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Phytophthora in natural and anthropic environments: new molecular diagnostic tools for early detection and ecological studies

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Phytophthora in natural and anthropic environments: new molecular diagnostic tools
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RIASSUNTO

Parole chiave:

qPCR; *Phytophthora*; invasive micro-organism; disease management; early detection, aerial spread, soilborn pathogen, Janzen-Connell negative density dependence hypothesis

Scopi

Scopo della tesi è stato l'analisi della patogenicità ed ecologia del genere *Phytophthora* attraverso differenti approcci molecolari.

Metodi e Risultati

L'efficacia di PCR quantitativa basata su una sonda TaqMan specifica per il genere è stata confermata come preciso strumento di rilevamento di DNA di *Phytophthora* su suolo artificialmente infettato, in suolo di invasatura contaminato proveniente da vivaio ed in campioni di trappole aeree. Nessuna quantificazione di DNA è avvenuta dopo due settimane dalla morte indotta del patogeno e, in confronto con i metodi di isolamento tradizionali, è stata dimostrata una significativa maggiore efficienza come strumento diagnostico. La fluttuazione stagionale di *Phytophthora* in aria è stata quantificata e descritta per il periodo di campionamento. La tecnica di sequenziamento 454 è stata utilizzata per identificare la tassonomia delle specie di *Phytophthora* in un hotspot biologico in Western Australia ed al fine di descrivere la patogenicità delle due specie sequenziate con maggior frequenza è stato realizzato un esperimento in serra.

Conclusioni

Le tecniche di laboratorio utilizzate in questo studio hanno fornito nuove nozioni sull'ecologia di *Phytophthora*. La PCR quantitativa basata su sonda TaqMan è testata e proposta come efficace strumento di prevenzione verso l'arrivo di specie invasive.

Importanza dell'impatto di questo studio

L'efficienza della gestione di *Phytophthora* dipende dalla conoscenza delle caratteristiche patogene di gruppi ristretti di specie o di una singola specie . Ricerche come queste forniscono le basi scientifiche per comprendere l'epidemiologia di una malattia ed applicare un controllo risolutivo.

Lavori correlati alla Tesi.

Migliorini, D., Ghelardini, L., Tondini, E., Luchi, N., & Santini, A., 2015, The potential of symptomless potted plants for carrying invasive soilborne plant pathogens, *Diversity and Distributions*, *21*(10), 1218-1229.*

* Articolo riprodotto con il permesso di Diversity and Distributions

ABSTRACT

Key words

qPCR; *Phytophthora*; invasive micro-organism; disease management; early detection, aerial spread, soil born pathogen, Janzen-Connell negative density dependence hypothesis

Aims

The purpose of my thesis was to investigate pathogenicity and ecological traits of the genus *Phytophthora* through molecular approaches.

Methods and Results

A quantitative PCR technique based on a genus specific TaqMan probe was confirmed as a precise method for detecting *Phytophthora* DNA in artificially infested soil under laboratory condition, in naturally infested soil and tissues of potted nursery plants and in the filters of air traps. No positive DNA quantification occurred in soil after two weeks from pathogen induced death and a significant higher efficiency as diagnostic tool was demonstrated compared to traditional isolation methods both in soil and plant tissues. Seasonal fluctuation of aerial spread of *Phytophthora* was also quantified and described. A 454 sequencing approach was used to identify the *Phytophthora* species present in a biological hotspot area in Western Australia, and a glass house experiment was performed in order to describe the pathogenicity traits of the two most frequently detected species.

Conclusions

The lab procedures used in this study provided a more precise knowledge of *Phytophthora* ecology. The quantitative PCR assay based on designed TaqMan probe was demonstrated to be very efficient and is proposed as a reliable early detection instrument of prevention against the income of invasive species.

Significance of impact of the study

Efficient management of Phytophthora depends to the knowledge of pathogenicity traits in restrict groups or single species. Investigations like those presented in this thesis contribute the scientific bases to understand the epidemiology of disease and to apply a successful control.

Papers related to the Thesis.

Migliorini, D., Ghelardini, L., Tondini, E., Luchi, N., & Santini, A., 2015, The potential of symptomless potted plants for carrying invasive soilborne plant pathogens, *Diversity and Distributions*, *21*(10), 1218-1229.*

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Contents

Introduction

Biological cycle of *Phytophthora*

The genus *Phytophthora* includes fungal-like microorganisms belonging to the Oomycetes (Peronosporaceae, Peronosporales, Peronosporidae, Peronosporea, Incertae sedis, Oomycota, Chromista). These microorganisms differ from fungi in as much as they produce zygotes, from which a diploid mycelium develops, and have cell walls composed of cellulose and glucans (Agrios, 2005).
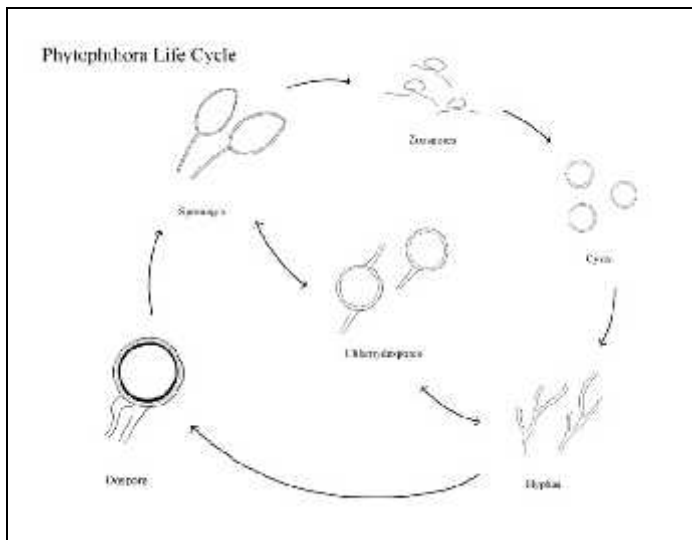

