isolation values of *B. nummularia* from healthy twig tissues collected in "La Faggeta" of Viterbo (Site 2) (Fig. 28). In particular, in Site 2 were obteined incidence values greater than "La Spianessa" (Site 1) (P<0,0001) excepted in June 2008, with number of positive twigs to fungus presence slightly higher in Site 1 than the other (χ^2=4.29, P<0,03). Whereas *B.*

nummularia was not detected from beech shoots collected at Site 1 during February 2008 (χ^2=60, p<0,001).

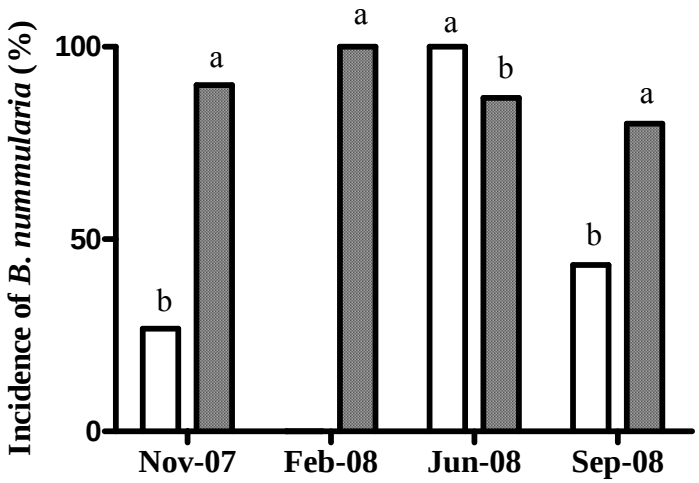


Fig. 28. Incidence of *B. nummularia* by real time PCR comparing detection data in 2 different beech forest sites. ▭= "La Spianessa" (Site 1); ▭= "La Faggeta" (Site 2).

16. Discussion: objective 4

The present study has confirmed the early detection of *B. nummularia* on symptomless European beech in Central Italy using quantitative Real time PCR, and greater ability of this molecular approach compared to traditional isolation technic on colture agar media. The study confirmed the endophytic habit of this fungus according to Luchi *et al*. (2006). In forest site of Viterbo, in comparison with isolation technique, quantitative Real time PCR enabled *B. nummularia* presence to be detected at a higher frequency in symptomless beech woody samples. After this study it will be easier to assess and quantify fluctuations of *B. nummularia* in its endophytic phase at different seasons. During February 2008, where frequency values was the highest using both methods, *B. nummularia* was detected in 100% with quantitative Rt PCR, but in only 53% using isolation on PDA, confirming the high specificity of the molecular method that is able to detect the presence of *B. nummularia* twice as much as isolation do (Fig 27). In particular results confirmed the great repeatability of the molecular assay, showing by simil incidence values always obteined during different periods sampling, compared to isolation method with strongly differences at different seasons, in meaning of detection of fungus, confirming presence of isolation boundary of fungal endophytes.

This method has always given important results with DNA extracted from woody samples collected in different areas of the Apennines (Fig. 28). Differences could be explained by the ecological behaviour of xylariaceous fungus that is more common in Central Italy in contrast to more northerly test areas (Luchi *et al*., 2005; 2006). This finding was consistent with Danti *et al*. (2002), who were looking for endophytic fungi in the European beech bark in the same region. It seems that in Central and South Apennines the occurrence of *B. nummularia* in asymptomatic beech tissue is more common, and not only limited to the infection sites from which fungi spread, probably because levels of environmental stress are higher than that in the northern sites (Capretti *et al.*, 2003; Granata and Sidoti, 2004). In particular, most presence of *B. nummularia* in forest site of Viterbo, with longer water stress conditions than forest site of Pistoia ($P<0,05$) and major altitude, confirmed higher risk associated with our beech forest in Central Italy than other equal phyto-coenosis located at most latitudes, in terms of declining damages and loss of biodiversity.

17. Conclusion: objective 4

Present study, based on use of quantitative Rt PCR for detection of *B. nummularia* in healthy beech plants, increases the understanding of presence and ecological behaviours of initial infection phases of this fungal pathogens in forests of Mediterranean basin. Again, this work confirms strategic position of Monti Cimini area in order to monitoring of declining infections caused by *B. nummularia* as prelude to prevention study about spreading of that damage disease.

Fungal endophytes comprise a diverse group of species that vary in symbiotic and ecological functions. It is clear that these fungi can have profound impacts on the survival and fitness of plants in all terrestrial ecosystems, and therefore likely play a significant role in plant biogeography, evolution and community structure. In addition, several new and emerging molecular technologies (Real time PCR, T-RFLP, Pyrosequencing) are now being applied to better characterize endophytes and their roles in plant ecophysiology (e.g. Arnold and Lutzoni, 2007; Macia'-Vicente *et al.*, 2008). These data can feed directly into studies of the systematics and taxonomy of these little-known fungi to better evalueted their nature "pathogenic" or "non-pathogenic". In addition, as we learn more about the contribution of endophytes to plant gene expression, it will be possible to profile gene expression patterns to assess the symbiotic status of both single fungal endophyte and plant communities. Ultimately it should be possible to determine metabolic activity of all fungal symbionts associated with plants across landscapes, and consequently the community structure. This may allow the development of new tools to assess changes in ecosystems resulting from natural fluctuations, climate change, and other anthropogenic features of environmental modification. More extensive characterization of different endophyte–plant associations, by combination of morphological and molecular approaches may provide greater insight into the evolution of mutualisms. Our limited understanding of such important plant–endophyte interactions is a testament to the fact that the 'age of discovery' is just beginning. Until we understand more about the significance of endophytes in plant biology and the genetic and ecological factors that affect switching to pathogenic fase, our understanding of plant community dynamics and ecosystem function will be limited. Before scientists typically followed fairly reductionist approaches to studying plant–microbe interactions. We are now poised to begin taking a

systems approach to plant symbiosis by studying multiple symbionts within individual host plants, and multiple hosts within a single habitat or across landscapes.

References

Adair S, Kim SH, Breuil C, 2002. A molecular approach for early monitoring of decay basidiomycetes in wood chips. FEMS Microbiol Lett 211, 117-122

Allen N and J Ngonga, 2003. "Building Kenya Together — Conference on Private Sector participation in Kenya's Infrastructure", Background Paper on Water and Sanitation, PricewaterhouseCoopers, Nairobi, Kenya

Allen TW, Burpee LL, Buck JW, 2004. In vitro attachment of phylloplane yeasts to *Botrytis cinerea*, *Rhizoctonia solani*, and *Sclerotinia homoeocarpa*. Canadian Journal of Microbiology 50, 1041-1048

Anderson C, 2001. The adaptive value of inactive foragers and the scout-recruit system in honey bee *Apis mellifera* colonies. Behavioral Ecology 121, 111-119

Anderson C, Rothermich JR, Bonabeau E, 2005. Modeling, Quantifying and Testing Complex Aggregate Service Chains 2005 IEEE International Conference on Web Services ICWS 2005, July 2005, Orlando, Florida

Andrews JH, 2006. Population growth and the landscape ecology of microbes on leaf surfaces. In: Bailey MJ, Lilley AK, Timms- Wilson TM, Spencer-PhillipsPTN eds, Microbial Ecology of Aerial Plant Surfaces. CAB International, Wallingford pp. 239-250

Anselmi N, Celerino GP, Franceschini A, Granata G, Luisi N, Marras F, Mazzaglia A, Mutto Accordi S, Ragazzi A, 2004. Geographic distribution of fungal endophytes of *Quercus* spp. in Italy, in: Ragazzi A, Moricca S, Dellavalle I Eds, Endophytism in forest trees, Accad It Sci For, Firenze pp 75-89

