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"Studies on the Pythiaceous fungi assemblage of Italian beech forest soils along altitudinal and latitudinal gradients under the climate change scenario"

by

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A Chiara e Riccardo

1- Introduction

1.1 - Climate Change (CC)

The IPCC (Intergovernmental Panel on Climate Change) defines CC as a statistically significant variation in the variables that define the climate of a region (such as temperature or precipitation) or in its variability persistent over an extended period of time, typically decades or longer periods. It refers to any change in climate, due to natural variability or as a result of human activity. Instead, UNFCCC (United Nations Framework Convention on Climate Change) ascribes CC to human activity only, by altering the composition of the global atmosphere.

The effects of CC are evident from the observation of the increase of global average air and ocean temperatures, the widespread melting of snow and ice and the rising global average sea level (IPCC, 2007). The temperature is greater at higher northern latitudes; global average temperature has increased by 0.8°C since 1900 (Hansen *et al.,* 2006) and the 12 hottest years observed globally since 1880 all occurred between 1990 and 2005. Over this period, precipitation increased in eastern parts on North and South America, northern Europe and central Asia, whereas precipitation declined in the Sahel, the Mediterranean, southern Africa and parts of southern Asia. Globally, the area affected by drought has likely increased since the 1970s. Observed effects in all continents and most oceans showed that many biological systems are been affected by CC, particularly temperature increasing (e.g. earlier timing of spring events, leaf-unfolding, poleward and upward shifts in ranges in plant and animal species) (IPCC, 2007).

1.2 - Effects of CC on European forests

Forests are particularly sensitive to CC, because the long life-span of trees does not allow for rapid adaptation to environmental changes**.** Associated with CC there are several factors affecting forest ecosystems, which can act independently or in combination (Lindner *et al.*, 2010).

Rising concentrations of CO_2 in the atmosphere increase photosynthesis rates, but vary with plant nitrogen status and species (Saxe *et al*., 1998; Norby *et al*., 1999; Ainsworth and Long, 2005). In addiction it induces a partial closure of stomata reducing water loss by transpiration. This results in an increase in the ratio of carbon gain to water loss, i.e. the water use efficiency at the leaf and whole stand level increases (Field *et al*., 1995; Picon *et al*., 1996).

Other changes in the chemical atmospheric environment affecting tree growth include tropospheric and ground-level concentrations of ozone, which may increase drought stress in trees (McLaughlin *et al*., 2007) and reduce tree biomass under current ambient compared to pre-industrial concentrations (Wittig *et al*., 2009). Also atmospheric nitrogen deposition has been a major factor

influencing forest growth and other ecosystem characteristics over the last decades (Magnani *et al*., 2007; Kahle *et al*., 2008).

Finally, since the 1970s, drought spells have become more frequent in the Mediterranean Basin (e.g. McCabe and Palecki, 2006), and natural forest productivity in the northern portion of the basin appears to have declined (Boisvenue and Running, 2006). In coming decades, changes in climate and land use are likely to cause water shortages, increased risk of forest fires, northward shifts in the distribution of species, and loss of agricultural products. According to Jump *et al*. (2006), increasing temperature caused growth decline of beech (*Fagus sylvatica* L.) stands located in the Montseny Mountains of Catalonia (northeast Spain) at the lower elevational limit for the species. Because a comparable fall in basal area increment (BAI) was reported for the central Apennines (Italy) by Piovesan *et al*. (2005), this climate-related decline is not an isolated phenomenon, but may be occurring at the southern edge of this species range elsewhere in Europe.

1.3 - Predictions of CC impact on European forests

CC will bring many and complex effects for forests over different European bioclimatic regions. Rising atmospheric CO_2 concentration, higher temperatures, changes in precipitation, flooding, drought duration and frequency will have significant effects on trees growth. These CC will also have associated consequences for biotic (frequency and consequences of pests and diseases outbreaks) and abiotic disturbances (changes in fire occurrence, changes in wind storm frequency and intensity) with strong implications for forests ecosystems. The outcomes of different CC scenarios showed regional variability of CC. The changes in average temperatures that forests will have to face over the next 100 years range, according to latest projections, between about 2° C increase in Ireland and the UK, up to about 3° increase in central Europe and 4°C – 5°C increase in northern Boreal and parts of the Mediterranean regions. Climate is expected to become more variable with greater risk of extreme weather events, such as prolonged drought, storms and floods. Forests will have to adapt to changes in mean climate variables but also to increased variability. Rising temperatures without increase in precipitation or with decreasing rainfall can lead to drought, especially in the Mediterranean and Continental Temperate conditions. Fire danger is expected to increase throughout Europe, especially in the already fire-prone Mediterranean region. Wind throws and storm damage are most relevant in central Europe, as well as in western and northern Europe. Changes in the seasonal distribution of precipitation will lead to higher amounts of rainfall, especially during winter and spring, considerably increasing the risk of flooding in Central and Northern Europe. CC affects the temporal and spatial dynamics of pest species,

influencing the frequency, intensity and consequences of outbreaks as well as their spatial patterns, size and geographical range. Coevolved relationships between hosts and their pests probably will be disturbed, hosts will come in contact with novel pathogens and herbivores, and changes of species composition of communities are to be expected (IPCC, 2007).

European forests are extremely variable with regard to their ecological and socio-economic conditions. The impacts of CC on European forests are likely to be unevenly distributed not only across different bioclimatic zones but also among different forest ecosystems of each zone. This is because forest ecological characteristics will affect forest ecosystem sensitivity and the inherent adaptive of capacity of forest ecosystems to respond CC. A forest typology classification reflecting this ecological variability is needed to facilitate the assessment of CC impacts. The European Forest Type scheme consisting of 14 main categories of European forests presented by the EEA (2006) but we can refer to the most frequent of them.

Table 1.3.1: most frequent European forest categories

10 most frequent European Forest Categories
Montane beech forest
Coniferous forests of the Meditarranean, Anatolian and Macronesian regions
Lowland to submontane beech forest
Broadleaved evergreen forest
Acidophylous oakwood and oak-birch forest
Termophilous deciduous forest
Alpine conipherous forest
Hemiboreal forest and nemoral coniferous and miwed broadleaved-coniferous forest
Non-riverine alder, birch or aspen forest
Boreal forest

1.4 - CC effects on forest diseases

CC influences the distribution of living organisms and biogeographical studies have mainly focused on higher plants or animals and, more generally, free-living organisms. Although parasites and pathogens are primarily dependent on the presence and density of their host, climate is also an important driver of their distribution pattern. This applies in particular to plant pathogenic fungi: they are heavily influenced by temperature and precipitation for their growth rate and dispersal (Agrios 2005).

Pathogens **directly affected by climate** can cause disease in a healthy vigorous host, if the pathogen's environmental requirements are met. Their life cycles are directly affected by temperature and moisture. Many pathogens are sensitive to precipitation and humidity and their rate of reproduction, spread and infection are greater when conditions are moist, so these changes in temperature and moisture more directly affect the pathogen regardless of their effects on the host (Sturrock *et al*., 2011). In some cases pathogens infection (e.g. *Phytophthora* sp.) can only occur following a particular sequence of dry and wet conditions (Desprez-Loustau *et al*., 2006). On the contrary secondary pathogens (e.g. fungal endophytes) take more advantages of indirect effects of CC enhancing host trees susceptibility producing damages during/after the most severe drought events.

Pathogens **indirectly affected by climate** tend to infect hosts that are stressed by (i) environmental factors, (ii) pathogens directly affected by climate, or (iii) insects. Such pathogens can sometimes infect a healthy host and remain latent until the host is stressed. Whilst the ability of these pathogens to sporulate, spread and infect new hosts is affected by temperature and moisture, factors that stress their hosts are often critical to their successful invasion of host tissues. For example, an increased incidence of summer drought will increase the probability that trees will be infected by pathogens whose activity is facilitated by host stresses, such as root pathogens, wound colonizers and latent colonizers of sapwood (Brasier and Scott, 1994; Lonsdale and Gibbs, 2002; Desprez- Loustau *et al*., 2006).

1.5 - *Pythiaceae* root rot and CC

Many species of *Pythiaceae*, are economically important plant pathogens. Diseases caused by both *Pythium* and *Phytophthora* species are generally favored by wet soil conditions, when their rapid dispersal is often achieved by asexual, flagellate zoospores (Pettitt *et al*., 2002). Species of *Pythium* and *Phytophthora* cause root and collar rot on many economically important plant species world-wide, herbaceous plants as well as trees and shrubs (Erwin and Ribeiro, 1996)

A number of soil-borne *Phytophthora* species are potentially harmful to woody plants. These pathogens are widespread in the natural forests and hardwood plantations in Europe. Some species are host specific, such as *Phytophthora quercina* Jung (Jung *et al.*, 2000). Others are known as ubiquitous parasites on a broad range of hosts and are frequently associated with declining individual trees in forest stands, natural environments and plantations (Jung *et al*.,

1996, 1999, 2000, 2002; Hansen and Delatour, 1999; Werres *et al*., 2001; Balci and Halmschlager, 2003a, b; Vettraino *et al*., 2002, 2003, 2005).

Pythium spp. seems to be restricted to young plant tissues, such as fresh fine roots or seedlings (Krober 1985; Martin 1992).

Changes in climatic conditions in the last 60 years, i.e. increased mean winter temperatures, seasonal precipitation shift from summer into winter and a tendency to heavy rains are favoring infection by several species of *Phytophthora* in Central Europe. A proliferation of *Phytophthora* root rots may be expected, increasing the instability and vulnerability of forest ecosystems dominated by beech and other susceptible tree species, including oak, alder, maple, fir and pine species (Jung, 2009).

1.6- European beech (*Fagus sylvatica* L.) distribution and ecology

Deciduous forests of the temperate bioclimatic zone are dominated by beech. European beech (*Fagus sylvatica* L.) is the most common and most frequently planted broadleaved tree species in central European forests (Ellenberg, 1996; Jung *et al*., 2005). It is due to its high shade tolerance and growth capacity, and its wide climatic and geological amplitude (e.g. Atlantic to continental climate, colline to mountainous zone, moderately dry to periodically wet soils with pH ranging from <3 to >7) (Kölling *et al*., 2005; Felbermeier and Mosandl, 2006). Beech extends from southern Scandinavia (under the 60^{th} parallel, except from two separated Norwegian stands) to northern Sicily and Greece and from Spain to Moldavia (Gellini and Grossoni, 1997). In the Mediterranean basin, it is confined to mountainous regions where rainfall is sufficiently high. In these sites, many beech stands can be considered as relicts, living under ecological conditions just barely within the limit of their requirements. Southern beeches show some morpho-physiological differences with northern beeches, such as sclerophylly and a superior tannin content (Bussotti *et al*. 1998; Grossoni *et al*. 1998).

In Italy beech forests characterize the mountain belt: they are mostly monospecific, so the European beech is only a dominant tree, otherwise it is linked to *Abies alba* Mill., *Picea abies* L. and *Larix decidua* Mill. on alpine valley (Gellini and Grossoni, 1997; Pignatti 1998).

1.7 - European beech, *Pythiaceae* root rot and CC

Fagus sylvatica growth and health in southern Europe are affected by CC. Recent dendro-climatological studies (Jump *et al*., 2006; Penuelas *et al*., 2006; Piovesan *et al*., 2008) reported a steep beech growth decline (lower basal area increment, BAI) and stands replacement due to drought since the 70s. This phenomenon is spread throughout all the southern edge of this species in

Europe. Piovesan *et al*., (2008) reported a decreasing BAI mainly at the lower altitudes of hills and mountains in Italian Apennines under the influence of long-term drought since 1970.

The synergistic interactions between CC, in particular climatic extremes, and root losses by soil-borne *Phytophthora* species are a major cause for the general decline of forests across Europe. The severe damages of beech trees are resulting from a fatal interaction between introduced root pathogenic *Phytophthora* species, the succession of an extremely wet and an extremely dry vegetation period and exploding populations of secondary parasites. Symptoms like increased crown transparency, abnormally small and yellowish foliage, dieback of the crown, necrosis of the inner bark and cambium with tarry spots on the bark surface and bleeding cankers are typical for Phytophthoras (Jung *et al*. 2005). A proliferation of *Phytophthora* root rots may be expected, increasing the instability and the vulnerability of forest ecosystems dominated by beech and other susceptible tree species (Jung 2009).

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2 - Aims

This study has been carried out within the European project "Biodiversity And Climate Change: A Risk Analysis" (BACCARA no 22000325).