Figure 1. http://forestphytophthoras.org/. Sexual reproductive long term survival spores (Oospores) are generated when the antheridium (female organ) is fertilized by the oogonium (male organ). Cross fertilization occurred in heterothallic species while self-fertilization in homothallic species. In the first case the reproduction organs belong from two distinct organisms (two different mating types) while in the second they are from the same organism (one mating type). Filamentous structures called hyphae developed from flagellate motile zoospores, unicellular released propagules produced and released by a specific structure formed on hyphae, the sporangium. Biological cycle of *Phytophthora* is characterized by hyphal growing in the host, sporangia formation and zoospores spreading and germination. Cysts (encysted zoospores) and chlamydospores are resting structures. The second are part of the asexual cycle only and they are more thick-walled, longer term survival structures; they developed in nature in order to survive to critical season (weeks/months).

Phytophthora life cycle includes both sexual and asexual reproduction. During the asexual phase, zoospores, i.e. ciliate spores able of active diffusion in water, are released in the environment by specific structures produced on hyphae: chlamydospores and sporangia. In *P. infestans*, sporangiophores are additional structures developed as a support for sporangia, while in *P. ramorum* the sporangia are often grouped in clusters. Sexual reproduction takes place by the union of Oogonium and Antheridium, the hyphal apexes aimed for breeding, belonging to two different mating types. After meiosis, parental haploid gametes are mixed through karyogamy and the Oogonium becomes a zygotic spore, the Oospore. The germination of an oospore produces a new diploid mycelium.

The hyphal mycelium found in infected hosts originates from zoospores, grows inside plant tissues causing symptoms such as loss of fine roots, root rots, steam roots, blights on twigs and leafs, seedling damping off. *In planta* the diffusion of these microorganisms is generally quick because they move throughout the cambium and vessels.

The most destructive, and most studied, *Phytophthora* diseases are characterized by a limited number of genetic lineages and sexual reproduction is nearly absent due to the presence of a single mating type. For instance, *P. cinnamomi* has long been introduced from either Papua New Guinea or Sumatra in agri-horticultural environments from where it migrated to forests. The greatest damages are currently produced on Australian native plants. Due of numerous separate introductions, several distinct groups of genotypes have been found to be responsible for disease outbreaks at regional and global scale. However each group comprised a single mating type. Introductions have been characterized by extremely simplified populations, which reproduced clonally, and the same genotype has appeared on the same host species in different parts of the world (Garbelotto, 2008). Another example is *P. ramorum*, the agent of Sudden Oak Death (SOD) disease, which has caused extensive damages to forests and nurseries in California and Oregon, has recently out-broken in plantations of Japanese larch and in plantations of many tree species in Britain and Northern Ireland, has been isolated in Norwegian natural Bilberry plants, and is signaled in 14 European countries (Brasier and Webber, 2010; Webber *et al.*, 2010; Herrero *et al.*, 2011; Santini *et al.*, 2013). There are two lineages *P. ramorum* in North America (NA1 and NA2) and two in Europe (EU1 and EU2) (Grünwald *et al.*, 2009; Van Poucke *et al.*, 2012) with only two mating types (A1 and A2) diffused in both continents (Chandelier *et al.*, 2014). Finally, another example of this mechanism of diffusion is *P. infestans*, the causal agent of potato and tomato late blight and possibly the most destructive Oomycete for agricultural crops, whose mating type A1 spread world-wide asexually. Only after the 1980s the compatible mating type A2 escaped from Mexico and sexual reproduction became possible in non-native countries.

The three *Phytophthora* species mentioned above are heterothallic, i.e. produce compatible male and female gametes on physiologically distinct mycelia, and when two compatible mating types come into contact, sexual reproduction occurs. Aspects of *Phytophthora* reproduction strategies has been discussed in Jung *et al.* (2011b). Heterothallism is a successful strategy for microorganisms that inhabit tropical rain forests, where the high degree of spatial plant diversity requires rapid adaptation to new host genotypes. However the constantly warm and wet conditions of the tropical environment are always favorable for continuous asexual multiplication, while sexual reproduction is possible but rare. In Mediterranean and temperate climates, ecological requirements are different. Quiescent resistant structures are necessary for *Phytophthora* to survive during dry summers and cold winters. Oospores are the perfect organs to survive harsh environmental conditions for long periods of time because they encapsulate viable zoospores that will only be released when favorable conditions occur. Oospores are produced through sexual reproduction in heterothallic or homothallic breeding systems. In homothallic species compatible male and female gametes are produced on the same mycelium. Homothallic reproduction is a compromise between the need for adaptability via sexual recombination and the necessity of regular and abundant production of resting structures for survival in a dormant state during the unfavorable season, and it is indeed more common in *Phytophthora* species inhabiting geographical regions with temperate and cold climates. Parasexual phenomena (heterokaryon formation, hyphal anastomosis and zoospore fusion within or between species, i.e. somatic hybridization) are also reported in Phytophthoras, but they are imperfectly described and not clearly understood (Jung *et al.*, 2011b).