Anselmi N, Mazzaglia A, Vannini A, 2000. The role of endophytes in oak decline, in: Ragazzi A, DellaValle I Eds, Decline of oak species in Italy problems and perspectives, Accad Sci For, Firenze pp 129-144

Anzalone B, 1961. *Sul limite altimetrico inferiore del faggio nella regione laziale*. *Ann. Bot.* 27, 80- 109

Anzalone B, 1980. *Escursione ai Monti Cimini. Atti e Resoconti Sociali. Informatore Botanico Italiano* 12, 32-39

Arnold AE, Lutzoni F, 2007. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? Ecology 88, 541-549

Arnold AE, Maynard Z, Gilbert GS, 2001. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. Mycological Research 105, 1502-1507

Arnold AE, Mejı´a LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA, 2003. Fungal endophytes limit pathogen damage in a tropical tree. PNAS, 100 15649-15654

Arnold, AE, Henk, DA, Eells, RL, Lutzoni, F and Vilgalys, R 2007. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99, 185-206

Arnold, AE, Maynard, Z, Gilbert, GS, Coley, PD and Kursar, TA 2000. Are tropical fungal endophytes hyperdiverse?. Ecology Letters 3, 267-274

Bahnweg G, Heller W, Stich S, Knappe C, Betz C, Heerdt C, Kehr RD, Ernst D, Langebartels C, Nunn AJ, Rothenburger J, Schubert R, Wallis P, Müller-Starck G, Werner H, Matyssek R, Sandermann H Jr 2005. Beech leaf colonization by the endophyte *Apiognomia errabunda*. Plant Biol 7, 659-669

Barengo N, Sieber TN, Holdenrieder O. 2000. Diversity of endophytic mycobiota in leaves and twigs of pubescent birch *Betula pubescens*. Sydowia 52, 305-320

Baum S, Sieber TN, Schwarze FWMR, Fink S, 2003. Latent infections of *Fomes fomentarius* in the xylem of European beech *Fagus sylvatica*. Mycological Progress 2, 141-148

Bayman P, Otero JT, 2006. Microbial endophytes of orchid roots: Diversity and effects on plants, pp 153-178 In B Schulz, C Boyle, and T Sieber eds Microbial Root Endophytes Soil Biology, Vol 9 Springer-Verlag, Berlin and New York

Bayman P, Angulo-Sandoval P, Baez-Ortiz Z, Lodge DJ, 1998. Distribution and dispersal of Xylaria endophytes in two species in Puerto Rico. Mycological Research 102, 944-948

Bazzaz FA, 1990. Response of natural ecosystems to the rising global CO2 level. Annu Rev Ecol Syst 21, 167-196

Bills GF, 1996. Isolation and analysis of endophytic fungal communities from woody plants. In Endophytic Fungi in Grasses and Woody Plants SC Redlin & LM Carris, eds 31-65. American Phytopathological Society Press, St Paul, MN

Biondi E, Casavecchia S, Frattaroli AR, Pirone G, Pesaresi S, Di Martino L, Galassi S, Paradisi L, Ventrone F, Angelini E, Ciaschetti G, 2008. Forest vegetation of the Upper Valley of the Vomano River central Italy Fitosociologia 45 1, 117-160

Biondi F, 1992. Development of a tree-ring network for the Italian Peninsula. Tree Ring Bull 52,15-29

Blackwell M, Hibbett DS, Taylor JW, Spatafora JW, 2006. Research coordination networks: a phylogeny of the kingdom Fungi Deep Hypha. Mycologia 98, 829-837

Bohn U, 1992 *Zum internationalen Projekt einer Karte der natürlichen Vegetation Europas im Maßstab* 1:2,5 *Mio. Konzept*, *Inhalt Erarbeitung*, *kartographische Darstellung und Anwendungsmöglichkeiten*. *Natur und Landschaft* 67 476-480

Brasier CM, Beales PA, Kirk SA, Denman S, Rose J, 2005. *Phytophthora kernoviae* sp. *nov*., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in Britain. Mycological Research 109, 853-859

Bray JR, Curtis JT, 1957. An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr 27, 325-349

Bréda N, Huc R, Granier A, Dreyer E, 2006. Temperate forest trees and stands under severe drought: a review of ecophysiological responses, adaptation processes and long-term consequences. Ann For Sci 63, 625-644

Brook RD, Brook JR, Urch B, Vincent R, Rajagopalan S, Silverman F. 2002. Inhalation of fine particulate air pollu-tion and ozone causes acute arterial vasoconstriction in healthy adults. Circulation 10513, 1534-1536

Buée M, Reich M, Murat C, Morin E, Nilsson RH, Uroz S, Martin F, 2009. 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol. 184, 449-456

Butin H, 1983. Krankheiten der Wald- und Parkbäume. Georg Thieme Verlag, Stuttgart.

Butin H, 1995. Tree Diseases and Disorders Oxford: Oxford University Press

Butler MJ, Day AW, 1998. Fungal melanins: a review. Can J Microbiol 44, 1115-1136

Capretti P, Menguzzato G, Maresi G, Luchi N and Moriondo F, 2003. *Fenomeni di deperimento e di moria in popolamenti artificiali misti di latifoglie e conifere* - Ann Acc Ital Sci For, LII, 3-30

Carroll G, 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology, 69, 2-9

Carroll G, 2006. Host specificity among endophytes in transient plant communities. In: 8th International Mycological Congress, Congress Handbook & Abstracts Book 1. SAPMEA Conventions, Australia, p. 310.

Carroll GC, 1995. Forest endophytes: pattern and process. Canadian Journal of Botany 73 suppl 1, S1316-S1324
Carroll GC, 1986. The biology of endophytism in plants with particular reference to woody perennials. In Microbiology of the Phyllosphere N Fokkema & J van den Heuvel, eds, 205-222 Cambridge University Press, Cambridge, UK
Carroll Ge, Carroll FE, 1978. Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. Canadian Journal of Botany 56, 3034-3048
Chao A, Shen TJ, Hwang WH, 2006. Application of Laplace's boundary-mode approximations to estimate species and shared species richness. Aust NZ J Stat 48, 117-128
Chapela IH, Boddy L, 1988. Fungal colonization of attached beech branches. I. Early stages of development of fungal communities. New Phytol 110, 39-45
Chapela IH, Petrini O, Bielser G, 1993. The physiology of ascospore eclosion in *Hypoxylon fragiforme*: mechanisms in the early recognition and establishment of an endophytic symbiosis. Mycol Res 97, 157-162
Chapela IH, Petrini O, Hagmann L, 1991. Monolignol glucosides as specific recognition messengers in fungus- plant symbioses. Physiol Mol Plant Path 39, 289-298
Clarke KR, Warwick RM, 2001. Change in Marine Communities: an Approach to Statistical Analysis and Interpretation, 2nd edn Primer-E Ltd, Plymouth, UK
Clay K, 1991. Fungal endophytes, grasses, and herbivores. In: Barbosa P, Krischik VA, Jones CG eds Microbial mediation of plant-herbivore interactions, Wiley, New York, pp 199-226
Clay K, Schardl C, 2002. Evolutionary Origins and Ecological Consequences of Endophyte Symbiosis with Grasses. Am. Nat. 160, 99-127.
Clay K, 1989. Clavicipitaceous endophytes of grasses: Their potential as biocontrol agents Mycol Res 92, 1-12
Clement BG, Kehl LE, DeBord KL, Kitts CL, 1998. Terminal restriction fragment patterns TRFPs, a rapid, PCR-based method for the comparison of complex bacterial communities. J Microbiol Methods 31, 135:142
Coddington JA, Agnarsson I, Miller JA, Kuntner M, Hormiga G, 2009. Undersampling bias: the null hypothesis for singleton species in tropical arthropod surveys. J Anim Ecol 78, 573-584
Colditz F, Braun H P, Jacquet J, Niehaus K, Krajinski F, 2005. Proteomic profiling unravels insights into the molecular background underlying increased *Aphanomyces euteiches*—tolerance of *Medicago truncatula*. Plant Molecular Biology 59, 387-406
Collado J, Platas G, Paulus B, Bills GF, 2007. High-throughput culturing of fungi from plant litter by a dilution-to-extinction technique. FEMS Microbiology Ecology 60, 521-533.
Collado J, Platas G, Pelaez F, 2001. Identification of an endophytic *Nodulisporium* sp. from *Quercus ilex* in central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequence analysis and effect of different ecological factors on distribution of the fungus. Mycologia 93, 875-886
Colwell RK, Coddington JA, 1994. Estimating terrestrial biodiversity through extrapolation. Philosophical Transactions of the Royal Society of London, Series B, 345, 101-118
Dai CC, Yu BY, Li X, 2008. Screening of endophytic fungi that promote the growth of *Euphorbia pekinensis*. Afr. J. Biotechnol. 7, 3505-3509
Dale VH, Joyce LA, McNulty S, Neilson RP, Ayres MP, Flannigan MD, Hanson PJ, Irland LC, Lugo AE, Peterson CJ, Simberloff D, Swanson FJ, Stocks BJ, Wotton BM, 2001. Climate change and forest disturbances. Bioscience 51 723-734.