The main goal of the project was to evaluate the risk of European forest biodiversity and productivity losses under a changing climate scenario. In particular the Working Package 2 (WP2) aimed to predict the impact of CC on tree-associated species diversity and performance. It focused on species with key roles in the functioning of forest in terms of net biomass production, such as antagonists (herbivorous animals and pathogens), natural enemies (predators and parasitoids), and mutualistic species (mycorrhiza).

The analysis of the *Pythiaceae* fungal community inhabiting Italian beech stands has been carried out as part of the WP2. Biological samples have been collected in experimental plots distributed along latitudinal and altitudinal gradients, in order to assess the influence of climatic variables on richness, composition and diversity of the pythiaceous soil-borne communities.

Two methodologies have been adopted:

- The traditional culture-based method
- The high-throughput DNA 454 sequencing (GS FLX Titanium)

3 - Sampling on climatic gradients

3.1 - Experimental design

3.1.1 - Study sites

Pythiaceous fungi assemblage analysis has been carried out on four different European beech stands located along the Italian territory (Fig. 3.1.1). According to the Worldwide Bioclimatic Classification System (www.globalbioclimatics.org) all the forests considered in this study are within the Temperate Macrobioclimate. Geographic location, stand description and bioclimate/vegetation classifications (tab. 3.1.1) of any site is provided below:

Fig. 3.1.1: Map showing the location of the 4 sites sampled in this study. Image created using Google Earth 2012 Tele Atlas, 2012 Europe Technologies, US Dept of State Geographer, 2012 Google.

- **Pian Cansiglio (PC)**

The Cansiglio forest is situated in the prealpine mountain zone of the Veneto Region (46° 06’ 50” N, 12° 40’ 81” E) at altitudes between 900 m and 1694 m.

Karst phenomena give the Cansiglio a typical bowl shape, with sinkholes and swallow holes. This morphology causes inversion on valley slopes, with differences in average temperatures of 2–3 °C with respect to other prealpine areas at the same altitudes. Minimum values during severe winters may reach –35 °C in the deeper valleys (e.g., year 2000).

The ground is snow-covered from the end of November until mid-March. Average annual snowfall is about 60–150 cm, although. Above 1250 m beech finds optimal growing conditions as fog is year-round present, precipitation is abundant and evenly dispersed through the year (on the average 1800-1900 mm/year mm) and an annual average temperature is 5.1°C (Stergulc, 2000; Caudullo *et a*l., 2003 Nascimbene *et al.*, 2007).

The prevailing parent rock is limestone. Soils are well developed and well drained brown earths, characterised by zoogenic humus (Marchisio *et al*., 1994).

- **Cimini Mountains (MC)**

Cimini mountains is a volcanic complex in centre-western Italy (Viterbo, Latium, lat. 42° 24' 27.501" N) encompassing the extinct Cimino Volcano and the Vico caldera, which originated after the volcanic cone collapsing and now occupied by the Vico lake. Beech stands grow on Fogliano mountain, the highest peak (961 m a.s.l.) around the lake, on Venere Mountain (851 m a.s.l.), a volcanic cone within the Vico crater and on Cimino Mountain (1,053 m a.s.l.). Beech grows from 507 m upward, such an altitude is largely underneath the normal lower altimetric limit of this species (therefore they are called 'depressed' beech forests; Hofmann 1991) in the Appennines (Montelucci, 1956). This is due to the proximity of the Tyrrhenian sea that keep the annual temperature range down and to the effect of occult precipitation and upslope fogs formed by western winds blowing up the sides of the mountains (Hofmann, 1991).

In Cimino Mountain beech ranges between 950 and 1050 m a.s.l., it forms an old-growth secondary forest as timber logging was stopped 1949 to preserve the social aesthetic value of the forest (Lo Monaco, 1983; Piovesan, 2008). Today the forest is in the demographic transition stage (Frelich, 2002) progressively turning from a single-layer canopy to a multi-cohort structure (Piovesan *et a*l., 2011). The dominant even-aged cohort can rise to 40 m in height and it is 120-150 years old (Piovesan, 2008), it suffers natural mortality allowing the regeneration establishment. Also Venere Mountain (beech range from 550 to 850 m a.s.l.) and Fogliano Mountain beech forest were left develop naturally after intensive exploitation, therefore dominant cohort is now 150 years old, with sporadic individuals reaching 200 years old. Also in these cases forest showed a sudden reactivation of natural processes leading to structure diversification especially in Venere Mountain where the fertile deep volcanic soil makes growth rate very high (Piovesan *et a*l., 2011).

- **Serralunga (SL)**

Serralunga is a mountain slope located within a 3000 ha community forest that is part of a wider forest area, included in the external belt of the Abruzzi National Park in central Italy (Collelongo,

L'Aquila, Abruzzi, lat. 41° 53' 11.41" N). The environmental and structural conditions of the stand are representative of central Apennine beech forests. Reported mean annual temperature range between 6,9 and 10°C while annual precipitation between 1230 and 1300 mm/year at the upper forest limit (Petriccione, 2002; Scartazza *et al*., 2004). At the lower altimetric (1300 m a.s.l.) limit beech stand encounters mixed forest with hornbeam, European turkey oak and maples. On the other hand at the highest altitudes (1550 m) upper limit has been lowered by grazing. The State-owned Serralunga beech forest has been exploited intensively in the past for firewood production and cattle grazing, therefore coppicing was a widespread woodland management method. But nowadays, as human pressure has lowered conversion of old coppices to high-forest is increasing especially in the most fertile sites.

- **Soro Mountain (MS)**

Soro mountain is in northern Sicily at the southernmost limit of Europe beech range (Nebrodi mountains, Cesarò, Messina, Sicily, lat. 37° 53' 60.00" N). The Region-owned beech forest grows on Monte Soro between 1200 m and the mountain peak at 1847 m a.s.l.. Forest has been overexploited with grazing and 15-year-cycle-coppicing until 1993 when a Regional Park was instituted. Since 2004 silvicultural practices aimed to convert part of the old 30/50-years-beech coppice with standards on the N slope to an high-forest divided it in multi-aged compartments. The mean annual precipitation in this site is about 1460 mm, but sometimes it can be also much more exiguous as in 1990 (1064 mm). Beech can grow at such low latitudes thanks to the lingering snow, to the occult precipitation formed by cold air coming from the Tyrrhenian sea and to the sandstones bedrock that has an high moisture retaining capacity (Schicchi *et al*., 2009).

Since 1990 Monte Soro beech forest has been reported to be affected by serious decline associated with infections caused by the ascomycete *Biscogniauxia nummularia* (Bull. :Fr.) O. Kunze. The most severe symptoms occur in the southern slope especially in lowest part of beech vertical distribution. Probably this is due to the prolonged drought and high temperatures observed over the last decades making *F. sylvatica* more susceptible to the disease and to the soil with low water retention capacity (low organic matter content). In the summer trees exhibited foliar yellowing, microphylly, wilting and drop, stem cankers, black carbonaceous stromata bursting through the bark. Decline can cause the death in most cases within one or two years of one or more sprouts of the same stump or even of the entire stump in the most severe cases. Generally growth after declining episodes is not recovered (Granata and Whalley, 1994; Mazzaglia *et al*., 2002; Sidoti and Granata, 2003; Granata and Sidoti, 2004).

3.1.2 - Altitudinal gradients (AG)

Pythiaceous community analysis has been carried out on two altitudinal gradients in central Italy. The gradient "AG2010" was located in site SL and was sampled in 2010 while "AG2011" was located both in site SL and in site MC during the year 2011. Each gradient included 4 altitudinal levels distributed on the symptomless pure beech forest making the gradient as wide as possible. While in SL all the four levels (L2, L3, L4 and L5) were on the same slope resulting in a 210-m-gradient, in MC it had been necessary to locate the levels on Cimino mountain (L7), on Fogliano mountain (L8) and on Venere mountain (L9 and L10) to obtain a 380-m-gradient (Tab. 3.1.2.1). Gradients AG2010 and AG2011 were sampled in summers 2010 and 2011 respectively.

At each level three groups (plots) of five trees were chosen for samplings. Their d.b.h. ranged between 5 and 20 cm. When trees of the dominant cohort had too large diameters, young trees from regeneration were included in the plots. Distance between plots ranged between 50 and 200 m according to local slope and stand features. One extra-plot (SL13) stand was added and designed in a beech declining area. In fact during the early on-the-spot investigation in SL, a notable beech decline has been observed at the lower limit of its vertical distribution (1300-1350 m a.s.l.). Observable symptoms were similar to those described for strip-cankering caused by *Biscogniauxia nummularia* (Bull. :Fr.) O. Kunze (Granata and Whalley, 1994; Hendry *et a*l., 1998). Diseased trees showed elongated cankers on stems (Fig. 3.1.2.3a), leaves yellowing, microphylly and wilting, progressive stem and branches death from the top of the tree downward, death of the whole tree, black stromata bursting from the bark of death wooden tissues. The diseased trees showed also damages by other pests, e.g. abundant *Mikiola fagi* galls on leaves and *Kretzschmaria deusta* fruit bodies bursting from necrotic wooden tissues. The plot SL13 included 5 symptomatic trees of this declining area.

Tab. 3.1.2.1: altitude (m a.sl.) of each levels (L) in Serralunga (SL) and Cimini Mountains (MC) surveyed in 2010 - 2011 and 2011, respectively

AG2010 - 2011 SL		
Level name	**Plots**	**Altitude (m a.s.l.)**
L2	SL4, SL5, SL6	1550
L3	SL1, SL2, SL3	1500
L4	SL7, SL8, SL9	1370
L5	SL10, SL11, SL12	1340
	SL13 (declining)	1340

AG2011 MC		
Level name	**Plots**	**Altitude (m a.s.l.)**
L7	MC1, MC2, MC3	1020
L8	MC4, MC5, MC6	950
L9	MC7, MC8, MC9	820
L10	MC10, MC11, MC12	640

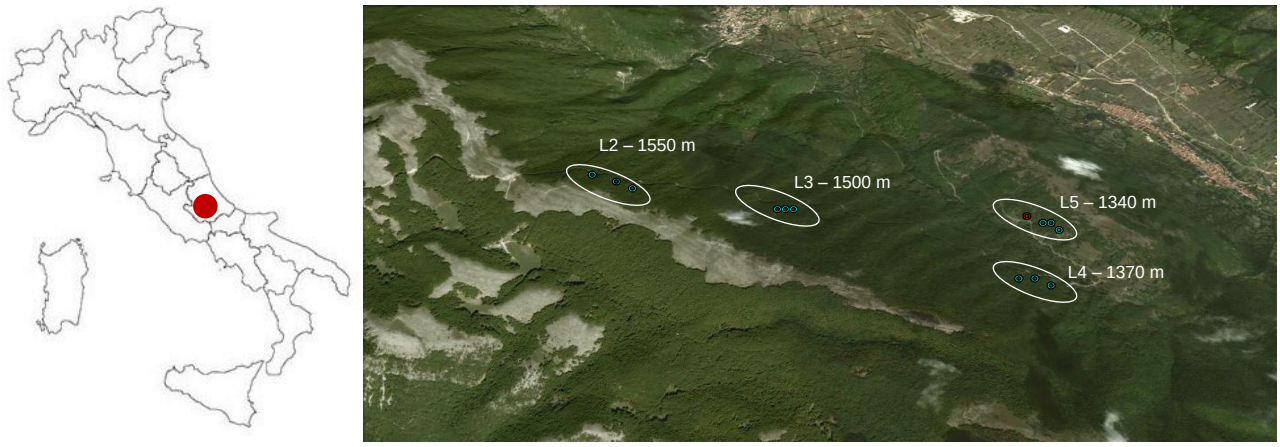

Fig. 3.1.2.1: Map showing the location and altitude of the 4 levels (L2, L3, L4, L5) of the altitudinal gradient (AG2010) sampled in 2010 and 2011 in SL site. Blue rings represent plot in healthy beech stand whereas the red ring the extra-plot in the declining forest. Image created using Google Earth 2012 and Microsoft Office Power point 2003.