In addition, in the majority of *Phytophthora* species, chlamydospores, which are resting structures that contain zoospores, are produced during the asexual phase of the life cycle. Persistence in nature of this type of spores is not well known, but they are surely more resistant than sporangia and zoospores. The formation of chlamydospores has been associated to occurrence of stress conditions for limited time, such as temporary lack or excess water, or temperature slightly outside the upper or lower limits of the growth range.

The study of Phytophthora as a plant pathogen

In 1996 the most important *Phytophthora* compendium published by Erwin and Ribeiro listed approximately 60 species. By 2008, around 90 species were described or proposed (Grunwald *et al.*, 2012) and according to an estimate by Brasier (2009), two to four times this number of species might be expected to exist. By

October 2011, the number of validly described and recognized species has risen to 101 (Kroon *et al.*, 2012) and to date, 123 species are formally described (phytophthoradb.org). The huge increase in the number of species is explained by increased effectiveness of modern detection methods, since classical isolation and identification of *Phytophthora* has always been difficult (Jung *et al.*, 2003; Maseko *et al.*, 2007; Rea *et al.*, 2011). Moreover, development of molecular tools has enabled distinction of different species within taxonomical groups previously thought to be uniform (Rytkönen, 2011; Jung and Burgess, 2009).

As a soil born pathogen, the transport of *Phytophthora* between continents occurs through the trade of live plants and the diffusion of new pathogenic species is a direct consequence. Potted plants for planting arrive to Europe principally from Central America and Eastern Asia. After a period of growth in European nurseries, they are shipped to the final destination as ornamental products, often being transported again over continental distances. While host plants are maintained in good conditions during the whole production chain, *Phytophthora* may easily survive as root infections or as resting structures like oospores or chlamydospores. Additionally, since zoospores are freely motile in water, the inoculum spreads quickly in nurseries through the drainage system and irrigation water. As far as Europe is concerned, some examples of the dimension of international trade provide the measure of the movement of pathogens. Plant imports from Morocco and Turkey, both countries being almost absent as traders of live trees in the global market in 2002, while nowadays they are both strong competitors of Costa Rica, USA and China, has jumped in a single decade (2002-2012) up to 4,217,988 US$ for Morocco and to 7,732,883 US$ for Turkey. These countries therefore are new leading plant providers at least for Europe. Cumulate US dollars amounts in the same time period (2002-2012) for the principal exporting partners, i.e. China, USA and Israel, were 12,876,059 US$, 4,361,931 US$, and 17,366,344 US$ respectively. Many plants coming from the largest plant producers in the world (China, USA, Costa Rica) reach Europe after a period in Turkey and Morocco (Santini, personal com.). The introduction of Oomycetes to Europe during the past two hundred years was reported by Santini *et. al.* (2013), who also underlined the dramatic increase of invasions by this group of pathogens occurred since the 1990s. To date, in the European continent there are 38 *Phytophthora* species, eight were present before 1950, eight were detected for the first time between 1950 and 1990, and 19 were found between 1990 and 2010. The major pathway of introduction was trade (20 species); four species are the result of interspecific hybridisation, and the remaining 13 species have unknown origin. It is important to note that 24 species (63%) are generalist pathogens, whose introduction caused the diffusion of disease to many host plants.

In the past few decades, many research efforts have been devoted to develop bio security protocols for preventing the introduction of new alien Phytophthoras, or confining the species already introduced to a limited area, normally the nursery that had imported the pathogens together with live potted plants. A preventative system approach to nursery plant production is reported by Parke and Grünwald (2012): the focus must be on identifying the phases of the plant production chain critical for *Phytophthora* contamination and undertaking incisive operations to minimize or possibly eliminate the hazard of plant contamination. Some simple precautions can strongly limit inoculum spreading, such as for instance to sterilize the soil contained in the pots of diseased plants before recycling it for new plants or to cultivate plants on benches elevated from the nursery floor, which reduces contamination by soil stored pathogens or infection by the inoculum floating in irrigation water. It is important to remark that, while importation of soil and growing media containing soil or organic matter from the majority of non-European countries is forbidden by EU legislation, the import of plants rooted in soil is allowed (EU 2000, 2014), practically nullifying the efficacy of the first prescription. An important branch of studies is aimed at optimizing the control of *Phytophthora* spread. The base for these studies is the knowledge of the actual presence of *Phytophthora* at the continental scale, i.e. a constantly updated list of species including diffusion data and real-time information about new detections. A wide view of the current situation about diversity of Phytophthoras in European nurseries and forests is available (Moralejo *et al.*, 2009; Rytkönen, 2011; Jung *et al.*, 2015). Moreover, species-specific investigation approaches are required in order to analyze organisms that such as *Phytophthora* species , often differ significantly in the epidemiology of infection even within the same genus. Reports about the techniques employed in the management of *P. cinnamomi* and *P. ramorum*