Danti R, Sieber TN, and Sanguineti, G 2002 Endophytic mycobiota in bark of European beech Fagus sylvatica in the Apennines Mycological Research 106, 1343-1348

Davis JE, Kubisiak TL, Milgroom MG, 2005. Polymorphic sequence-characterized codominant loci in the chestnut blight fungus, *Cryphonectria parasitica*. Molecular Ecology Notes 5, 195- 197.

Del Favero R, 2008. *I boschi delle regioni meridionali e insulari d'Italia. Tipologia*, *funzionamento*, *selvicoltura. CLEUP Padova*, Italy.

Desprez-Loustau ML, Marcais B, Nageleisen LM, Piou D, Vannini A, 2006. Interactive effects of drought and pathogens in forest trees. Ann For Sci 63, 597-612

Di Filippo A, Biondi F, Cûfar K, De Luis M, Grabner M, Maugeri M, Presutti Saba E, Schirone B, Piovesan G, 2007. Bioclimatology of beech *Fagus sylvatica* L. in the Eastern Alps: spatial and altitudinal climatic signals identified through a tree-ring network. J Biogeogr 34, 1873-1892

Di Filippo A, 2005. *Le foreste vetuste: ecosistemi chiave per la conservazione della biodiversità e lo sviluppo di una selvicoltura a basso impatto ambientale. Linea Ecologica* 2, 22-25

Dickie IA, Reich PB 2005 Ectomycorrhizal fungal communities at forest edges. J Ecol 93:244-255

Dickie IA, FitzJohn RG, 2007. Using terminal-restriction fragment length polymorphism T-RFLP to identify mycorrhizal fungi; a methods review Mycorrhiza 17, 259-270

Dittmar C, Zech W, Elling W, 2003. Growth variations of Common beech *Fagus sylvatica* L. under different climatic and environmental conditions in Europe-a dendroecological study. For Ecol Manage 173, 63-78

Docherty M, Salt DT, Holopainen JK, 1997. Impacts of climate change and pollution on forest insect pests. In: Forests and insects eds Watt AD, Stork NE, Hunter MD, pp 229-247. Chapman and Hall, London.

Dorworth CE, Macey DE, Sieber TN, Woods TAD, 1996. Biological control of red alder *Alnus rubra* with indigenous pathogenic Ascomycotina. Canadian Journal of Plant Pathology 18, 315-324

Dunbar J, Ticknor LO, Kuske CR, 2000. Assessment of microbial diversity in four southwestern united states soils by 16S rRNA gene terminal restriction fragment analysis. Appl Environ Microbiol 66, 2943-2950

Dunbar J, Ticknor LO, Kuske CR, 2001. Phylogenetic specificity and reproducibility and new method for analysis of terminal restricttion fragment profiles of 16S rRNA genes from bacterial communities Appl Environ Microbiol 67, 190-197

Eckstein D, Richter K, Aniol RW, Quiehl G, 1984. *Dendroklimatologische Untersuchungen zum Buchensterben im su¨dwestlichen Vogelsberg. Forstwiss Cent* 103, 274-289

Edwards IP, Cripliver JL, Gillespie AR, Johnsen KH, Scholler M, Turco RF, 2004. Nitrogen availability alters macrofungal basidiomycete community structure in optimally fertilized loblolly pine forests. New Phytol 162, 755-770

Edwards IP, Turco RF, 2005. Inter- and intraspecific resolution of nrDNA TRFLP assessed by computer-simulated restriction analysis of a diverse collection of ectomycorrhizal fungi. Mycol Res 109, 212-226

European Environment Agency EEA 2004 Impacts of Europe's changing climate. EEA, Copenhagen, 100 p.

Fahey JW, Dimock MB, Tomasino SF, Taylor JM, Carlson PS, 1991. Genetically engineered endophytes as biocontrol agents: a case study in industry, p.401-411. In

Andrews JH, Hirano SS eds Microbial ecology of leaves. New York, Springer, Verlag, 499p.
Fenaroli L, 1966. *Il Gargano, suoi aspetti vegetazionali e floristici. Annali Accademia Italiana di Scienze Forestali* 15, 107-135.
Fisher MC, Koenig GL, White TJ, Taylor JT, 2002. Molecular and phenotypic description of *Coccidioides posadasii* sp. *nov.*, previously recognized as the non-California population of *Coccidioides immitis*. Mycologia 94, 73-84
Fisher PJ, Petrini O, 1990. A comparative study of fungal endophytes in xylem and bark of *Alnus* species in England and Switzerland. Mycol. Research. 94, 313-319
Franzen-Reuter I, Gehrmann J, Frahm JP, 2006. *Veränderungen des Epiphytenbewuchses zwischen* 2002 *und* 2004 *auf Dauerbeobachtungsflächen in Nordrhein-Westfalen. LÖBF-Mitteilungen* 2, 06, 2-7
Freeman S, Rodriguez JR, 1993. Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science 260, 75- 78
Frelich LE, 2002. Forest dynamics and disturbance regimes. Cambridge University Press, Cambridge, UK
Gamboa MA, Laureano S, Bayman P, 2002. Measuring diversity of endophytic fungi in leaf fragments: does size matter?. Mycopathologia 156, 41-45
Gao FX, Dai CC, Liu XZ, 2010. Mechanisms of fungal endophytes in plant protection against pathogens. African Journal of Microbiology Research 413, 1346-1351, 4 July, 2010.
Gerten D, Schaphoff S, Lucht W, 2007. Potential future changes in water limitations of the terrestrial biosphere. Climatic Change 80, 277-299
Giménez C, Cabrera R, Reina M, Coloma A, 2007. González. Fungal endophytes and their role in plant protection. Curr. Org. Chem. 11, 707-720
Gleick PH, Chalecki EL, 1999. "The Impacts of Climatic Changes for Water Resources of the Colorado and Sacramento-San Joaquin River Basins" Journal of the American Water Resources Association 35, 6, 1429-1441, December 1999
Gotelli NJ, RK Colwell, 2001. Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecology Letters 4, 379-391
Grabherr G, Gottfried M, Pauli H, 1994. Climate effects on mountain plants. Nature 369 448.
Granata G, Sidoti A 2004. *Biscogniauxia nummularia*: pathogenic agent of a beech decline. Forest patology 34, 6, 363-367 - Blackwell publishing
Granata G, Whalley AJS, 1994. Decline of beech associated to *Biscogniauxia nummularia* in Italy. Petria 4, 111-116.
Gregory D, Kershaw R, Inness PM, 1997. Parameterization of momentum transport by convection II: Tests in single-column and general circulation models QJR Meteorol Soc 123, 1153-1183
Grünig CR, Brunner PC, Duò A, Sieber TN, 2007. Suitability of methods for species recognition in the *Phialocephala fortinii- Acephala applanata* species complex using DNA analysis. Fungal Genetics and Biology 44, 773-788
Guglielmo F, Gonthier P, Garbelotto M, Nicolotti G, 2008. A PCRbased method for the identification of important wood rotting fungal taxa within *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l. FEMS Microbiology Letters 282, 228-237
Gunatilaka AAL, 2006. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J. Nat. Prod, 69, 509-526