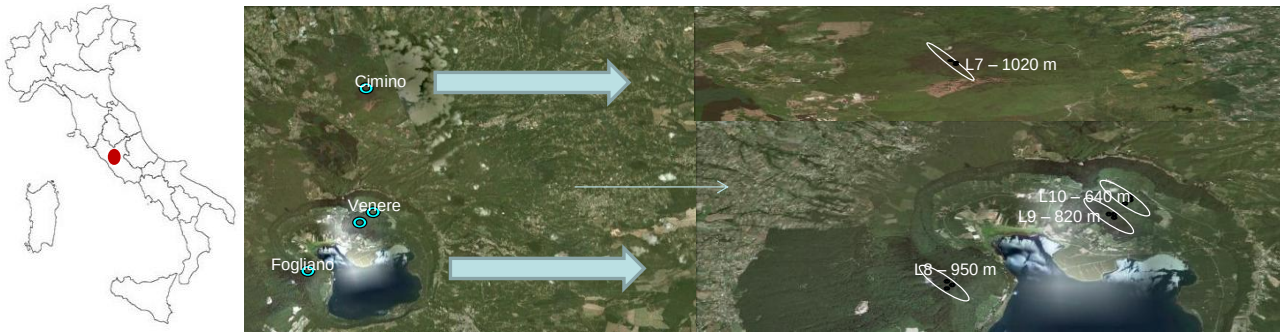

Fig. 3.1.2.2: Map showing the location and altitude of the 4 levels (L7, L8, L9, L10) of the altitudinal gradient (AG2011) sampled in 2011 in MC site. Image created using Google Earth 2013 and Microsoft Office Power Point 2010.

Climatic data were obtained from the Latium Regional Agency for Agriculture Development & Innovation (ARSIAL) and from the National Network for the Control of Forest Ecosystems (CONECOFOR) databases for site MC and SL respectively. In the former case monthly data for the years 2004-2011, recorded in Soriano nel Cimino (Poggio di Chia, 309 m a.s.l.) weather station, are available online at ARSIAL website (http://www.arsial.it/portalearsial/default.htm). In the latter case daily data for the years 2000-2010, recorded in Collelongo weather station (1560 m a.s.l.), were kindly released by CONECOFOR.

3.1.3 – Latitudinal gradient

A similar criterion was used to design the latitudinal gradient (LG2010, Fig.3.1.2.3) that was sampled in summer 2010 and 2011. The gradient included 3 latitudinal levels covering on the whole 920 kilometres, each one made up of 3 plots located at the same altitude in the healthy forest. The northern level (L1) is in PC site, the central level is in SL (L5, belonging to AG2010 too) and the

southern level (L6) is in MS. Levels latitude, altitude and plots name are provided in Tab. 3.1.3.1 As well as for altitudinal gradient an extra-plot (MS4, 1680 m a.s.l) in the MS declining forest area (Granata & Whalley, 1994; Mazzaglia *et al*., 2002; Sidoti & Granata, 2003; Granata & Sidoti, 2004) had been added to samplings.

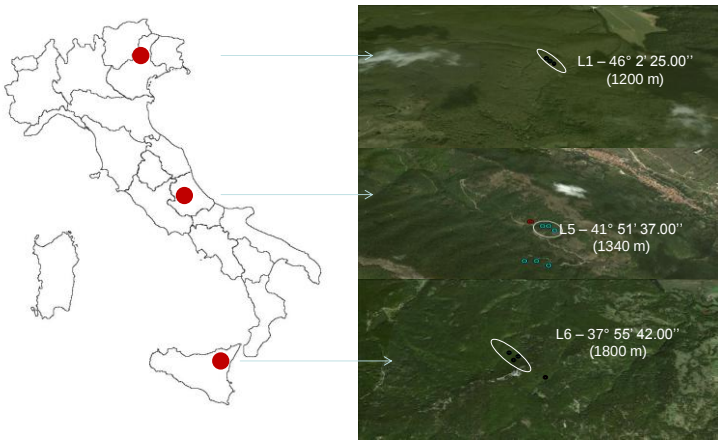

Fig. 3.1.2.3: Map showing the locations and latitude of the 3 levels (L1- Pian Cansiglio-, L5 – Serralunga- and L6 – Soro Mountain -) of the latitudinal gradient (LG2010-2011) along Italy. Blue rings represent plot in healthy beech stand whereas the red ring the extra-plot in the declining forest. Image created using Google Earth 2013 and Microsoft Office Power point 2010.

Tab. 3.1.3.1: Latitude, altitude and plots of the 3 levels of the latitudinal gradient LG2010-2011 on PC, SL and MS sites.

LG2010 - 2011			
Level name	**Plots**	**Latitude**	**Altitude (m a.s.l.)**
L1	PC1, PC2, PC3	46° 2' 25.00'' N	1200
L5	SL10, SL11, SL12	41° 51' 37.00'' N	1340
L6	MS1, MS2, MS3	37 °55' 42.00'' N	1800
	MS4 (declining)	38 °55' 42.00'' N	1680

Daily climatic data related to the years 2004-2011 were given by CONECOFOR for PC (Vittorio Veneto weather station, 1100 m a.s.l.), whereas monthly records for the years 2000-2011 for MS site where requested to Sicilian Regional Department of Water and Waste - Water Observatory Service (Cesarò weather station, 1100 m a.s.l.).

3.2 – Samplings

Samplings on gradients were carried out according to the calendar reported in Tab. 3.1.3.2 . From any tree one soil sample was collected: it contained fine and coarse beech roots and resulted from a mix of four monoliths of soil (25 x 25 x 25 cm) collected at the compass points around a tree at a distance of 50 cm from the collar (Jung *et al.*, 1996). Finally samples were quickly delivered to Forest Pathology laboratory -University of Tuscia- Viterbo.

Table 3.1.3.2: Period of sampling on PC, SL, MC and MS sites carried out in summer 2010 and in spring/summer 2011.

Climatic gradient	Sampling date	Site
LG2010	7/7/2010	MS
LG2010	20/07/2010	PC
AG2010/LG2010	7/9/2010	SL
AG2011a/LG2011	1/8/2011	SL
LG2011	1/6/2011	MS
LG2011	1/7/2011	PC
AG2011b	25/05/2011	MC

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4 - Chapter 1 - Analysis of biodiversity of European beech *Pythiaceae* community detected along climatic gradients: culture-dependent

4.1 – Materials and methods

4.1.1 – Isolation procedures of *Phytophthora* and *Pythium*

Soil samples were processed by the **baiting technique**. Baiting is a simple and specific method to detect Phytophthoras from environmental samples. It represents an important stage of diagnostic process and it is based on the employ of different biological baits (Erwin and Ribeiro, 1996).

Soils were gently mixed and a 200 ml aliquot of each sample was placed in a 15 x 25 cm polyethylene box and flooded with approximately 500 ml of tap water 2–3 cm above the soil surface. Debris was carefully removed from the water surface. *Azalea*, *Fagus* and *Rhododendron* leaves and carnation petals were floated on the surface as baits. Three baits of each species were used. The bait boxes were incubated at 20°C (Fig.4.1.1.1).

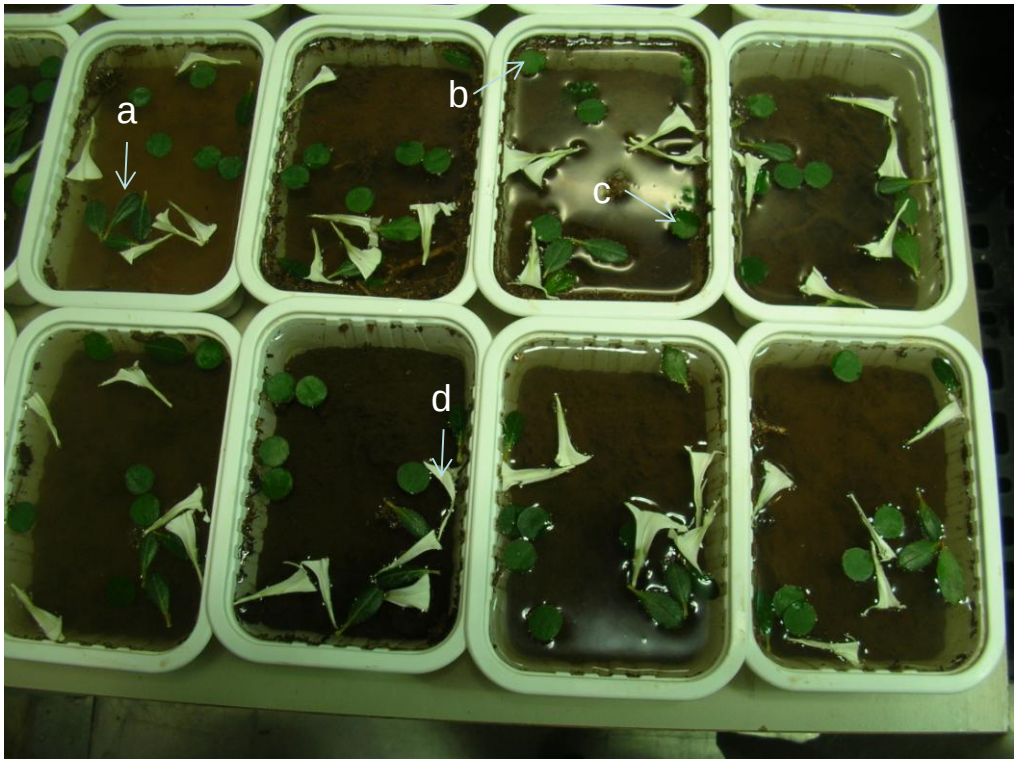

Fig.4.1.1.1: soil baiting set up of a set of beech soil samples from SL sampling carried out in 2010. *Azalea* (a), *Fagus* (b), *Rhododendron* (c) leaves and carnation petals (d) were used as baits.

Baits developing visible necrosis, which normally appeared after 3±7 days, were removed, washed twice in sterile distilled water to remove soil particles and dried on paper towel. Baits were sectioned into eight pieces and plated on Selective Malt Agar (Malt-Extract 15g/l, Agar 20g/l, Pimaricin 0,01 g/l, Sodium-Ampicillin 0,25 g/l, Hymexazol 0,05 g/l, Methyl 1-(butylcarbomoyl)-2-

benzimidazolecarbamate 95% 15 mg/l). After 9 days all baits were removed and plated as stated before. *Phytophthora* and *Pythium* colonies emerging from baits pieces were sub-cultured on Carrot-Agar (200 g/l sliced carrots, 20 g/l Agar).

Initially all the *Phytophthora* and *Pythium* isolates were grouped according to morphological characters.

Identification of *Pythiaceae* was based on their cultural and morphological characteristics (morphological features of sporangia, oogonia, antheridia, chlamydospores and hyphal swellings) and on comparisons with species descriptions provided in literature (e. g. Stamps *et al*., 1990; Erwin and Ribeiro, 1996; Van der Plaats-Niterink, 1981; Dick, 1990). Colony morphology was described on 10-day-old cultures (14-day-old cultures if growth was slow) grown on CA and potato dextrose agar (PDA) in 90 mm Petri dishes at 20°C in darkness. Sporangia were produced after 24-48 hours of incubation at 20°C by placing a disk of mycelium from a 7-day-old culture grown on CA into soil extract prepared according to Chee and Newhook (1965). Morphology was assessed by light microscopy and the length and breadth of 30 sporangia were measured for each isolate (200X or 400X).

4.1.2 – DNA extraction, amplification and sequencing

In order to confirm the identifications derived from the morphological studies, a subset of isolates of each species and morphotype was selected for DNA sequence analysis.

DNA of representative isolates was obtained from liquid culture grown on Carrot juice (20% concentration) (Brasier *et al*., 2010). They were lyophilized and their genomic DNA extracted using Qiagen's DNeasy Plant Mini-kit (Qiagen GmbH). Amplification and sequencing of the ITS1 – 5.8S – ITS2 rDNA region, using primers ITS6 and ITS4 (White *et al*., 1990) were carried out as described by Cooke *et al.* (2000). PCR products were cleaned using PEG precipitation (Applied Biosystems, 1994) and sequenced (Macrogen Inc.). Sequences were edited with BioEdit Software (BioEdit, Version 7.0, Hall, 1999) and compared with those existent on NCBI database (http://www.ncbi.nlm.nih.gov/Blast.cgi).

4.1.3 - Statistical analysis

Pythium and *Phytophthora* recovery percentages were expressed as total of pieces witch were positive to the isolation on the total of pieces plated.

Data elaboration was performed with Microsoft Excel 2010 (Microsoft Corp., Redmond, USA), GraphPad 31 Prism 5 (GraphPad, San Diego, CA, USA). PAST 2.06 (http://folk.uio.no/ohammer/past/index.html) was used for species accumulation curve construction

(Krebs algorithm; Krebs, 1989) and diversity (Shannon_H), dominance (Berger-Parker) indices calculation.