in forests have been published by Australian and US authors (bibliography reported and discussed in article **II**). Furthermore, host penetration dynamics, chemical control, causes and time of propagules dispersion have been investigated in *Phytophthora* species that are severe crops pathogens such as *P. infestans* (Erwin and Ribeiro, 1996; Vleeshouwers *et al.*, 2000; Fall *et al.*, 2015) and *P. palivora* (Hunter and Kunimoto, 1974; Sing *et al.*, 1975), and strategies of infection have been studied in *Phytophthora* species attacking woody plants (Oßwald *et al.*, 2014). In *P. ramorum*, a forest species that received much attention during the past two decades, classical isolation and molecular diagnostic tools were deeply exploited to study aspects of the biological cycle related to wind ad water dispersion as well as periodical resting in soil and litter (Hayden *et al.*, 2006; Fichtner *et al.*, 2007; Eyre and Garbelotto, 2015). The review of these aspects (Martin *et al.*, 2012; O'Brien *et al.*, 2009) has a primary role in supporting management decisions about disease containment adopted mainly by plant producers. However both the extended surveys of *Phytophthora* distribution and taxonomy, and the analyses of different biological aspects of the life cycle that have been done to support management decisions, are not a sufficient source of information for an adequate control of Phytophthoras. Effective prevention policies, such as to stop infected/contaminated plants prior to entrance in a pathogen-free area, would be the key measure for successful results. An important aspect in order to define effective biosecurity measures and successful prevention strategies is to understand which epidemiological background is linked to migration of *Phytophthora* inoculum during the initial steps of the process or during escape from the original habitat. Many ecological studies have originated from the appearance of new and invasive forest pathogens, studies that aimed at investigating the original range of these microorganisms. For instance and most recently, Eastern Asia was recognized as the origin of the ash dieback agent, *Hymenoscyphus fraxineus* that was introduced to Europe in the 1990s (Zhao *et al.*, 2013). Also in the case of *Phytophthora* species, some hypotheses have been proposed. According to Brasier (2010b) Taiwan may be the native range of *P. lateralis*, and the same applies to *P. ramorum,* which have probably arrived also from tropical habitats of South Eastern Asia. Widespread distribution, large number of isolations and high genetic diversity suggest that *P. multivora* may be endemic to the south west of Western Australia (Burgess T., personal comm.). In general, results in this research field are rarely fully efficient because of the huge effort required for sampling and isolating microorganism such as *Phytophthora* from the natural environment. More profitable is the screening of new species in the natural environment and the comprehension of breeding mechanisms that, by generating new genotypes even from homothallic species, are the putative source for new potentially invasive pathogens. During a large multiple spots survey in Taiwan, Jung *et al.* (2014) isolated 12 known species, 4 designated taxa and 17 unknown species of *Phytophthora***.** While this is just a valid example of how tropical countries might be potential prolific exporters of indigenous Phytophthoras, equally significant is the continuous creation of new genotypes in nature. In the genus *Phytophthora*, Clade VI includes species with high attitude to potential hybridization This taxonomical group received much attention in the past few years due to the progressive discovery of closely related but distinct species (*P. rosacearum* (Hansen *et al.*, 2009), *P. litoralis*, *P. thermophile*, *P. paludosa* (Jung et al 2011a), *P. amnicola*, *P. fluvialis* (Burgess, 2012; Jung et al 2011a), *P. mooyotj* (*Crous et al. 2014*), *P. bilorbang*, (Aghighi *et al.*, 2012). *P. taxon pg chlamydo*, *P. lacustris* (Nechwatal *et al.*, 2013), *P. riparia* (Hansen et al., 2012)). However, while there is evidence to support hybridization between reunited sister taxa in *Phytophthora*, there are few studies about natural hybridization between related sympatric species in their indigenous range (Burgess *et al.*, 2015). The most notorious case, with the most manifest consequences, is the *P. alni* complex, which had origin at first in European nurseries by hybridization between the clade VII *P. cambivora* and *P. fragariae* (Brasier *et al.*, 2004; Ioos *et al.*, 2006, Husson *et al.*, 2015) and nowadays causes alder destruction in the wild in central Europe. A recent investigation of this phenomenon in clade VI is provided by Burgess (2015), who found twelve (out of a possible number of 30) two-way hybrid combinations among six related indigenous waterways species in Western Australia: *P. amnicola*, *P. fluvialis*, *P. litoralis*, *P. moyootj* and *P. thermophile* and *P. sp. nov.*.

The deeper the knowledge of this group of Oomycetes becomes, the more evident it appears from the ecology of both the recently described species and the unknown closely related taxa that these microorganisms are able to exploit many biological mechanisms that render them dangerous plant

pathogens. It is out of question therefore that both ecological studies and diagnostic methods need to be further developed

Aim

Molecular analyses are powerful methods to evaluate the presence/absence of a pathogen and measure its incidence by estimating the amount of inoculum in the screened material. The employment of quantitative real –time PCR (qPCR), which is a highly sensitive diagnostic technique able to detect minimal traces of pathogens even in the absence of symptoms, is the most effective tool available today to detect a disease before entrance in a new country. Prevention of entrance seems the only reasonable mean to reduce the damage by exotic *Phytophthoras*, since the application of eradication techniques after introduction often requires relevant financial resources and rarely is successful, especially when operations take place in forests or in the open environment. However, *Phytophthora* easily escapes sanitary inspections as these are based mainly on visual screening of disease symptoms in the plant crowns. This genus has a cryptic infection stage during which it remains in the form of a resting structure in the growing medium or as mycelium in infected roots of still asymptomatic plants. Once the plants are placed in the suitable and stable environmental conditions of nurseries or plantations, the hidden pathogen may spread out according to its virulence, triggering disease symptoms.