Guo LD, Huang GR, Wang Y, 2008. Seasonal and tissue age influences on endophytic fungi of *Pinus tabulaeformis* Pinaceae in the Dongling Mountains, Beijing. J Integr Plant Biol 50, 997- 1003

Gutiérrez E, 1988. Dendroecological study of *Fagus sylvatica* L. in the Montseny mountains Spain. Acta Oecol 9, 301-309

Haeberli W, Beniston M, 1998. Climate change and its impacts on glaciers and permafrost in the Alps. Ambio 27, 258-265

Hamilton JG, DeLucia EH, George K, Naidu SL, Finzi AC, Schlesinger WH, 2002. Forest carbon balance under elevated CO2. Oecologia 131, 250-260

Hämmerli UA, Brändle UE, Petrini O, McDermott JM, 1992. Differentiation of isolates of *Discula umbrinella* Teleomorph, *Apiognomonia errabunda* from beech, chestnut, and oak using randomly amplified polymorphic DNA markers. Molecular Plant-Microbe Interactions 5, 479-483

Harrington TC, Wingfield BD, 1995. A PCR-based identification method for species of *Armillaria*. Mycologia 87, 280-288

Haskins KE, Gehring CA, 2004. Interactions with juniper alter the abundance and composition of pinyon pine ectomycorrhizal fungal communities. Ecology 85, 2687-2692

Heath J, 1998. Stomata of trees growing in CO2-enriched air show reduced sensitivity to vapour pressure deficit and drought. Plant Cell Environ 21, 1077-1088

Heath J, Kerstiens G, 1997. Effects of elevated CO2 on leaf gas exchange in beech and oak at two levels of nutrient supply: consequences for sensitivity to drought in beech. Plant Cell Environ 20, 57-67

Helander ML, Neuvonen S, Sieber T, Petrini O, 1993. Simulated acid rain affects birch leaf endophyte populations. Microbial Ecology 26, 227

Hendry SJ, Boddy L, Lonsdale D, 2002. Abiotic variables effect differential expression of latent infections in beech *Fagus sylvatica.* New Phytol. 155, 449-460

Hendry SJ, Lonsdale D, Boddy L, 1998. Strip cankering of beech *Fagus sylvatica*: pathology and distribution of symptomatic trees. New Phytol. 140, 549-565

Heungens, K, De Dobbelaere I, Maes M, 2006. Fungicide control of *Phytophthora ramorum* on rhododendron, 241-257, in SJ Frankel, PJ Shea, MI Haverty tech. coord.. Proceedings of the Sudden Oak Death Second Science Symposium: The State of Our Knowledge. US Dept. Agric. For.Serv. Gen. Tech Rep. PSW-GTR-196.

Hibbett DS Binder, Bischoff JF, Blackwell M, Canon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch HT, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson KH, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo JM, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schuessler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N, 2007. A higher-level phylogenetic classification of the Fungi. Mycological Research 111, 509-547

Hofmann A, 1960. *Il faggio in Sicilia. Flora et vegetatio italica*, *Memoria* n. 2, *Gianasso Ed.*, *Sondrio*, Italy.

Hofmann A, 1961. La *faggeta depressa del Gargano. Delpinoa* 3, 375-406

Hofmann A, 1991. *Il faggio e le faggete in Italia. MAF CFS*, *Collana Verde*, 81

Holland PM, Abramson RD, Watson R, Gelfand DH, 1991. Detection of specific polymerase chain reaction product by utilizing the 5′ to 3′ exonuclease activity of *Thermus acquaticus* DNA polymerase. Proc Natl Acad Sci USA 88, 7276-7280

Hughes RM, Kaufmann PR, Herlihy AT, Intelmann SS, Corbet SC, Arbogast MC, *et al*. 2002. Electrofishing distance needed to estimate fish species richness in raftable Oregon rivers North American. Journal of Fisheries Management 22, 1229-1240

Hungate BA, Chapin III FS, Zhong H, Holland EA, Field CB, 1997. Stimulation of grassland nitrogen cycling under carbon dioxide enrichment. Oecologia 109, 149-153

Hunt J, Boddy L, Randerson PF, and Rogers HJ, 2004. An evaluation of 18S rDNA approaches for the study of fungal diversity in grassland soils Microb Ecol 47, 385-395

Huntley ME, Karl DM, Niiler P, Holm-Hansen O, 1991. Research on Antarctic Coastal Ecosystem Rates RACER: an interdisciplinary field experiment. Deep Sea Res 38, 911-941

Hyde KD, Soytong K, 2007. Understanding microfungal diversity - a critique Cryptogamie Mycologie 28, 1-9

INFC, 2005. *Inventario Nazionale delle Foreste e dei Serbatoi Forestali di Carbonio. Ministero delle Politiche Agricole Alimentari e Forestali*, *Ispettorato Generale - Corpo Forestale dello Stato*, *CRA - Istituto Sperimentale per l'Assestamento Forestale e per l'Alpicoltura.* [online]
URL:http://www.sian.it/inventarioforestale/jsp/ home.jsp

Intergovernmental Panel on Climate Change, IPCC, 2007. Fourth assessment report in summary for policymakers., Geneva. Available from
http://www.ipcc.ch/pub/spm22- 01.pdf.

International Energy Agency IEA Oil Crises and Climate Challenges — Geothermal Energy Annual Report 2005.
http://www.ieagia.org/documents/GIA2005AnnualReportDraftWairakei4Dec2006Gina5Dec06_000.pdf

Johannesson H, Stenlid J, 1999. Molecular identification of wood inhabiting fungi in an unmanaged *Picea abies* forest in Sweden. Forest Ecology and Management 115, 203-211

Johnson NC, Graham JH, Smith FA, 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum New Phytol. 135, 575-586

Joughin I, Abdalati W, Fahnestock M, 2004. Large fluctuations in speed on Greenland's Jakobshavn Isbrae glacier. Nature 432, 608-610

Ju Y-M, Rogers JD, San Martin F, Granmo A, 1998. The genus *Biscogniauxia*. Mycotaxon 66, 1-98

Ju Y-M, Rogers JD, 1996. A revision of the genus *Hypoxylon.* Mycologia Memoir 20 APS Press, St Paul, MN 365p.

Jumpponen A, Jones KL, 2009. Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. New Phytologist.