4.2 – Results overview

The Pythiaceous community results to be composed mainly by *Pythium* spp. in all the different European beech stands. Isolates from SL belonged exclusively to the genus *Pythium*, whereas rare isolates belonging to the genus *Phytophthora* were isolated from PC, MC and MS (only in the declining plot). *Pythium* isolates were not identifiable at species level in most cases: indeed the genus is considered extremely difficult to identify (Mugnier and Grosjean, 1995) due to variation in morphological traits within the same species (Garzon *et al*., 2005; Van Der Plaats-Niterink, 1981).

Tab. 4.2.1: *Pythiaceae* species isolated for each bait type. CP = carnation petals; BD = beech leaves disks; RD= rhododendron leaves disks; AL = azaleas leaves.

Species	CP	BD	RD	AL
Py. intermedium	+	+	+	+
Py. macrosporum	+	+	+	+
Py. sp. (A)	+	+	+	+
Py. sp (B)	+	+	+	+
Py. sp. (SL4)	+	+	+	+
Py. sp. (MS3)*	+	+	-	-
Ph. plurivora	+	+	+	-
Ph. cactorum	+	-	+	+
*Ph. cambivora**	+	-	+	+

*recovered only in declining plots

4.2.1 - Healthy forests

Overall 150 healthy beech trees were investigated for the Pythiaceous fungi assemblage analysis with culture–dependent method. Isolates were grouped in 10 morphotypes according to their macroscopic and microscopic morphology. Two ITS consensus sequences per morphotype with a length ranging from 700 to 850 bp were obtained to confirm the morphological identification. Community composition was driven by few abundant species, *Pythium intermedium* and *Py. macrosporum*, isolated from all the study sites. No *Phytophthora* spp. was isolated from samples taken from SL and MS. Species accumulation curve for the observed richness (Fig. 4.2.1.1) revealed that the community richness does not likely exceed the richness captured by samplings.

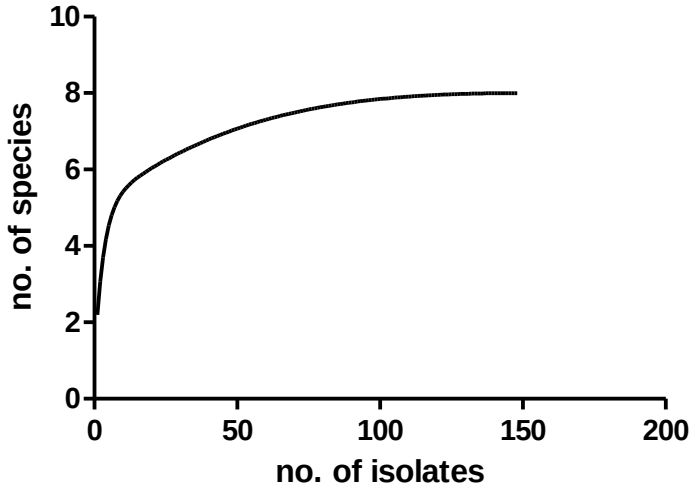

Fig. 4.2.1.1: species accumulation curve for Pythiaceous fungal community from 150 healthy trees of the 4 study sites.

4.2.1.1 – Altitudinal Gradient 2010 (Abruzzo)

Overall 379 Pythiaceous colonies were isolated from soil samples from the 4 altitudinal level in SL in September 2010 (60 healthy beech trees) (Fig. 4.2.1.1.1). The general average of *Pythium* spp. isolation percentage in each altitudinal level varied markedly, ranging from a minimum of 2% (L3) to a maximum of 17% (L4).

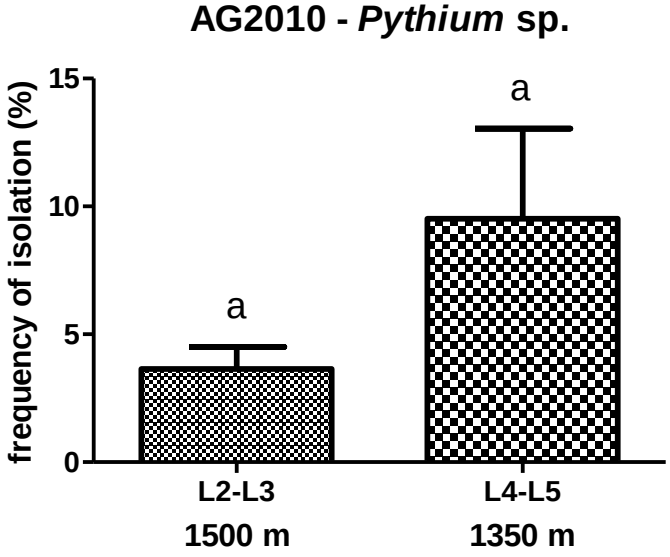

Fig.4.2.1.1.1: *Pythiaceae* isolates frequency in SL soil samples collected in 2010 sampling at two grouped altitudes. Data were analyzed with Student's t-test. Equal letters indicate no significant differences with the Mann-Whitney test (P>0.05).

A total of 5 *Pythium* species has been recovered; 2 *Pythium* (*Pythium* sp. (A) and *Pythium* sp. (B)) were common to all four levels. *Pythium intermedium* was common to L3, L4 and L5, *Pythium macrosporum* to L4 and L5 and *Pythium* sp. (SL4) to L2 and L4 (Tab.4.2.1.1.1).

Tab. 4.2.1.1.1: presence (+)/absence (-) of *Pythium* species in AG2010 in L2, L3, L4 and L5.

AG2010				
Species	**L2**	**L3**	**L4**	**L5**
Py. intermedium	-	+	+	+
Py. macrosporum	-	-	+	+
Py. sp. (A)	+	+	+	+
Py. sp. (B)	+	+	+	+
Py. sp. (SL4)	+	-	+	-

L4 was characterized from the highest recovery of *Pythium macrosporum* with a percentage of 74% and *Pythium. intermedium* (13%).

Pythiaceae diversity (Shannon index) increased with altitude whereas dominance (Berger-Parker index) decreased.

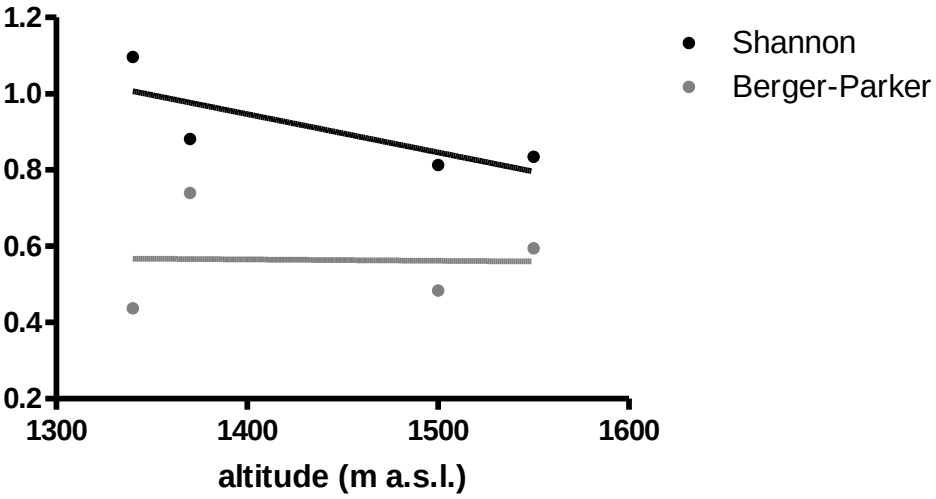

Fig. 4.2.1.1.2: Diversity indices along AG2010 in SL at 4 different altitudes.

4.2.1.2 – Altitudinal Gradient 2011 (Abruzzo)

Overall 304 Pythiaceous colonies were isolated from soil samples from the 4 altitudinal levels in SL in August 2011 (60 healthy beech trees). The general average of *Pythium* spp. isolation percentage in each altitudinal level varied markedly, ranging from a minimum of 2.2% (L2) to a maximum of 11% (L4) (Fig. 4.2.1.2.1)

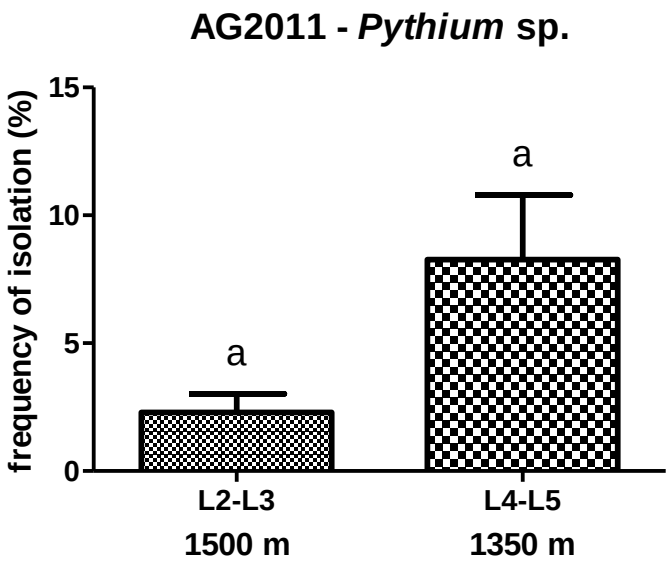

Fig. 4.2.1.2.1: *Pythiaceae* frequency in SL soil samples collected in 2011 sampling at two grouped altitudes. Data were analyzed with Student's t-test. Equal letters indicate no significant differences with the Mann-Whitney test (P>0.05).

A total of 4 *Pythium* species has been recovered; 2 *Pythium* (*Pythium intermedium* and *Pythium macrosporum*) are common to all four levels. *Pythium* sp. (A) was common to L3, L4 and L5, *Pythium* sp. (B) to L3 and L5.

Tab. 4.2.1.2.1: presence (+)/absence (-)of *Pythium* species in AG2011 in L2, L3, L4 and L5.

	AG2011			
Species	**L2**	**L3**	**L4**	**L5**
Py. intermedium	+	+	+	+
Py. macrosporum	+	+	+	+
Py. sp. (A)	-	+	+	+
Py. sp. (B)	-	+	-	+

L4 was characterized from the highest recovery of *Pythium intermedium* with a percentage of 90%. *Pythiaceae* diversity (Shannon index) increased with altitude whereas dominance (Berger-Parker index) decreased (Fig. 4.2.1.2.2).

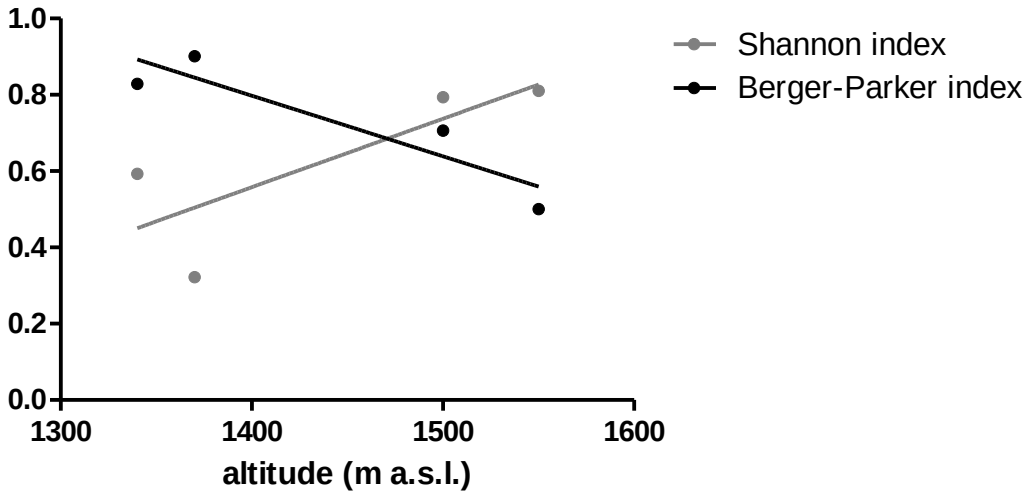

Fig. 4.2.1.2.2: Diversity indices in soil samples collected in 2011 at different altitudes in SL.

4.2.1.3 – Altitudinal Gradient 2011 (Latium)

Overall 1716 Pythiaceous colonies were isolated from soil samples from the 4 altitudinal level in MC in May 2011 (60 healthy beech trees).

The general average of *Pythiaceae* isolation percentage in each altitudinal level varied markedly, ranging from a minimum of 10% (L10) to a maximum of 39% (L8) and it increases with altitude (Fig. 4.2.1.3.1 and 4.2.1.3.2).