In this work a qPCR assay was used in order to achieve the following purposes:

I To detect the presence and quantify *Phytophthora* DNA in ornamental potted plants traded to, from and within Europe. In this part of the project a qPCR TaqMan probe to detect the genus of *Phytophthora* was designed, fine-tuned and applied. The study includes a comparison of diagnosis efficiency between the qPCR method and classical isolation methods. The role of plants for planting as a reservoir of inoculum of *Phytophthora* species is discussed.

II To develop a lab procedure to selectively detect viable *Phytophthora* inoculum in soil samples by means of qPCR, reducing therefore the risk of false positives due the presence of dead mycelium. To this aim an *in vitro* experiment was designed where *Phytophthora* DNA from treated (dead) and untreated (viable) mycelium in soil was quantified during 4 weeks. The study showed that the dead mycelium possibly present in a soil sample is rapidly degraded in the experimental conditions and become undetectable by qPCR in a short time. Instead, in a soil sample containing live mycelium the qPCR signal is stable or increases in the same time period. The experiment was designed in order to simulate the latest phases of *Phytophthora* management, when the soil of treated areas must be analyzed to determine if the pathogens are still viable.

III To quantify the dispersal of *Phytophthora* species in the air and the seasonal variation of the aerial inoculum in a sampling site close to an important nursery area in central Italy. Besides employing the genus-specific qPCR assay developed in the first part of this thesis (I), a new assay designed to uniquely detect *P. lateralis* and *P. ramorum* was developed and used. The European and Mediterranean Plant Protection Organization recommend these species for regulation as quarantine pests. In addition, *P. ramorum* is the object of emergency measures taken by EU to prevent spread of the pathogen in Europe. The levels of inoculum detected in the course of the year by qPCR were associated with climatic variables.

In addition, an ecological approach was used to analyze the taxonomy of *Phytophthora* in a natural environment characterized by high plant diversity, the Western Australia Kwongan bush. Deep sequencing and a glass house trial were employed. Aim of the work:
IV To evaluate, by means of next generation sequencing (NGS), the diversity of *Phytophthora* species in the sandy soil of a natural dune system that form a geological chronosequence on the coast of Western Australia (Jurien Bay) in relation to the local variation in geological age. To verify, by means of a glass house experiment, pathogenicity and incidence in woody plants native to Western Australian, of two important

generalist species, *Phytophthora multivora* and *Phytophthora cinnamomi*, that were frequently detected during sequencing in the soil of the dunes in Jurien Bay.

Materials and methods

Development of a TaqMan MGB probe specific for the genus *Phytophthora* (**I, II, III**)

Primers and TaqMan® MGB probe to amplify *Phytophthora* DNA were designed using Primer Express® Software 3.0 (Applied Biosystems, Forster City, CA, USA) using the ITS2 (Internal Transcribed Spacer) region of *Phytophthora palmivora* (isolate Ph32SA from the culture collection of the Institute for Sustainable Plant Protection IPSP-CNR, Italy; GenBank accession number KT148922). The upstream and downstream primers were PhyF (5'-TCGGCTGTGAGTCCTTTGAA-3'Forward primer) and PhyR (5'- GCCACGCTTTTGGAGCAA-3' Reverse primer). The TaqMan® MGB probe (PhyPr: 5'- ACTGAACTGTACTTCTC -3') was labelled with 6-carboxy-fluorescein (FAM) at the 5' end, and a non-fluorescent quencher (NFQ) with minor groove binder (MGB) ligands, at the 3' end. Homology of *P. palmivora* amplicon sequence with the sequences of other 35 *Phytophthora* species belonged to all ten clades was performed using Standard nucleotide-nucleotide BLAST (BLASTN) in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The qPCR assay was tested on DNA from axenic cultures of 13 *Phytophthora* species belonging to six genetic clades and commonly isolated from nurseries (Kroon *et al.*, 2012) (*P. cambivora, P. cinnamomi, P. citrophthora, P. cactorum, P. citricola, P. cryptogea, P. gonapodyides, P. lateralis, P. nicotianae, P. palmivora, P. quercina, P. ramorum* and *P. syringae*). Multiple isolates per species were tested. The possible cross-reaction of the qPCR assay with DNA of closely related (*Pythium* spp.) and unrelated (*Fusarium* sp., *Mortariella*, sp.) species, which are common in nurseries, was also tested. All isolates tested belong to the culture collection of the IPSP-CNR.