Kaplan CW, Astaire JC, Sanders ME, Reddy BS, Kitts CL, 2001. 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed *Lactobacillus acidophilus.* NCFM Appl Environ Microbiol 67, 1935-1939

Kogel, K-H; Franken, P; Huckelhoven, R 2006 Endophyte or parasite - what decides? Current Opinion in Plant Biology 9:358-363

Kowalski T, Kehr RD, 1997. Fungal endophytes o living branch bases in several European tree species in Endophytic fungi in grasses and woody plants: systematics, ecology and evolution SC Redlin, LM Carris, ed. APS Press USA, 67-86

Kowalski T, Kehr RD, 1996. Fungal endophytes of living branch bases in several European tree species. In Endophytic Fungi in Grasses and Woody Plants SC Redlin, LM Carris, eds 67-86 American Phytopathological Society Press, St Paul, MN

Kullman L, 2001. 20th century climate warming and tree-limit rise in the southern Scandes of Sweden. Ambio 30, 72-80

Kumar S, Tamura K, Jakobsen IB, Nei M, 2001. MEGA2: Molecular Evolutionary Genetics Analysis software Bioinformatics, 17, 1244-1245

La Porta N, Capretti P, Korhonen K, Kammiovirta K, Karjalainen R, 1997. The relatedness of the Italian F intersterility group of *Heterobasidion annosum* with the S group, as revealed by RAPD assay. Mycol Res 101, 1065-1072

Læssøe T, Granmo A, Scheuer C, 1999. *Biscogniauxia granmoi* Xylariaceae in Europe - Österr Z Pilzk 8, 139-147

Law BE, Falge E, Gu L, Baldocchi DD, Bakwin P, Berbigier P, Davis K, Dolman AJ, Falk M, Fuentes JD, Goldstein A, Granier A, Grelle A, Hollinger D, Janssens IA, Jarvis P, Jensen NO, Katul G, Mahli Y, Matteucci G, Meyers T, Monson R, Munger W, Oechel W, Olson R, Pilegaard K, Paw KT, Thorgeirsson H, Valentini R, Verma S, Vesala T, Wilson K, Wofsy S 2002 Environmental controls over carbon dioxide and water vapour exchange of terrestrial vegetation. Agr Forest Meteorol 113, 97-120

Lebourgeois F, Breda N, Ulrich E, Granier A, 2005. Climate-treegrowth relationship of European beech *Fagus sylvatica* L. in the French Permanent Plot Network RENECOFOR. Trees 19, 385-401

Lee DH, Veizer J, 2003. Water and carbon cycles in the Mississippi River basin: potential implications for the northern hemisphere residual terrestrial sink. Global Biogeochem Cy 17 1037

Leser TD, Lindecrona RH, Jensen TK, Jensen BB, Møller K, 2000. Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with *Brachyspira hyodysenteriae* Appl Environ Microbiol 66, 3290-3296

Leugnerová G, 2007. *Fagus sylvatica* L. - European Beech. http://botany.cz/en/fagus-sylvatica/print/.pdf.

Lewis GC, 2004. Effects of biotic and abiotic stress on the growth of three genotypes of *Lolium perenne* with and without infection by the fungal endophyte *Neotyphodium lolii*. Ann. Appl. Biol. 144, 53-63

Lindroth RL, Kinney KK, Platz CL, 1993. Responses of deciduous trees to elevated atmospheric CO2: productivity, phytochemistry, and insect performance. Ecology 74, 763-777

Livak K, Jeff M, Flood S, 1995. Guidelines for designing TaqMan fluorogenic probes for 5′ nuclease assay Perkin Elmer Applied Biosystems Research News

Lonsdale D, 1983. Some aspects of the pathology of environmentally stressed trees. Yearbook of the International Dendrology Society 1982, 90-97

Lord NS, Kaplan CW, Shank P, Kitts CL, Elrod SL, 2002. Assessment of fungal diversity using terminal restriction fragment TRF pattern analysis: comparison of 18S and ITS ribosomal regions. FEMS Microbiol Ecol 42, 327-337

Lorenzoni P, Mirabella A, Bidini D 1995 Soil genesis on trachytic and leucititic lavas of Cimini volcanic complex Latium, Italy. Geoderma 68, 79-99

Luchi N, Capretti P, Surico G, Orlando C, Pazzagli M, Pinzani P 2005 A realtime quantitative PCR assay for the detection of *Sphaeropsis sapinea* from inoculated *Pinus nigra* shoots. Journal of Phythopathology 153, 37-42

Luchi N, Capretti P, Vettraino A M, Vannini A, Pinsani P e Pazzagli M, 2006.- Early detection of *Biscogniauxia nummularia* in symptomless European beech *Fagus sylvatica* L. by TaqMan™ quantitative real-time PCR. Applied Microbiolgy 43, 1, 33-38

Maciá-Vicente JG, Jansson HB, Abdullah SK, Descals E, Salinas J, Lopez-Llorca LV, 2008. Fungal root endophytes from natural vegetation in Mediterranean environments with special reference to Fusarium spp. FEMS Microbiol Ecol 64, 90-105

Malinowski DP, Zuo H, Belesky DP, Alloush GA, 2004. Evidence for copper binding by extracellular root exudates of tall fescue but not perennial ryegrass infected with *Neotyphodium* spp. endophytes. Plant Soil 267, 1-12

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, *et al.*, 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64,795-799

Marsh H, 2000. Evaluating management initiatives aimed at reducing the mortality of dugongs in gill and mesh nets in the Great Barrier Reef World Heritage Area. Marine Mammal Science 16, 684-694

Marsh TL, 1999. Terminal restriction fragment length polymorphism T-RFLP: an emerging method for characterizing diversity among homologous populations of amplification products. Curr Opin Microbiol 2, 323-327

Mazzaglia A, Anselmi N, Gasbarri A, Vannini A, 2001. Development of a polymerase chain reaction PCR assay for the specific detection of *Biscogniauxia mediterranea* living as endophyte in oak tissue Mycol Res 105, 952-956

McArdle BH, Anderson MJ, 2001. Fitting multivariate models to community data: A comment on distance-based redundancy analysis Ecology 82 1, 290-297

Meier IC, Leuschner C, 2008. Belowground drought response of European beech: fine root biomass and carbon partitioning in 14 mature stands across a precipitation gradient. Glob Change Biol 14, 2081-2095

Meier IC, Leuschner C, 2008. Genotypic variation and phenotypic plasticity in the drought response of fine roots of European beech. Tree Physiol 28, 297-309

Meier IC, Leuschner C, 2008. Leaf size and leaf area index in *Fagus sylvatica* forests: competing eVects of precipitation, temperature, and nitrogen availability. Ecosystems 11, 655-669

Meister B, Krauss J, Härri SA, Schneider MV, Müller CB, 2006. Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. Basic and Applied Ecology 7, 244-252

Meshinev T, Apostolova I, Koleva E, 2000. Influence of warming on timberline rising: a case study on *Pinus peuce* Griseb. in Bulgaria. Phytocoenologia 30, 431-438

Miller JH, 1961. A monograph of the world species of *Hypoxylon* Athens: Univ Georgia Press

Miller SL, 1986. Hypogeus fungi from the South-eastern United States I The genus Rhizopogon Mycotaxon 27, 193-218

Moeseneder MM, Arrieta JM, Muyzer G, Winter C, Herndl GJ, 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis Appl Environ Microbiol 65, 3518-3525