A total of 2 *Phytophthora* and 4 *Pythium* have been recovered; the 4 *Pythium* are common to all four levels. *Phytophthora plurivora* and *Phytophthora cactorum* have been recovered only in L8, with a percentage of isolation of 0.3% and 6% respectively (Tab. 4.2.1.3.1).

The frequency of isolation of *Pythium intermedium* and *Pythium macrosporum* increased with altitude.

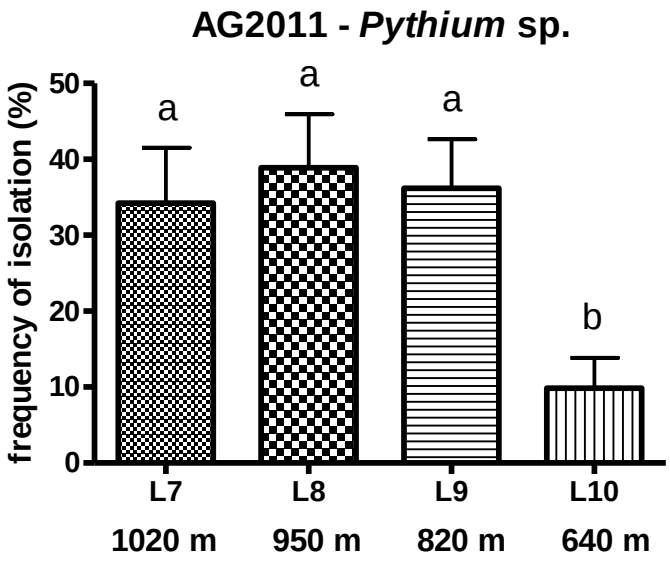

Fig. 4.2.1.3.1: *Pythiaceae* isolates frequency in SL soil samples collected in 2011 sampling at four different levels L7, L8, L9 and L10. Data were analyzed with Student's t-test. Different letters above bars indicate significant differences (P>0.05) between groups (Dunn's Multiple Comparison Test).

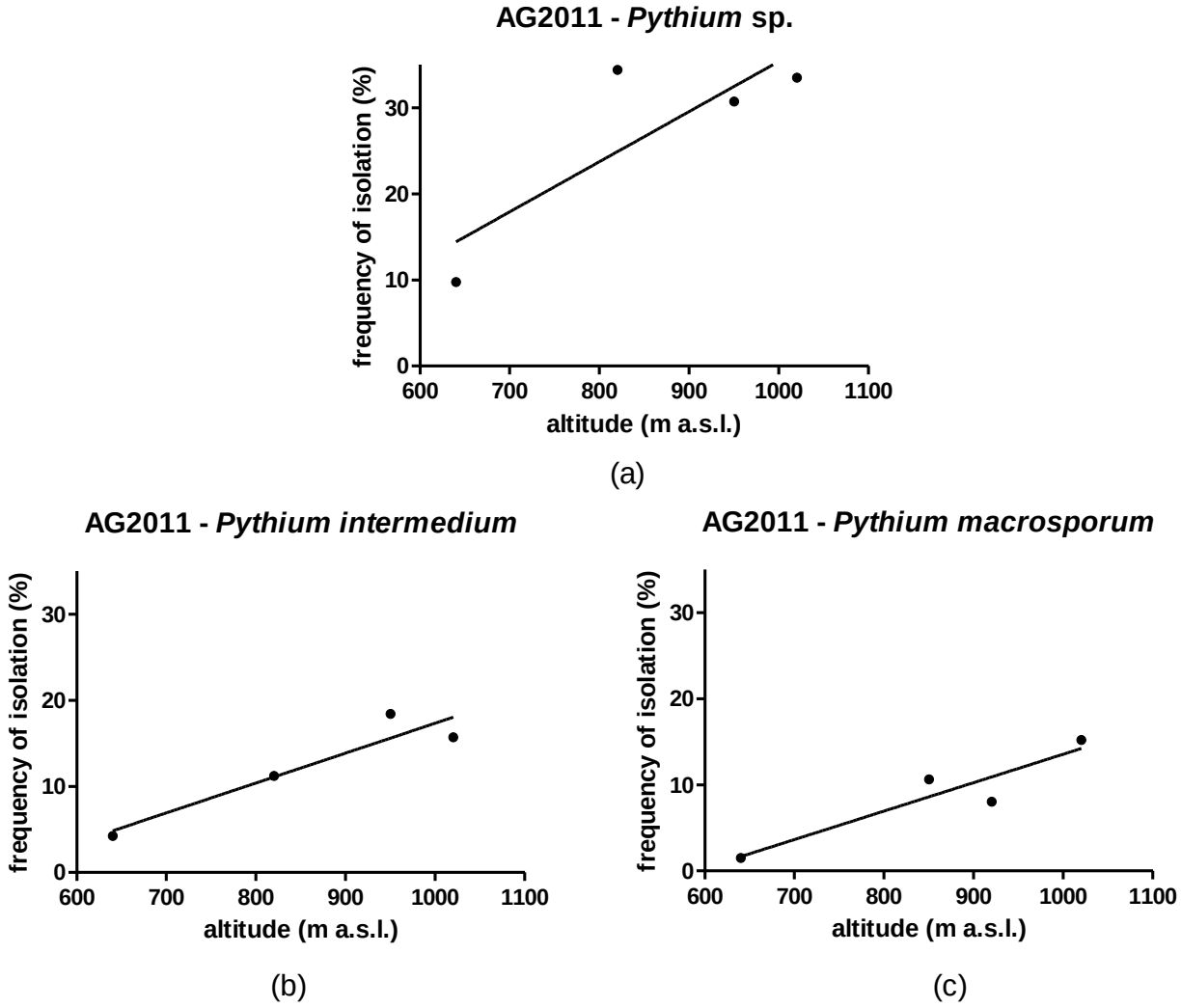

Fig. 4.2.1.3.2 (a), (b) and (c): frequency of isolation of *Pythium* species: *Pythium* spp. (a), *P. intermedium* (b) and *P. macrosporum* (c) in AG2011 (Latium).

Tab. 4.2.1.3.1: presence (+)/absence(-) of *Pythiaceae* species in AG2011 (Latium) in L7, L8, L9 and L10.

	AG2011			
Species	**L7**	**L8**	**L9**	**L10**
Py. intermedium	+	+	+	+
Py. macrosporum	+	+	+	+
Py. sp. (A)	+	+	+	+
Py. sp. (B)	+	+	+	+
Ph. plurivora	-	+	-	-
Ph. cactorum	-	+	-	-

Pythiaceae diversity (Shannon index) decreased with altitude whereas dominance (Berger-Parker index) increased (Fig. 4.2.1.3.3).

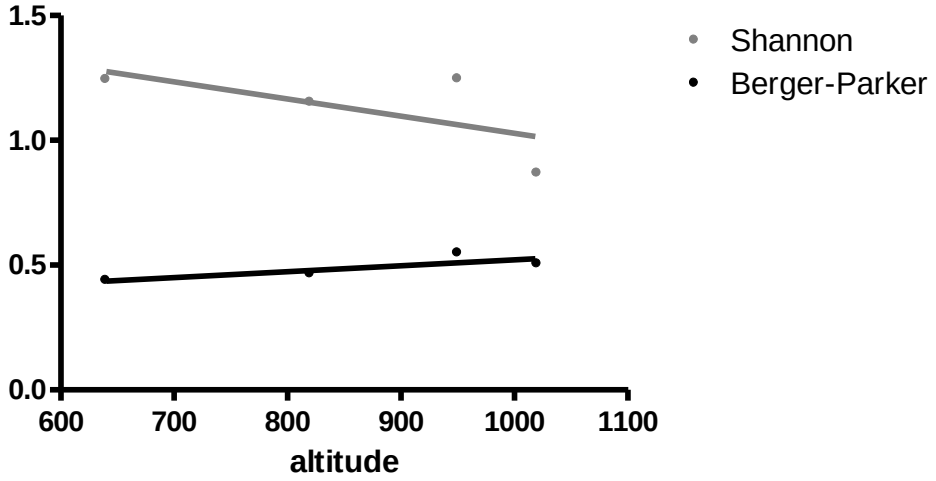

Fig. 4.2.1.3.3: Diversity indices in soil samples collected in 2011 at different altitudes in MC (Latium).

4.2.1.4 – Latitudinal Gradient 2010

Overall 614 Pythiaceous colonies were isolated from soil samples from PC and MS in July 2010 and in SL in September 2010 (45 healthy beech trees).

The general average of *Pythiaceae* isolation percentage in each altitudinal level varied markedly, ranging from a minimum of 2% (SL) to a maximum of 22% (PC) (Fig. 4.2.1.4.1).

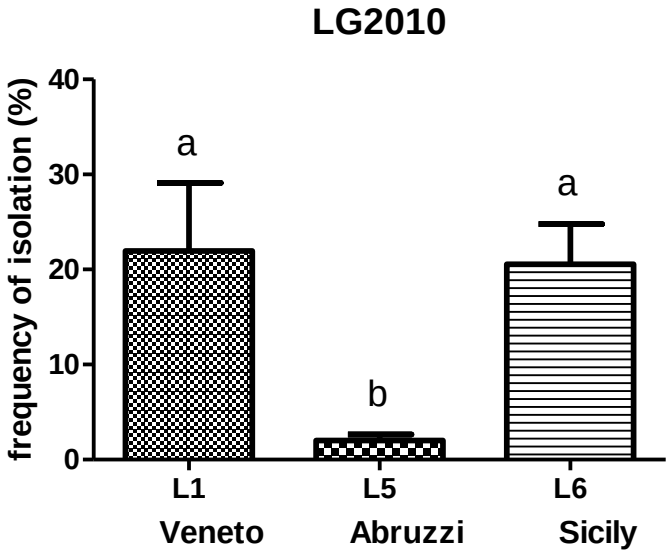

Fig. 4.2.1.4.1: *Pythiaceae* isolates frequency in Veneto, Abruzzo and Sicily soil samples collected in 2010 at three latitudinal different levels L1, L5 and L6. Data were analyzed with Student's t-test. Different letters above bars indicate significant differences (P>0.05) between groups (Dunn's Multiple Comparison Test).

A total of 4 *Pythium* and 1 *Phytophthora* have been recovered; *Phytophthora plurivora* has been recovered from PC, with only 1 isolate. *Pythium intermedium* was common to PC and SL, *Pythium macrosporum*, *Pythium* sp. (A) and *Pythium* sp. (B) were common to SL and MS (Tab 4.2.1.4.1).

Tab 4.2.1.4.1: presence (+)/absence (-) of *Pythiaceae* species in LG2010 in PC, SL and MS.

LG 2010			
species	**PC**	**SL**	**MS**
Ph. plurivora	+	-	-
Py intermedium	+	+	-
Py macrosporum	-	+	+
Py. sp. (A)	-	+	+
Py. sp. (B)	-	+	+

Pythiaceae diversity (Shannon index) decreased with latitude whereas dominance (Berger-Parker index) increased (Fig. 4.2.1.4.2).

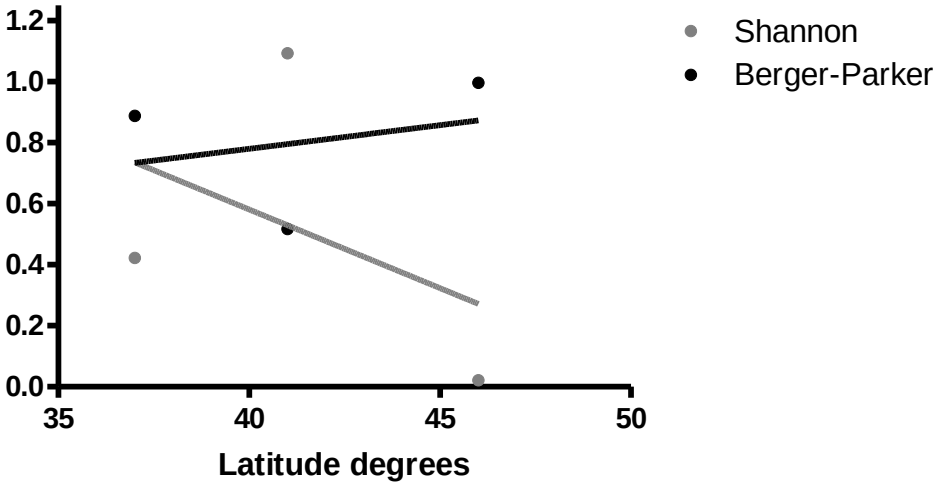

Fig. 4.2.1.4.2: Diversity indices in soil samples collected in 2010 at different latitudes.