I The potential of symptomless potted plants for carrying invasive soil-borne plant pathogens

The study was carried out at two large European retail nurseries which import and resale from and to European and extra-European partners. Seventy-two potted plants belonging to 17 woody ornamental species were collected. Root tissues and soil from the pot of each individual plant (both symptomatic and asymptomatic; the two classes included an equal number of plants) were collected for isolation and molecular analyses. Since above-ground symptoms of infection by *Phytophthora* species may not appear until root decay is advanced, the roots of asymptomatic plants were carefully examined to detect incipient symptoms (i.e. rotten fine and feeder roots; major roots showing reddish-brown lesions, internal brown or black tissues). The compost produced in the nursery was also tested for the presence of *Phytophthora*, as was irrigation water from ponds and drainage systems. Samples for DNA extraction were collected in 1.5ml Eppendorf tubes and stored at -20°C.

Isolation of *Phytophthora*

Phytophthora species were isolated from roots, soil, compost, and irrigation water. Soil samples and compost were processed using apple baits while irrigation water was processed using 1-month-old leaves of *Quercus robur* as baiting (Erwin and Ribeiro, 1996). *Phytophthora* isolates were grown on 90 mm Petri dishes covered by cellophane discs (Celsa, Varese, Italy) of the same diameter. After an additional ten days' growth, the mycelium was scraped from the surface of cellophane and placed in 1.5ml Eppendorf tubes for DNA extraction.

DNA extraction

Mycelium and root samples (ca. 100 mg fresh weight) DNA was extracted from all samples using the EZNA Plant DNA Kit (Omega Bio-tek), following the manufacturer's protocol. Water samples (40ml each) were placed in 50ml tubes (Sarstedt, Verona, Italy) and centrifuged for ten minutes at 10,000 rpm. After that, 1ml of water containing the pellet was transferred to a new 2ml microtube (Sarstedt) and centrifuged at 16,000 g for a further ten minutes. The supernatant was discarded and the pellet used for DNA extraction with the EZNA Plant DNA Kit (Omega Bio-tek).

From compost and soil (c.a. 80 mg for each sample), DNA was extracted with the EZNA Soil DNA Kit (Omega Bio-tek).

Real-time qPCR detection of *Phytophthora* species

DNA extracts from roots, soil, compost, irrigation water, and mycelium as control, were assayed in MicroAmp Fast 96-well Reaction Plates (0.1mL) closed with Optical Adhesive, and by using the StepOnePlus™ Real Time PCR System (Applied Biosystems, Life Science, Foster City, CA). Each DNA sample was assayed in two replicates.

PCR Identification of *Phytophthora* species

Pure cultures of *Phytophthora* were obtained and identified through amplification of the Internal Transcribed Spacer (ITS, including both ITS1 and ITS2) of the ribosomal DNA with the ITS6/ITS4 primer pair (White *et al.*, 1990). Sequences were blasted in the NCBI database in order to identify the most similar available sequences.

Data analyses

Chi-square tests were applied to identify significant differences between the frequencies of detection of *Phytophthora* species (by isolation on selective medium or by qPCR) in symptomatic vs. asymptomatic plants, and in different substrates (soil, roots, compost, water). Kruskall-Wallis test was applied to identify significant differences in *Phytophthora* DNA quantity estimated by qPCR, between symptomatic and asymptomatic plants, and between substrates. The variation in frequency of isolation of *Phytophthora* species between substrates (roots vs. soil), pots carrying plants with or without external symptoms (symptomatic vs. asymptomatic leaves), and between plant species, was illustrated by heatmaps accompanied by dendrograms obtained through hierarchical clustering. Statistical tests were performed in R (R Development Core Team 2013) and Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA). Heatmaps, bar plots and boxplots were produced with the GPLOTS package in R (http://cran.rproject.org/web/packages/gplots/gplots.pdf).

II A reliable lab procedure to detect the presence of viable Phytophthora inoculum in soil samples through qPCR.

Inoculum preparation

Three *Phytophthor*a species, *P. nicotianae* (Genebank accession number KT148933)*, P. ramorum* (sample P. ram44RG) *and P. citrophthora* (Genebank accession number: KT148897) from IPSP-CNR collection, were incubated for five weeks in eight flasks (2 flasks per species plus 2 negative controls) containing 400 ml of vermiculite, 4 g of millet, 240 ml of V8 broth, and 0,8 g CaCO3 (melted in V8 broth) each.

Substrate inoculation and treatments

Inoculation was carried out in two substrates: river sand (S) and forest organic soil (OS). For each substrate, 5 thesis were set up: 1) untreated growth medium with untreated pathogen (UU); 2) untreated growth medium with treated pathogen (UT); 3) treated growth medium with untreated pathogen (TU); 4) treated growth medium with treated pathogen (TT); 5) Mock-inoculation consisting of untreated growth medium with mock-flasks inoculum (CTR). The treatment applied for cleaning the substrates was autoclaving (121°C for 20 minutes) repeated 3 times at 24h intervals. A single autoclave cycle (121°C for 20 minutes) was used to treat inoculum in flasks. For each thesis 100 ml volume of substrate (S and OS) were placed in different 30 glass Petri dishes (12 cm diameter). For each Petri dish 25ml of inoculum (described above) was added and gently mixed to the substrate to promote uniform distribution. Inoculated dishes were incubated at 20°C in the dark.