Montelucci G, 1956. *Aspetti della faggeta depressa del monte Fogliano. Nuovo Giornale Botanico Italiano* 63, 507-530
Mooney HA, Drake BG, Luxmoore RJ, Oechel WC, Pitelka LF 1991 Predicting ecosystem responses to elevated CO2 concentrations. Bioscience 41 96-104.
Morelet M, 1989. *L'anhracnose des chênes et du hêtre en France. Revue Forestière Française* 41, 488-496
Moricca S, Ragazzi A, 2008. - Fungal endophytes in Mediterranean oak forests: a lesson from *Discula quercina.* Phytopathology 98, 380-386
Moriondo F, Menguzzato G, Maresi G, Danti R, 1999. *Un caso di moria del faggio nel bosco della Ferdinandea sulle Serre Calabresi. Informatore Fitopatologico* 9, 45-51
Murali TS, Suryanarayanan TS, Venkatesan G, 2007. Fungal endophyte communities in two tropical forests of southern India: diversity and host affiliation. Mycological Progress 6, 191-199
Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J, 2000. Biodiversity hotspots for conservation priorities Nature, 403, 853-858
Nemani R, White M, Thornton P, Nishida K, Reddy S, Jenkins J, Running S, 2002. Recent trends in hydrologic balance have enhanced the terrestrial carbon sink in the United States. Geophys Res Lett 29 1468
Nocentini S, 2009. Structure and management of beech *Fagus sylvatica* L. forests in Italy. iForest 2, 105-113
Norby RJ, Luo Y 2004 Evaluating ecosystem responses to rising atmospheric CO2 and global warming in a multi-factor world. New Phytol 162 281-293.
Norby RJ, Hanson PJ, O'Neill EG, Tschaplinski TJ, Weltzin JF, Hansen RA, Cheng WX, Wullschleger SD, Gunderson CA, Edwards NT, Johnson DW, 2002. Net primary productivity of a CO2-enriched deciduous forest and the implications for carbon storage. Ecol Appl 12, 1261-1266
Nugent LK, Sihanonth P, Thienhirun S, Whalley AJS, 2005. Biscogniauxia: a genus of latent invaders. Mycologist 19, 40-43
Opik M, Metsis M, Daniell TJ, Zobel M, Moora M, 2009. Large-scale parallel 454 sequencing reveals host ecological group specificity of arbuscular mycorrhizal fungi in a boreonemoral forest New Phytol 184, 424-437
Orlando C, Pinzani P, Pazzagli M, 1998. Developments in quantitative PCR. Clin Chem Lab Med 36, 255-269
Osborne AM, Moore ERB, Timmis KN, 2000. An evaluation of terminal-restriction fragment length polymorphism T-RFLP analysis for the study of microbial structure and dynamics Environ Microbiol 2, 39-50
Osono T, Mori A, 2003. Colonization of Japanese beech leaves by phyllosphere fungi. Mycoscience 44, 437-441
Osono T, Takeda H, 1999. A methodological survey on incubation of fungi on leaf litter of *Fagus crenata* in Japanese with English summary. Appl For Sci Kansai 8, 103-108
Pandey AK, Reddy MS, Suryanarayanan TS, 2003. ITS RFLP and ITS sequence analysis of foliar endophytic fungus *Phyllosticta* from different tropical trees in India. Mycological Research 107, 439-444
Paoletti MG, Sommaggio D, Bressan M, Celano V, 1996. Can sustainable agricultural practices affect biodiversity in agricultural landscapes? A case study concerning orchards in Italy Acta Jutlandica 71, 241-254
Parfitt G, Shepherd P, Eston R 2007. Reliability of effort production using the children's CALER and BABE perceived exertion scales. J Exerc Sci Fitness 5, 49-55.

Parfitt D, Hynes J, Rogers HJ, Boddy L, 2005. New PCR assay detects rare tooth fungi in wood where traditional approaches fail Mycol Res 109, 1187-1194

Parmesan C, Yohe G, 2003. A globally coherent fingerprint of climate change impacts across natural systems. Nature 421 37-42.

Pehl L, Butin H, 1994. *Endophytische Pilze in Blättern von Laubbäumen und ihre Beziehungen zu Blattgallen Zoocecidien Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem* 297, 1-52

Peñuelas J, Boada M, 2003. A global change-induced biome shift in the Montseny mountains NE Spain. Glob Change Biol 9, 131-140

Petrini O, 1991. Fungal endophytes of tree leaves - Andrews and Hirano Editors, Microbial Ecology of Leaves, 179-197

Petrini O, Carroll G, 1981. Endophytic fungi in foliage of some Cupressaceae in Oregon. Canadian Journal of Botany 59, 629-636

Petrini O, Petrini LE, Rodrigues K, 1995. Xylariaceous endophytes: an exercise in biodiversity. Fitopatol Bras 20, 531-539

Phillips R, 1981. Mushrooms and other fungi of Great Britain and Europe. Pan books

Pignatti S, 1998. *I boschi d'Italia. UTET*, *Torino*, Italy

Piovesan G, 1998. Analisi di problemi assestamentali nella foresta montana dell'Appennino centrale. PhD Thesis, University of Tuscia, Viterbo, Italy

Piovesan G, Biondi F, Bernabei M, Di Filippo A, Schirone B, 2005. Spatial and altitudinal bioclimatic zones of the Italian peninsula identified from a beech Fagus sylvatica L. tree-ring network. Acta Oecol 27, 197-210

Piovesan G, Biondi F, Di Filippo A, Alessandrini A, Maugeri M, 2008. Drought-driven growth reduction in old beech *Fagus sylvatica* L. forests of the central Apennines, Italy. Global Change Biology 14, 1-17

Piovesan G, Bernabei M, di Filippo A, Romagnoli M, Schirone B, 2003. A long-term tree ring beech chronology from a high-elevation old-growth forest of Central Italy Dendrochronologia 21, 1, 13-22

Pirttilä AM, Pospiech H, Laukkanen H, Myllylä R, Hohtola A, 2003. Two endophytic fungi in different tissues of Scots pine buds *Pinus sylvestris* L.. Microb Ecol 45, 53-62

Podani J, 1994. - Multivariate data analysis in ecology and systematics. Ecological Computation Series, 6. SPD Academic Publishing, The Hague, 120 pp.

Pouzar Z, 1979. Notes on taxonomy and nomeclature of *Nummularia.* Pyrenomycetes Ceskà Mykologie 33, 207-219

Pouzar Z, 1986. A key and conspectus of Central European species of *Biscogniauxia* and *Obolarina.* Pyrenomycetes Ceská Mykol 40, 1-10

Prljincevic MB, 1982. Economic signicance of the infection of beech forests by *Hypoxylon deustum* HoffmEtFr Grev at Sara mountain - Eur J For Pathol 12, 7 n°10

Promputtha I, Lumyong P, Dhanasekaran V, McKenzie EHC, Hyde KD, Jeewon R, 2007. A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. Microbial Ecology 53, 579-590

Rayner ADM, 1986. Water and the origins of decay in trees. In "Water, Fungi and Plants" eds PG Ayres and L Boddy, pp. 321-341 Cambridge University Press

Rayner ADM, L Boddy, 1986. Population structure and the infection biology of wood-decay fungi in living trees. Adv. Pl. Pathol. 5, 119-160.

Redman CA, Coen PG, Matthews L, Lewis RM, Dingwall WS, Foster J D, Chase-Topping ME, Hunter N, Woolhouse ME J, 2002. Comparative epidemiology of scrapie outbreaks in individual sheep flocks. Epidemiology and Infection, 128 513-521.