4.2.1.5 – Latitudinal Gradient 2011

Overall 688 Pythiaceous colonies were isolated from soil samples from PC in July, MS in June and SL in August 2011 (45 healthy beech trees).

The general average of *Pythiaceae* isolation percentage in each altitudinal level varied markedly, ranging from a minimum of 5% (SL) to a maximum of 25% (PC). *Phytophthora plurivora* has been recovered in PC only (Fig. 4.2.1.5.1).

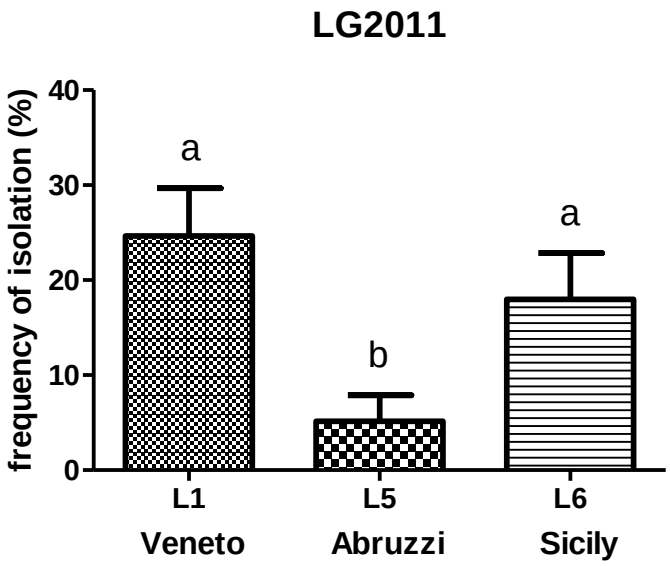

Fig. 4.2.1.5.1: *Pythiaceae* isolates frequency in Veneto, Abruzzo and Sicily soil samples collected in 2011 at three latitudinal different levels L1, L5 and L6. Data were analyzed with Student's t-test. Different letters above bars indicate significant differences (P>0.05) between groups (Dunn's Multiple Comparison Test).

Tab. 4.2.1.5.1: presence (+)/absence (-) of species in LG2011in PC, SL and MS.

	LG2011		
species	**PC**	**SL**	**MS**
Ph. plurivora	+	-	-
Py. intermedium	+	+	+
Py. macrosporum	+	+	+
Py. sp. (A)	-	+	+
Py. sp. (B)	-	+	+
Py. sp. (SL4)	-	-	+

The frequency of isolation of *Pythium intermedium* increased with latitude (Fig. 4.2.1.5.2).

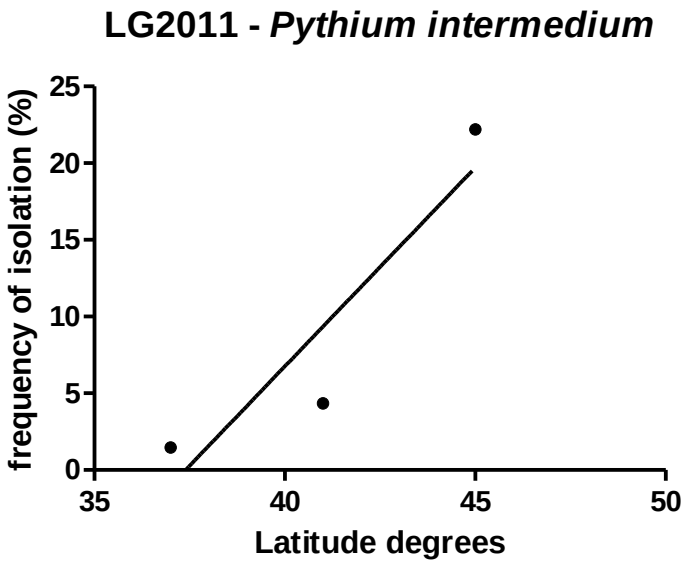

Fig. 4.2.1.5.2: Frequency of isolation of *Pythium intermedium* at different latitudes in LG2011.

Pythiaceae diversity (Shannon index) decreased with latitude whereas dominance (Berger-Parker index) increased (Fig. 4.2.1.4.2).

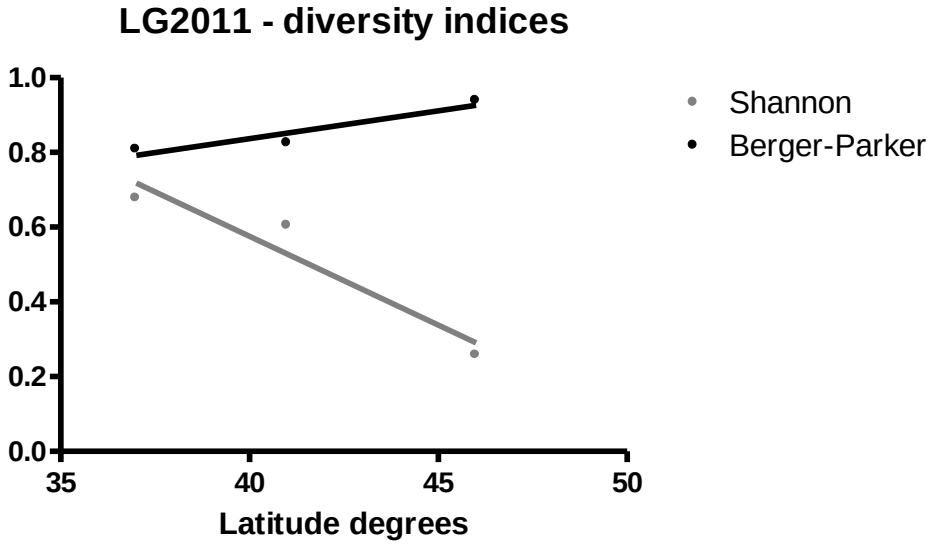

Fig. 4.2.1.5.3: Diversity indices in soil samples collected in 2010 at different latitudes.

4.2.2 – Declining forests

Overall 88 and 171 Pythiaceous colonies were isolated from soil samples from declining forests in 2010 and 2011 respectively (10 declining beech trees) (Fig.4.2.2.1).

In SL only *Pythium* spp. has been recovered during the two surveys in 2010 and 2011. *Phytophthora cambivora* has been detected in MS declining plot in 2010 and 2011 (Tab. 4.2.2.1 and Fig. 4.2.2.1).

Tab. 4.2.2.1: presence (+)/absence (-)of *Pythiaceae* in healthy and declining plots in MS (2010-2011).

MS - 2010		
species	**healthy**	**declining**
Ph.cambivora	-	+
Py.intermedium	+	-
Py.macrosporum	+	+
Py.sp. (A)	+	-
Py.sp. (B)	+	-
Py.sp. (MS3)	-	+
Py.sp. (SL4)	-	+

MS - 2011		
species	**healthy**	**declining**
Ph.cambivora	-	+
Py.intermedium	+	+
Py.macrosporum	+	+
Py.sp. (A)	+	-
Py.sp. (B)	+	-
Py.sp. (MS3)	-	-
Py.sp. (SL4)	+	+

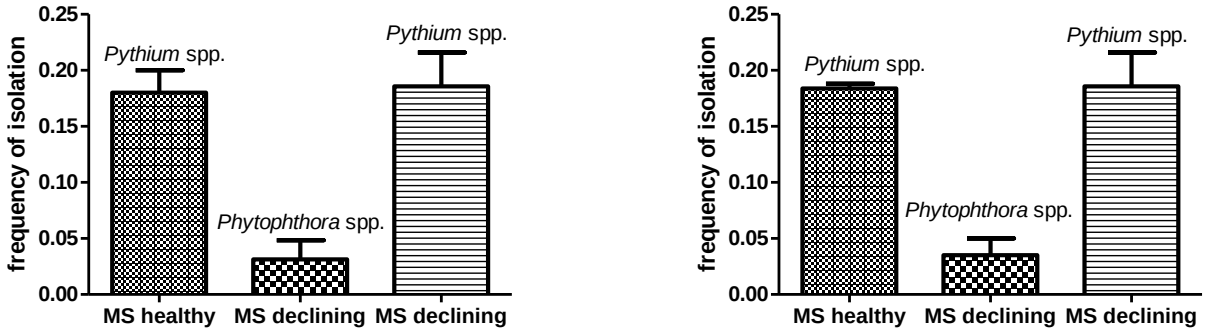

Fig. 4.2.2.1: *Pythium* isolates frequency in declining plots in MS (2010 and 2011).

4.3 – Discussion

Culture-dependent method revealed a high isolation frequency of *Pythium* spp. compared to *Phytophthora* spp. A moderate number of soil samples baiting-threaten was negative to the presence of *Pythiaceae*. This is probably due to the low number of sampling carried out on the study sites. It is generally accepted that failure to detect *Pythiaceae*, (especially *Phytophthora* spp.) with baiting techniques does not necessarily indicate their absence (Old, 1979; Erwin and Ribeiro, 1996; Vettraino *et al*., 2001). In fact baiting assays are affected by different biases, increasing the risk of false negative results; the success of isolation depends on several variables, such as the time of sampling, the type of media used and the presence of inhibitors in soil (Tsao, 1983).

Pythium species often referred to as mere pathogens of seedlings, root tips and succulent plant tissues (Hendrix and Campbell 1973) or as ubiquitous soil inhabitants, may have a contributing role in decline phenomena of woody plants, causing root damage on old trees. Furthermore, a study

conducted in Germany where *Pythium*-inoculated beech plants have revealed severe leaf necrosis, supports this theory (Nechwatal and Oβwald, 2001).

Pythium intermedium has been recovered with the highest percentage of isolation, suggesting a remarkable quantity of inoculum in beech stands investigated. Furthermore it had a very fast growth rate on plate, overlaying other *Pythiaceae* species, *Phytophthora* spp. in particular. *Pythium intermedium* is a typical soil inhabitant, it has a worldwide distribution (Van Der Plaats-Niterink, 1981) and it is associated with coniferous seedlings damping-off on nurseries and forest soils (Li *et al*., 2010; McLeod *et al*., 2009; Nechwatal, and Oβwald, 2001; Hocking, 1970; Campbell and Hendrix, 1968; Griffin, 1965). In this study it was found to increase at upper altitude in central Italy (MC) and latitude (LG2011). In AG2010-2011 (SL) *Pythium intermedium* had no response to climatic gradient: it is probably due to the limited range of altitude of the Abruzzi beech forest investigated. Also *Pythium macrosporum* has been recovered from all study sites: this species is distributed worldwide (Uzuahashi *et al*., 2008; Van der Plaats-Niterink, 1981) and it is associated with root disease of flower bulbs (Westover and Bever 2001), grasses (van Os *et al*., 1999) and carrot (Allain-Boulé *et al*., 2004). It had a good response along AG2011 (MC), with an increased frequency of isolation with altitude. Four additional *Pythium* strains have been recovered (*Pythium* sp. (A), *Pythium* sp. (B), *Pythium* sp. (SL4) and *Pythium* sp. (MS3)) that couldn't be identified: neither morphological identification nor analysis of sequences allowed to find a correspondence with species already described, therefore they are probably new species. Their distribution in the sites investigated did not reveal a connection with altitudinal and latitudinal gradients.

The low frequency of isolation of *Phytophthora* spp. (*P. plurivora, P. cactorum* and *P. cambivora*) prevented to have a response along gradients. However the recovery of *Phytophthora plurivora* and *P. cactorum*, already known to be particularly aggressive on beech, give a further contribution to our knowledge about the occurrence of this genus on healthy beech stands. *Phytophthora plurivora* has been recovered in PC and MC sites, whereas *Phytophthora cambivora* has been detected in MS (declining plot), where a widespread beech decline has been reported since the 90s. This beech forest dieback has been attributed to *Biscogniauxia nummularia*, a secondary pathogen normally found in beech wood (Granata and Whalley, 1994; Granata and Sidoti, 2004); however the sampled area revealed the presence of several beech trees with typical symptoms ascribed to *Phytophthora* spp. such as severe collar rot, stem with bleeding cankers and upper crown transparency. *Phytopthora cambivora* is considered as one of the most aggressive *Phytophthora* species involved in beech decline in Europe (Jung *et al*., 2005). *Phytophthora* spp. has been detected mainly in MC, where the presence of *Phytophthora cactorum* has already been reported by Vettraino *et al*. (2008).