Sample collection, DNA extraction and Phytophthora DNA quantification

For each thesis, 0.4 g of substrate were collected weekly for one month from ten spots within the plate. DNA extraction was carried out using the EZNA Soil DNA Kit (Omega Bio-tek). Quantitation of *Phytophthora* DNA was carried out by using the real-time TaqMan® MGB probe described in Migliorini *et.al.* (2015). The amount of the pathogen in the soil was expressed as pg *Phytophthora* DNA/µg DNA total.

Statistical analysis

Kruskal–Wallis test was applied to identify significant differences in *Phytophthora* DNA quantity estimated by qPCR, between experimental theses. Statistical tests were performed in R (R Core Team, 2015). Boxplots were produced with the GPLOTS package in R (http://cran.r- project.org/web/packages/gplots/gplots.pdf).

III Real Time PCR detection of airborne *Phytophthora* species in a plant nursery area

Aerobiological samples were collected from May to December 2012 with a Hirst-type spore trap (VPPS 2000; Lanzoni, Bologna, Italy) placed on top of a building 35 m above ground level. DNA was extracted from filters with the EZNA Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacture's short protocol.

Aerial DNA (aDNA) of *Phytophthora* species was detected and quantified by the same real time PCR (qPCR) TaqMan® MGB assay as described in Migliorini *et.al.* (2015). Groups of 24-hour DNA samples were pooled together to obtain twenty 10-day DNA samples (May-December). In periods when Phytophthora DNA was especially abundant, 24-hour samples were processed separately.

In this study, a second qPCR assay based on a TaqMan MGB probe specific to *Phytophthora ramorum* and *P. lateralis* DNA was designed with PRIMER EXPRESS Software 3.0 (Applied Biosystems, Forster City, CA, USA) on the internal transcribed spacer 2 (ITS2) region of *Phytophthora ramorum* (KC473522). The upstream and downstream primers were PramF (5'-GCAGGGCTTGGCTTTTGA-3' forward primer) and PramR (5'-GCCGAACCGCCACTCTACT-3 ' reverse primer). The TaqMan[R] MGB probe (Pram_PR: 5'-TCGACGGTGTTGTGCG-3') was labelled with 6-carboxy-fluorescein (FAM) at the 5' end and a non-fluorescent quencher (NFQ) with minor groove binder (MGB) ligands, at the 3' end. Homology of the *P. ramorum* amplicon sequence with the sequences of other species in the NCBI database was performed using standard nucleotide–nucleotide BLAST (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Meteorological data for studying the effect of climatic variables on the air dispersal of Phytophthoras were supplied by CESPEVI web site (Experimental Center for the Nursery Sector, Pistoia, Italy).

IV Phytophthora distribution, taxonomy and pathogenicity within a hyperdiverse bush plant hotspot in a dune system of Western Australia

454 sequencing

Sixty soil samples were collected from six dunes (ten samples per dune) that form a dune system located North to Perth City, West Australia. Each sample (70 grams of sand), corresponding to a single plot, was obtained by mixing 10 grams from seven different sand cores (one per subplot of 2 m X 2 m). Location of plots has been previously reported (Laliberté *et al.*, 2012; Hayes *et al.,* 2014). DNA extraction from samples was performed by the use of MO BIO Laboratories, Inc. PowerSoil DNA Isolation Kit. According to methodology reported by Català *et al.* (2015), amplicon libraries were generated using a nested PCR approach. EmPCR of pooled amplicons was performed using the GS Junior Titanium emPCR Kit(Lib-L) according to the GS Junior emPCR Amplification Method manual – lib-L, (March 2012) . Sequencing was realized according to GS Junior Titanium Sequencing Kit; the method is described in the GS Junior Sequencing Method Manual March 2012. The observed variation in *Phytophthora* species composition and abundance in the dune system was first analyzed through non-metric multidimensional scaling (NMDS). Multiple regression of environmental variables (soil age, available N, available P, Carbonate, Organic C, Total N, Total P, pH, C to N ratio, C to P ratio, N to P ratio) with ordination axes (environmental variable is used as dependent and selected ordination axes as explanatory variables) was performed by using the 'envfit ' function on a reduced number of samples for which environmental data were available. Significance was tested by permutation test. In order to discretize the continuous variation observed along the dune chronosequence, aiding structure detection and hypothesis generation, hierarchical cluster analysis was also applied.

Pathogenicity trial of *P. multivora* and *P. cinnamomi*

Sterilized river sand was put in 1.8 dm^3 volume plastic pots in the bottom of which it was previously set a layer of fly web in order to limit sand losses. 20 WA range forest and bush plants species were included in the experiment. Two isolates of *P. multivora* (in the following referred to as *P. multivora a* and *P. multivora b*) and an isolate of *P. cinnamomi* (collection of CPSM, Murdoch University, Perth) were inoculated each in six flasks while six flasks were left as control. The content of flasks (the inoculum of the experiment) was composed by 400 ml of vermiculite, 4g of millet, 240 ml of V8 broth (0.8g of CaCo3). Two PVC irrigation tubes 1.5 cm large and 15 cm long were planted in each pot at 5-8 cm depth and at a distance of 10 cm from each other. Four weeks after preparation of the flasks, PVC tubes were moved out of the pots and 5 g of inoculum were placed in each of the two tubes room (10 g per pot) with sterilized spoons. Pathogen diffusion was induced by a 24-hour fluting in distilled water, repeated every two weeks for 12 weeks in total (6 flutings for the entire experiment). After the first fluting and for the remaining duration of the trial, a complete randomized block design for plants inoculation was realized. Five weeks after inoculation, the content of pots was plated on NARPH media (Nystatin, Ampicillin, Rifamicin, Pentachloronitrobenzene, Hymexazol, Cornmeal agar) in order to check inoculum viability. After the last fluting plant roots were washed under running water and weighted. Below ground organs were assigned to classes of health condition (from 0=healthy to 4= dead) on the base of visual assessment of symptoms.