Redman RS, Dunigan DD, Rodriguez RJ, 2001. Fungal symbiosis: from mutualist to parasitism, who controls the outcome, host or invader?. New Phytologist 151, 705-716

Rignot E, 2006. Changes in ice dynamics and mass balance of the Antarctic ice sheet. Philos T R Soc A 364, 1637-1655

Rogers JD, 2000. Thoughts and musings on tropical Xylariaceae. Mycol Res 104, 1412-1420

Rouault G, Candau JN, Lieutier F, Nageleisen LM, Martin JC, Warzee N, 2006. Effects of drought and heat on forest insect populations in relation to the 2003 drought in western Europe. Ann For Sci 63, 613-624

Rozas V, 2001. Detecting the impact of climate and disturbances on tree-rings of *Fagus sylvatica* L. and *Quercus robur* L. in a lowland forest in Cantabria, Northern Spain. Ann For Sci 58, 237-251

Saikkonen K, 2007. Forest structure and fungal endophytes. Fungal Biology Reviews 21, 67-74

Saikkonen K, Faeth SH, Helander M, Sullivan TJ, 1998. Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 29, 319-343

Santrucek J, Sage RF, 1996. Acclimation of stomatal conductance to a CO2-enriched atmosphere and elevated temperature in *Chenopodium album*. Aust J Plant Physiol 23, 467-478

Schröter D, Cramer W, Leemans R, 2005. Ecosystem service supply and vulnerability to global change in Europe. Science 5752, 1333 - 1337

Schulz B, Boyle C, 2005. The endophytic continuum. Mycol. Res. 109, 661-686

Schwarze FWMR, Lonsdale D, Mattheck C, 1995. - Detectability of wood decay caused by Ustulina deusta in Comparison with other tree-decay fungi - Eur J For Pathol. 25, 327 341

Scoppola A, Caporali C, 1997. *I boschi caducifogli mesofili con faggio della Provincia di Viterbo: aggiornamento sulla distribuzione. Annali Accademia Italiana di Scienze Forestali* 45, 167- 188

Scoppola A, 1999. *Tipologie vegetazionali delle faggete appenniniche. In Ecologia Strutturale e Funzionale di Faggete Italiane* G Scarascia Mugnozza, ed: 21-31 *Edagricole*, *Bologna*

Searle M P, Waters D J, Rex DC, Wilson RN, 1992. Pressure, temperature and time constraints on Himalayan metamorphism from eastern Kashmir and western Zanskar J Geol Soc Lond 149, 753-773

Seifert KA, 2008. Recent developments and progress on fungal dna barcoding. In: Towards a Barcoded Planet, Canadian Barcode of Life Network 2008 Scientific Symposium, p. 24 Royal Ontario Museum, Toronto, Canada.

Seifert KA, Samuels GJ, 2000. How should we look at anamorphs?. Studies in Mycology 45, 5-19

Sieber TN, Hugentobler C, 1987. “Endophytische pilze in blattern und asten gesunder und geschadigter buchen *Fagus sylvatica* L.” - European Journal of Forest Pathology 17, 411-425

Sieber TN, Sieber -Canavesi F, Dorworth CE, 1991. Endophytic fungi of red alder *Alnus rubra* leaves and twigs in British Columbia. Canadian Journal of Botany 69, 407-411

Sinclair WA, Lion HH, Johnson WT, 1987. Disease of Trees and Shrubs. Ithaca and London - Cornell University Press

Sinclair WA, Lion HH, Johnson WT, 1987. Disease of Trees and Shrubs Ithaca and London: Cornell University Press

Stambaugh WJ, 1989. Annosus root disease in Europe and the southeastern United States: Occurrence, research, and historical perspective Pages 3-9 in: Proc Symp Res Manag Annosus Root Dis *Heterobasidion annosum* in Western North Am W J Otrosina and R F Scharpf, eds US Dep Agric, For Serv, Monterey, CA

Stanosz, GR, Blodgett JT, Smith DR, Kruger EL, 2001. Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings New Phythologist 149, 531-538

Idso SB, 1999. The long-term response of trees to atmospheric CO2 enrichment. Glob Change Biol 5, 493-495

Stone JK, 1987. Initiation and development of latent infections by *Rhabdocline parkeri* on Douglas fir. Canadian Journal of Botany 65, 2614-2621

Stone EM, Reifsnyder C, McVey M, Gazo B, Pillus L, 2000. Two classes of sir3 mutants enhance the sir1 mutant mating defect and abolish telomeric silencing in *Saccharomyces cerevisiae*. Genetics 155, 509-522

Sturrock RN, 2007. Climate change effects on forest diseases: an overview. In Proceedings of the 54th Annual Western International Forest Disease Work Conference, 2-6 Oct. 2006, Smithers, B.C. Compiled by M.B. Jackson. US Department of Agriculture, Forest Service, Forest Health Protection, Missoula, Montana. pp. 51-55

Swofford DL, 1999. PAUP 4.0: Phylogenetic Analysis Using Parsimony And Other Methods. Sunderland, MA, Sinauer Associates, Inc.

Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B, 2005. A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol Microbiol 57,1036-1050

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24,4876-4882

Tiedje JM, Asuming-Brempong S, Nüsslein K, Marsh TL, Flynn SJ, 1999. Opening the black box of soil microbial diversity. Appl Soil Ecol 13, 109-122

Toti L, Viret O, Horat GE, Petrini O, 1993. Detection of the endophyte *Discula umbrinella* in buds and twigs of *Fagus sylvatica* - European Journal of forest pathology 23, 147-152

Toti L, Viret O, Chapela IH, Petrini O, 1992. Differential attachment by conidia of the endophyte, *Discula umbrinella* Berk & Br Morelet, to host and non-host surfaces. New Phytol 121, 469-475

Toti L, Viret O, Horat G, Petrini O, 1993. Detection of the endophyte Discula umbrinella in buds and twigs of *Fagus sylvatica* European Journal of Forest Pathology 23, 147-152

Tripathi S, Kamal S, Sheramati I, Oelmuller R, Varma A, 2008. Mycorrhizal fungi and other root endophytes as biocontrol agents against root pathogens. Mycorrhiza, 3 edition, pp. 281-306

Trowbridge J, Jumpponen A, 2004. Fungal colonization of shrub willow roots at the forefront of a receding glacier. Mycorrhiza 14, 283-293

Tuomi J, Niemalä P, Chapin III FS, Bryant JP, Sirin S 1988 Defensive responses of trees in relation to their carbon/nutrient balance. In: Mechanisms of woody plant defences against insects. Search for pattern eds Mattson WJ, Levieux J, Bernard-Dagan C, pp. 57-72. Springer, New York.

Unterseher M, Schnittler M, Dormann C, Sickert A, 2008. Application of species richness estimators for the assessment of fungal diversity. FEMS Microbiology Letters 282: 205-213.