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5 - Chapter 2 - Analysis of biodiversity of European beech *Pythiaceae* community detected along climatic gradients through massively parallel 454 sequencing (GS FLX Titanium) and comparison with baiting technique.

5.1 – Introduction

In this chapter the pythiaceous fungi assemblage diversity along climatic gradients is evaluated through high-throughput DNA sequencing.

Pyrosequencing is a straightforward, non-electrophoretic DNA sequencing method using the luciferase–luciferin light release as a signal for nucleotide incorporation into a PCR template DNA.

It offers a means to more extensively sample molecular diversity in microbial populations. It is possible to generate hundreds of thousands of short (100-400 bp) DNA sequence reads in a few hours without requiring the preparation of sequence templates by conventional cloning (Nilsson *et al.*, 2009). 454 sequencing uses a large-scale parallel pyrosequencing system capable of sequencing roughly 400-600 megabases of DNA per 10-hour run on the Genome Sequencer FLX with GS FLX Titanium series reagents (Voelkerding *et al*., 2009).

5.2.1 – Experimental design

Pythiaceous fungi assemblage analysis trough massively parallel sequencing involved the latitudinal gradient 2010 (LG2010) and the 2 declining plots situated in SL and MS (SL13 and MS4 respectively). Assuming the plot as experimental unit, 11 samples (9 for LG2010 and 2 for declining stands) were run at the same time on 1/16th of the Pico TiterPlate (PTP, 454 Life Science) device loaded on the 454 Life Sciences Genome Sequencer FLX Titanum (Roche Applied Biosystems, Nutley, NJ, USA) at Macrogen Korea. According to Roche indications, each 1/16th region usually yields between 40,000 and 60,000 sequences (reads) on the whole, therefore a sequence depth varying from 2000 to 3000 reads per sample was expected for samples run.

5.2.2 – DNA extraction, amplification and 454 sequencing

For each plot, the stored soil samples were bulked and a 5g-sub-sample was randomly selected and used for DNA extraction according to manufacturer's instructions (NucleoSpin Plant II, Macherey-Nagel, Düren, Germany; the kit allows a maximum of 5 g of soil to be processed). For each sample total DNA concentration was assessed running 3 µl DNA on 1.5% agarose gels containing 1xTBE buffer and 0.005% ethidium bromide.

For the Lib-L Unidirectional Sequencing (GS FLX Titanium emPCR Kits, Lib-L, 454 Life Science) of fungal internal transcribed spacer 1 (ITS1) amplicons forward primers were designed including

GS FLX Titanium Primer A (or adaptor A), a Multiplex Identifier (MID) tag and the oomycetes specific primer ITS6 (5'-A*xxx*GAAGGTGAAGTCGTAACAAGG-3'). The reverse primer was designed including GS FLX Titanium Primer B (or adaptor B), a Multiplex Identifier (MID) tag and the primer ITS7 (5'B*xxx*AGCGTTCTTCATCGATGTGC-3') (Cooke *et al.*, 2000), where A and B represent the two pyrosequencing adaptors (CGTATCGCCTCCCTCGCGCCATCAG and CTATGCGCCTTGCCAGCCCGCTCAG) and *xxx* represents the MIDs. In order to enable reads segregation of each of the samples after sequencing, one different forward primer per sample was designed by inserting a different FLX Titanium MID.

Template DNA (10 ng) was PCR amplified in a 25 µl reaction containing 1x MyTaq TM HS Mix (Bioline), 1 µl forward primer, 1 µl reverse primers and making up to 25 µl adding GIBCO® DNase/RNase free distilled water (Invitrogen, Carlsbad, CA, USA). Negative (water) control was included in the PCR reactions. The PCR cycle parameters, according to MyTaq TM manufacturer's instructions and primers annealing temperature, consisted of an initial denaturation of 95°C for 1 min, then 35 cycles consisting of a denaturation of 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, followed by 10 min at 72°C. Amplicons were purified using the Agencourt AMPure XP system (Beckam Coulter Inc., Milan, Italy), and quantified with Qubit Quantitation Kit (Invitrogen, USA) by using Quant-it^{TM} ds DNA HS Assay Kit (Invitrogen).

110 ng of purified DNA were pooled in a unique final sample. The sample was evaporated to dryness at 30°C on a Savant DNA 110 Speedvac (Savant, Farmingdale, NY, USA); finally pellet was resuspended in 50 µl 10 mM TrisHCl, pH 8, before GS FLX sequencing (454 Life Science/Roche Applied Biosystems) at Macrogen Korea (Seoul, Korea).

5.2.3 – Molecular Bioinformatics and Operational Taxonomic Units (MOTUs) designation

FLX-generated reads were trimmed for low quality and adaptors sequence and then sorted by tag sequence into separate sample-specific files by Macrogen Korea (Roche GS FLX software 2.6). A further reads trimming for low quality and ambiguous bases (0 ambiguous nucleotides allowed in sequence) was then performed with CLC Genomics Workbench 4.9, reads shorter than 150 bp were excluded from further analysis. In order to identify Molecular Operation Taxonomic Units (MOTUs), the reads were compared with a non-redundant-custom-curated database (CCD) derived from GenBank and a laboratory reference database, for a total of 2317 sequences addressing 280 *Pythiaceae* species. The 98% barcoding threshold and a 70% of query length were assumed to assign sequences to MOTUs (Vettraino, *et al.*, 2012). The overall MOTUs richness (S) was calculated by summing the number of MOTUs of each sample, including singletons.

5.2.4 – Statistical analysis

All the statistical analyses were carried out with the Prism5 package (GraphPad Software Inc., San Diego, CA, USA). PAST 2.06 (http://folk.uio.no/ohammer/past/index.html) was used for species accumulation curve construction (Krebs algorithm; Krebs, 1989) and diversity (Shannon_H), dominance (Berger-Parker) indices calculation.

5.3 – Results

5.3.1 – 454 sequencing output – Latitudinal gradient 2010 and declining plots

Overall 28056 rough reads resulted from the sequencing of 1/16 of PicoTiter Plate; 26707 reads passed quality control with the number of reads ranging from 347 to 4147. A total of 18320 reads (69%) (4070 in site PC, 5810 in site SL –healthy-, 1667 in site MS –healthy-, 2626 in site SL –declining- and 4147 in site MS –declining-) matched the CCD on the basis of the 98% identity criterion. The number of unmatched accounted to 3999 (49.6%), 338 (5.5%) and 972 (36.8%) in site PC, SL and MS respectively, for a total of 5309 reads (31.5%). On declining sites the number of unmatched accounted to 2997 (53.3%) and 81 (1.9%) in site SL and MS respectively.

The final sequence depth did not resolve completely the resident population: the species accumulation curve revealed that community richness of *Pythium*, *Phytophthora* and *Phytopythium* spp. is likely to exceed the richness captured by samplings (Fig. 5.3.1.1).

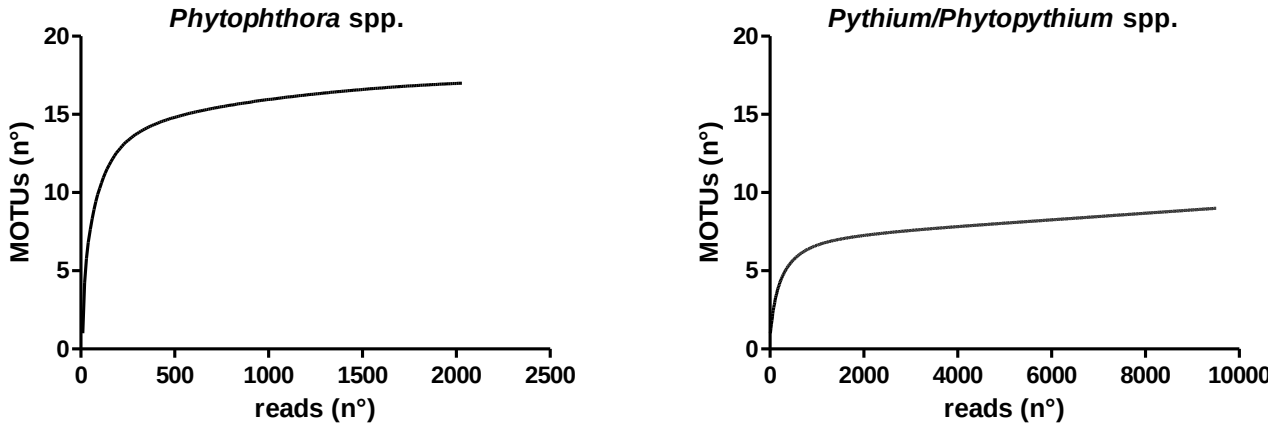

Fig. 5.3.1.1: rarefaction curves of number of reads versus number of MOTUs of 150 healthy trees of the 4 study sites: *Phytophthora* spp (left) and *Pythium* and *Phytopythium* spp. (right). Curves generated using PAST 2.06 and GraphPad Prism 5.

The pythiaceous community was characterized by 26 species, 17 belonging to the genus *Phytophtora*, 7 to the genus *Pythium* and 2 to the genus *Phytopythium* (Tab. 5.3.1.1 and 5.3.1.2)

with a predominance of *Pythium intermedium* (74%) in all soil samples at all sites, except for the declining plot of Serralunga.

A total of 10 *Phytophthora* and 4 *Pythium* species were common to the 3 healthy sites and the richness of species ranged between 18 (SL) and 21 (PC). *Phytophthora nemorosa*, *Phytopythium litorale* (PC2), *Pythium apiculatum* (PC2) and *Py.chamaehyphon* (SL11) were present as singletons (Tab. 5.3.1.1).

Table 5.3.1.1: Number of reads per soil sample attributed to *Phytophthora* and *Pythium* species in LG2010 (PC, SL and MS).
LL = latitudinal level; PC = Pian Cansiglio; SL: Serralunga; MS = Monte Soro

	n° reads											
species	**soil samples LL1**				**soil samples LL5**				**soil samples LL6**			
	PC1	PC2	PC3	**Total**	SL10	SL11	SL12	**Total**	MS1	MS2	MS3	**Total**
Phytophthora cactorum	0	1	0	**1**	0	0	0	**0**	1	0	0	**1**
Ph. cambivora	7	10	0	**17**	0	7	0	**7**	0	1	0	**1**
Ph. capsici	5	2	0	**7**	0	4	0	**4**	2	4	5	**11**
Ph. cinnamomi	12	5	1	**18**	5	3	1	**9**	82	4	11	**97**
Ph. citrophthora	8	135	8	**151**	11	4	0	**15**	28	38	10	**76**
Ph. cryptogea	14	9	0	**23**	2	0	1	**3**	4	4	1	**9**
Ph.europaea	0	0	0	**0**	1	0	1	**2**	0	0	0	**0**
Ph.gonapodyides	0	0	0	**0**	2	0	0	**2**	0	0	5	**5**
Ph.ilicis	0	0	0	**0**	14	0	0	**14**	3	0	0	**3**
Ph. lateralis	5	16	591	**612**	538	7	0	**545**	4	16	5	**25**
Ph. megasperma	4	3	1	**8**	0	0	0	**0**	3	1	3	**7**
Ph.nemorosa	0	0	0	**0**	1	0	0	**1**	0	0	0	**0**
Ph. palmivora	17	3	0	**20**	3	0	0	**3**	0	2	0	**2**
Ph. plurivora	4	2	0	**6**	0	0	0	**0**	1	0	1	**2**
Ph. pseudosyringae	1	3	0	**4**	222	0	0	**222**	8	4	0	**12**
Ph. ramorum	0	0	5	**5**	5	3	0	**8**	0	11	1	**12**
Ph. taxon Pgchlamido	3	1	14	**18**	6	2	0	**8**	16	3	15	**34**
Phytopythium litorale	0	1	0	**1**	0	0	0	**0**	0	0	0	**0**
Phytopy.vexans	4	12	1	**17**	0	0	0	**0**	5	1	4	**10**
Pythium.aff.monospermum	0	5	1	**6**	0	0	0	**0**	0	2	0	**2**
Py.apiculatum	0	1	0	**1**	0	0	0	**0**	0	0	0	**0**
Py.attrantheridium	50	14	2	**66**	0	4	0	**4**	19	19	12	**50**
Py.chamaehyphon	0	0	0	**0**	0	1	0	**1**	0	0	0	**0**
Py.heterothallicum	60	11	0	**71**	1	5	0	**6**	0	0	0	**0**
Py.intermedium	2469	362	142	**2973**	123	2478	2353	**4954**	193	305	804	**1302**
Py.macrosporum	19	8	0	**27**	0	0	0	**0**	0	1	0	**1**
Py. sp.	12	5	1	**18**	0	2	0	**2**	1	2	2	**5**

Table 5.3.1.2: Number of reads per soil sample attributed to *Phytophthora* and *Pythium/Phytopythium* species in declining plots 2010 (SL and MS). SL: Serralunga; MS = Monte Soro.

species	n° reads soil samples site SL (declining)	soil sample site MS (declining)
Phytophthora cactorum	6	0
Ph. capsici	4	0
Ph. cinnamomi	13	0
Ph.citrophthora	10	4
Ph.cryptogea	1	0
Ph.europaea	3	0
Ph.lateralis	2248	1
Ph.megasperma	7	0
Ph.palmivora	2	0
Ph.pseudosyringae	5	0
Ph.ramorum	11	0
Ph.taxon Pgchlamydo	65	0
Pythium attrantheridium	6	0
Py.heterothallicum	2	0
Py.intermedium	239	4142
Py.macrosporum	1	0
Phytopythium vexans	3	0

The most represented species was *Pythium intermedium* (74%), particularly frequent in SL11 and MS (declining) samples; its abundance increased with latitude (Fig. 5.3.1.2). Among Phytophthoras, *P. lateralis* (19%), *P. citrophthora* (1,4%) and *P. pseudosyringae* (1.3%) were the most abundant.

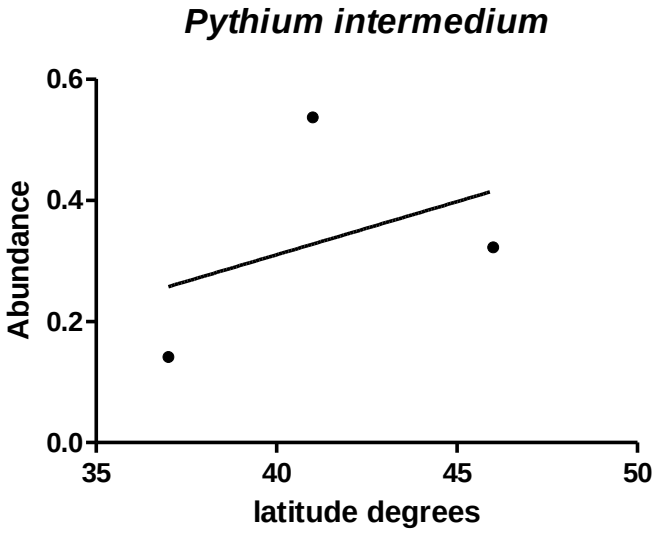

Fig. 5.3.1.2: abundance of *Pythium intermedium* along at different latitudes in 2010.

The dominance (Shannon index) increased with latitude, while the diversity decreased (Fig. 5.3.1.3).

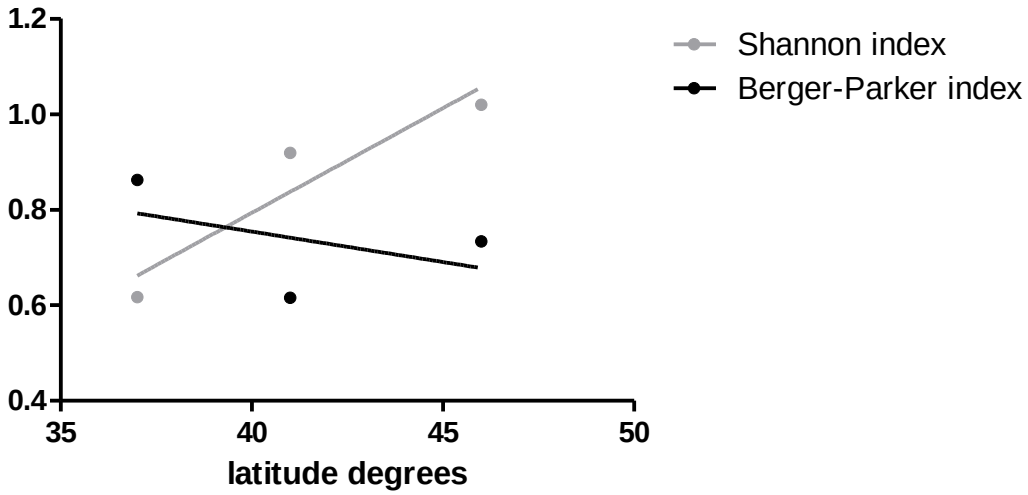

Fig. 5.3.1.3: Diversity indices in soil samples collected in 2010 at different latitudes.

5.4 – Pyrosequencing assay (PA) vs. baiting technique in LG2010-2011 and declining plots

Of the 26 pythiaceous species detected by PA, 2 *Pythium* and 2 *Phytophthora* spp. were isolated by baiting in the study sites (LG2010 and declining plots) during the 2010-2011 surveys. PAs confirmed the presence of *Pythium intermedium*, *Pythium macrosporum*, and *Phytophthora plurivora*. *Phytophthora cambivora* was isolated by baiting in declining plot in MS but PAs revealed its presence only one healthy plot of the same sampling site (as a singleton). *Pythium* sp. (A), *Pythium* sp. (B), *Pythium* sp. (SL4) and *Pythium* sp. (MS3) were detected only by baiting, whereas *Phytophthora cactorum, Ph. capsici, Ph. cinnamomi, Ph. citrophthora, Ph. cryptogea, Ph. europaea, Ph. gonapodyides, Ph. europaea, Ph. ilicis, Ph. lateralis, Ph. megasperma, Ph. nemorosa, Ph. palmivora, Ph. pseudosyringae, Ph. ramorum, Ph.* Taxon PgChlamydo, *Phytopythium litorale, Phytopythium vexans, Pythium* aff. *monospermum, Py.apiculatum, Py. attrantheridium, Py. chamaeyphon* and *Py. heterotallicum* were detected by PA but not by baiting (Tab. 5.4.1)

Table 5.4.1: comparison of the detection of *Pythiaceae* with baiting and PA in the three sites of latitudinal gradient.

	Site PC		Site SL		Site MS	
Pythiaceae	Baiting	PAs	Baiting	Pas	Baiting	Pas
Ph. cactorum	No	Yes	No	No	No	Yes
Ph. cambivora	No	Yes	No	Yes	No	Yes
Ph. capsici	No	Yes	No	Yes	No	Yes
Ph. cinnamomi	No	Yes	No	Yes	No	Yes
Ph. citrophthora	No	Yes	No	Yes	No	Yes
Ph. cryptogea	No	Yes	No	Yes	No	Yes
Ph.europaea	No	No	No	Yes	No	No
Ph.gonapodyides	No	No	No	Yes	No	Yes
Ph.ilicis	No	No	No	Yes	No	Yes
Ph. lateralis	No	Yes	No	Yes	No	Yes
Ph. megasperma	No	Yes	No	No	No	Yes
Ph.nemorosa	No	No	No	Yes**	No	No
Ph. palmivora	No	Yes	No	Yes	No	Yes
Ph. plurivora	No	Yes	No	No	No	Yes
Ph. pseudosyringae	No	Yes	No	Yes	No	Yes
Ph. ramorum	No	Yes	No	Yes	No	Yes
Ph. taxon Pgchlamido	No	Yes	No	Yes	No	Yes
Phytopythium litorale	No	Yes**	No	No	No	No
Phytopythium vexans	No	Yes	No	No	No	Yes
Py.aff.monospermum	No	Yes	No	No	No	Yes
Py.apiculatum	No	Yes**	No	No	No	No
Py.attrantheridium	No	Yes	No	Yes	No	Yes
Py.chamaehyphon	No	No	No	Yes**	No	No
Py.heterothallicum	No	Yes	No	Yes	No	No
Py.intermedium	Yes	Yes	Yes	Yes	Yes	Yes
Py.macrosporum	Yes	Yes	Yes	No	Yes	Yes
Py. sp.(A)	No	No	Yes	No	Yes	No
Py. sp. (B)	No	No	Yes	No	Yes*	No
Py. sp. (SL4)	No	No	No	No	Yes	No
Py. sp. (MS3)	No	No	No	No	Yes	No

*Detected by baiting only on declining plot.
**Detected by PA as a singleton.

Differences in diversity indices among Pythiaceous communities detected by PA and baiting in LG2010, and baiting of soil samples collected in LG2011 surveys were consisted at the Kluskal-Wallis tests (Tab. 5.4.1.2)

Tab. 5.4.1.2: comparison of diversity indices of the Pythiaceous community assemblage based on PA (9 soil samples on LG2010) and baiting of 135 soil samples collected in LG2010 and LG2011 respectively.

	Soil samples LG2010 (9)	**Soil samples LG2010 (135)**	**Soil samples LG2011 (135)**
Diversity index	PA	Baiting	Baiting
Shannon diversity	0,78a	0,19b	0,25b
Berger-Parker dominance	0,78a	0,90b	0,93b

Data were analyzed with Kluskal-Wallis test. Different letters along rows indicate significant differences with the Dunn's multiple comparison tests (P>0.05).

5.5 – Discussion

The most widely used technique to identify *Pythiaceae* in environmental samples relies upon culture-based morphological approaches (selective media and baiting technique). Since it is not easy to identify or quantify *Pythiaceae* species the need arise to find a rigorous technique, which is rapid, reliable and highly reproducible to evaluate their diversity in living in European beech. Next-generation sequencing (NGS) technologies offer an opportunity to overcome most limitations. The use of PA analyses opens new perspectives in diagnostic applications and also for issues related to the movement of alien species into new environments or along pathways of introduction (Vannini *et al*., 2013).

The results of rarefaction curves indicate that PA of soil samples was not sufficient to describe the *Pythiaceae* community in this study.

Phytophthora cinnamomi in Sicily is an interesting element because it is a thermophile species, even if it isn't known to be aggressive on beech.

The occurrence of *Phytophthora pseudosyringae* (just known to be particularly aggressive on European beech (Jung *et al*., 2003; Jung *et al*., 2005; Jung 2009)) in SL stands is a recurring result: this species has been already isolated on beech stands in Abruzzi (Cacciola *et al*., 2005). The recovery of *Phytopthora pseudosyringae* even with NGS is very important to confirm the effectiveness of pyrosequencing.

The high abundance of *Phytophthora lateralis* is a very unexpected result. This species is considered one of the most destructive among the invasive species and it was first isolated from roots and collars of dying ornamental cedar (*Chamaecyparis* spp.) in nurseries and gardens in USA (Tucker and Millbrath, 1942) but it probably has an Asiatic origin (Brasier *et al*., 2010). Since then it has continued to spread to the native range of *C. lawsoniana* and it has spread in Europe through nurseries with its first confirmation on Lawson's cypress in France (Hansen *et al*., 1999). Moreover an unpublished study conducted in France with NGS technology confirmed the presence of this species on beech forests, but this data was not supported by other analyses.

The high abundance of reads of *Pythium intemedium* confirmed the results of baiting technique along the latitudinal gradient in terms of elevated isolation and response to biodiversity indices. Except for *P. intermedium* only *Pythium macrosporum* has been revealed by PA, but uncultered method does not give a real quantitative value.

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6- Conclusions

This study, conducted along altitudinal and latitudinal gradients with two different approaches (baiting technique vs. pyrosequencing assays), helped to identify efficient bioindicators able to describe beech stands located in different areas.

Baiting technique analysis allowed the identification of a small number of Pythiaceous species with a low abundance. The relative abundance of *Pythium intemedium* (the most represented species) changed along latitudinal (2011) and altitudinal (2011, Latium) gradients. It was assumed that its presence and distribution along the study sites was mainly driven by climate. *Pythium intermedium* resulted associated to colder climate in northern Italy and high elevations (Latium altitudinal gradient). Similarly, according to 454 sequencing results, it responded positively to latitude. In the next decade CC is predicted to impact on European forests fungal communities so target species may be taken into account to contribute to the monitoring studies along gradients.

Pythiaceous community resulted highly rich and diverse in PA but baiting technique showed a low diversity and abundance. This is probably due to the low number of samplings and to the different biases affecting baiting technique, such as the time of sampling, the type of media used and the presence of inhibitors in soil (Tsao, 1983). On the other hand 454 sequencing detect DNA of any *Pythiaceae* species in soil but the vitality is almost impossible to determine (Jumpponen and Jones, 2010).

In conclusion the combination of these two methods gave us an efficient tool to determine a good bioindicator of climate change, such as *Pythium intermedium*.

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