Unterseher M, Schnittler M, 2009. Dilution-to-extinction cultivation of leaf-inhabiting endophytic fungi in beech *Fagus sylvatica* L - different cultivation techniques influence fungal biodiversity assessment. Mycological Research 113, 645-654

Unterseher M, Schnittler M, 2010. Species richness analysis and ITS rDNA phylogeny revealed the majority of cultivable foliar endophytes from beech *Fagus sylvatica* L.. Fungal Ecology 34, 366-378

Vainio EJ, Hantula J, 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis. Mycological Research 104, 927-930

Van Brummelen J, 1967. A world-monograph of the genera Ascobolus and Saccobolus Ascomycetes. Pezizales Persoonia Suppl 1, 1-260

Vannini A, Valentini R, 1994. Influence of water relations on *Quercus cerris* - *Hypoxylon mediterraneum* interaction: a model of drought-induced susceptibility to a weakness parasite. Tree Physiology 14, 129-39

Vannini A, Lucero G, Anselmi N, and Vettraino AM, 2009 Response of endophytic Biscogniauxia mediterranea to variation in leaf water potential of *Quercus cerris.* For Pathol 39, 8-14

Vannini A, Paganini R, Anselmi N, 1996. Factors affecting discharge and germination of ascospores of *Hypoxylon mediterraneum* (De Not.) Mill.. Eur J For Pathol 26, 12-24

Vega FE, Posada F, Aime MC, Pava-Ripoll M, Infante F, Rehner SA, 2008. Entomopathogenic fungal endophytes. Biol. Control, 46, 72- 82

Vettraino A M, Morel O, Perlerou C, Robin C, Diamandis S, Vannini A 2005: Occurrence and distribution of *Phytophthora* species in European chestnut stands, and their association with ink disease and crown decline - Eur J Plant Pathol 111, 169-180

Viret O, Scheidegger C, Petrini O, 1993. Infection of beech leaves *Fagus sylvatica* by the endophyte *Discula umbrinella* teleomorph: *Apiognomonia errabunda* : low-temperature scanning electron microscopy studies. Canadian Journal of Botany 71: 1520-1527

Von Wuehlisch, G 2006 EUFORGEN Technical Guidelines for genetic conservation and use for European beech *Fagus sylvatica* International Plant Genetic Resources Institute, Rome, Italy
http://wwwipgricgiarorg/networks/euforgen/ Euf_Distribution_Mapsasp

Wang Y, Buermann W, Stenberg P, Smolander H, Häme T, Tian Y, Hu J, Knyazikhin Y, Myneni RB, 2003. Hyperspectral remote sensing of vegetation canopy: Leaf area index and foliage optical properties. Remote Sensing of Environment, 85, 304- 315

Watanabe T, 2002. - Pictorial Atlas of soil and seed fungi Morphologies of cultured fungi and key to species second edition - CRC PRESS pp 486

Wei CZ; Harada Y, 1998. *Didymella fagi* sp nov and its anamorph *Ascochyta fagi*, causing the yellow leaf spot disease of *Fagus crenata* and *Quercus mongolica* var grosseserrata in Japan. Mycoscience 39, 63-69

Wennström A, 1994. Endophyte - the misuse of an old term. Oikos 71, 535-536

Wetherald RT, Manabe S, 2002. Simulation of Hydrologic Changes Associated with Global Warming. J Geophys Res 107, 4379-4394

Whalley AJS, 1996. The xylariaceaous way of life. Mycological Research 100, 897-922

White Thomas J, Bruns TD, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p 315-324 In MA Innis, DH Gelfand, JJ Sninsky, and TJ White ed, PCR protocols: a guide to methods and applications Academic Press, Inc, San Diego, Calif

Wilson D, 1995. Endophyte - The evolution of a term and clarification of its use and definition.- Oikos 72, 274-276

Wilson D, Carroll GC, 1997. Avoidance of high-endophyte space by gall-forming insects. Ecology 78, 7, 2153-2163

Wilson D, 1993. Fungal endophytes: out of sight but should not be out of mind. Oikos 68, 379-384

Xiao JF, Moody A, 2004. Photosynthetic activity of US biomes: responses to the spatial variability and seasonality of precipitation and temperature. Glob Change Biol 10, 437-451.

Yang YH, Watanabe M, Wang ZP, Sakura Y, Tang CY, 2003. Prediction of changes in soil moisture associated with climatic changes and their implications for vegetation changes: waves model simulation on Taihang Mountain, China. Climatic Change 57. 163-183

Publications and proceedings

Bayoud disease in central Algeria: history, distribution and strategies for control (20-25 Giu 2010). A.M. Vettraino, F. Abed, F. Bessedik, H. Khelafi, S. Franceschini, B. Ceccarelli, A. Prodi, A. Vannini. 13th Congress of the Mediterranean Phytopathological Union, Rome *PETRIA Giornale di Patologia delle Piante* 20 (2), 116-117 (presentazione poster).

***Fagus sylvatica*: diversity and composition of fungal endophyte communities** (20-25 Giu 2010). A.M. Vettraino, B. Ceccarelli, A. Vannini. 13th Congress of the Mediterranean Phytopathological Union, Rome. *PETRIA Giornale di Patologia delle Piante* 20 (2), 131-132 (presentazione poster).

Detection of *Biscogniauxia nummularia* in asymptomatic beech trees of the Italian Apennine Mountains (20-25 Giu 2010).N. Luchi, B. Ceccarelli, A.M. Vettraino, A. Vannini. 13th Congress of the Mediterranean Phytopathological Union, Rome. *PETRIA Giornale di Patologia delle Piante* 20 (2), 209-210 (presentazione poster).

Effect of essential oils on the population density of *Fusarium oxysporum* f. sp. *pisi* in soil and control of fusarium wilt (20-25 Giu 2010). A.M. Vettraino, B. Ceccarelli, S. Franceschini, A. Vannini. 13th Congress of the Mediterranean Phytopathological Union, Rome *PETRIA Giornale di Patologia delle Piante* 20 (2), 526-527 (presentazione poster).

Bayoudh desease: effectiveness of plant extracts for controlling Fusarium oxysporum f. sp. albedinis (20-25 Giu 2010). A.M. Vettraino, B. Ceccarelli, S. Franceschini, A. Tomao, F. Abed, A. Vannini(20-25 Giu 2010). A.M. Vettraino, B. Ceccarelli, S. Franceschini, A. Vannini. 13th Congress of the Mediterranean Phytopathological Union, Rome *PETRIA Giornale di Patologia delle Piante* 20 (2), 575-576 (presentazione poster).

Fungal endophytes of *Castanea sativa*: indifferent or opportunistic organisms? (25 – 28 Set 2008)**.** A.M. Vettraino, G. Di Carlo, B. Ceccarelli, A. Vannini. IV International Chestnut Congress - Beijing, China. Symposium Abstracts S4-14, 39-40 (presentazione poster).

Bayoudh disease: monitoring of microflora present in the soil of an Algerian oasis (24 – 29 Ago 2008). A.M. Vettraino, A. Prodi, S. Franceschini, B. Ceccarelli, F. Abed, F. Bessedik, H. Khelafi, P. Nipoti and A. Vannini. 9th International Congress of Plant Pathology, Torino. Journal of Plant Pathology 90 (2, Supplement), S2 458-459 (presentazione poster).

Risk of establishment and spread of *Phytophthora ramorum* in the Mediterranean and forest areas in Italy and Spain (18 – 21 Set 2007). A M Vettraino, E Moralejo, B Ceccarelli e A Vannini. *XIV Congresso Nazionale SIPaV, Perugia*. Journal of Plant Pathology 89 (3, Special Issue), S24 (presentazione orale).

Potential risk of spread of *Phytophthora ramorum* in Mediterranean wild environments and nurseries (26 – 31 Ago 2007). A M Vettraino, B Ceccarelli e A Vannini. 4th Meeting of the International Union of Forest Research Organizations (IUFRO) Phytophthoras in Forests & Natural Ecosystems, Monterey, California http://www.cnr.berkeley.edu/IUFRO2007/phytophthora/abstracts/21_vettraino.pdf (presentazione orale).

Susceptibility of Some Italian Ornamental and Forestry Species to *Phytophthora ramorum* (26 – 31 Ago 2007). A M Vettraino, B Ceccarelli e A Vannini. 4th Meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07.02.09, 137-139. Phytophthoras in Forests & Natural Ecosystems, Monterey, California (presentazione orale).

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