Results

I The potential of symptomless potted plants for carrying invasive soil-borne plant pathogens

Real time PCR was in general much more efficient than isolation for detecting *Phytophthora* species, especially in soil and environmental samples.

Phytophthora was isolated on selective medium from nearly half of the potted plants, while the pathogen was not isolated from water or compost. 59 isolates for a total of eight species: *P. cinnamomi, P. palmivora, P. nicotianae*, and *P. citrophthora* were isolated both from roots of various plants, and from soil of pots containing different plants; *P. cactorum* was isolated both from roots and from soil of *Viburnum; P. citricola* was isolated only from roots of *Viburnum; P. cryptogea* and *P. syringae* were isolated only from soil.

The frequency of isolation did not differ significantly between symptomatic and asymptomatic plants (54 vs. 46% $X^2_{(df1)}$=0.05, ns), nor did it differ between soil and roots (39 vs. 44% $X^2_{(df1)}$=0.54, ns).

Detection of *Phytophthora* in potted plants, water and compost through qPCR

Phytophthora was detected in 87% of the pots tested through qPCR with the specific TaqMan MGB probe. All samples from irrigation water and a quarter of the compost samples were positive to the assay. The difference in detection frequency between pots containing symptomatic plants and pots containing asymptomatic plants (90 vs. 84 % positive samples) was non-significant ($X^2_{(df1)}$=0.08). The frequency of detection of *Phytophthora* by qPCR in soil was twice as high as the frequency in root tissues (82 vs. 40% $X^2_{(df1)}$=14.27 p <0.005). More than half of the plants with asymptomatic leaves had symptomatic roots upon visual inspection. All plants with asymptomatic leaves and damaged roots were positive to the *Phytophthora*-specific TaqMan assay and in all the pots containing a plant of this kind the pathogen was detected both in roots and in soil. Also positive to the qPCR assay were the large majority (70%) of the asymptomatic potted plants with roots that seemed healthy to visual inspection. In nearly half of the plants with both asymptomatic leaves and healthy roots, *Phytophthora* was detected in root tissues, and from about half of those plants, *Phytophthora* was also isolated on selective medium. The difference in DNA quantity was non-significant between pots containing symptomatic plants and pots containing asymptomatic plants (Kruskall-Wallis$_{(1,89)}$ = 0.11, p = 0.74), and between root and soil samples (Kruskall-Wallis$_{(1,89)}$ = 0.01, p = 0.92).

II A reliable lab procedure to detect the presence of viable Phytophthora inoculum in soil samples through qPCR.

The quantity of target DNA detected in the theses with untreated (viable) *Phytophthora* (**UU** and **TU)** was much higher possibly due to active colonization of the plate content. On the contrary, the quantity of *Phytophthora* DNA detected in treated samples (**TT** and **UT**) where the pathogen was killed by heat, was extremely low. In **UT**, *Phytophthora* remained detectable in quantity comparable to those found in the environmental and control samples. When pathogen and soils were both treated **(TT),** a small quantity of DNA was detected a week post inoculation, but all TT samples became negative from two weeks on.

III Real Time PCR detection of airborne *Phytophthora* species in a plant nursery area

No positive quantification occurred by the use of *P. ramorum-P. lateralis* specific TaqMan probe. The resolution of this tool, tested during standard curve generation (0.00128 pg/μl total DNA), is high enough to affirm that none of these two aerial pathogens was present in the aerial flux intercepted by the trap.

The genus specific probe detected *Phytophthora* in the air during the whole observation period. Higher quantities of *Phytophthora* DNA were found in spring and autumn in correspondence with rainy periods. After a peak of detection in November, the pathogen quickly dropped to levels comparable to those observed in mid-summer, despite an increase in precipitation. The sudden decrease of temperature down to values below 10°C was reasonably the cause. *Phytophthora* inoculum decreased in early summer when relative humidity decreased as a consequence of both disappearing of rain and rising of air temperatures. Differently, in September the progressive increase of relative humidity due to both rainfall and, more incisively, decrease of air temperature, did not produce an increase in *Phytophthora* quantity comparable to that observed in spring: under the same relative humidity in early and late summer periods different quantities of *Phytophthora* were detected.

IV Phytophthora distribution, taxonomy and pathogenicity within a hyperdiverse bush plant hotspot in a dune system of Western Australia

454 sequencing

A total of 141882 good quality reads were considered for the analysis. There were 34 *Phytophthora* species included in Clades 1, 2, 3, 4, 5, 6, 7 and 8 from the 60 soil samples of the dune chronosequence. The results of NMDS and multiple regression of environmental variables with ordination axes for a reduced number of samples showed that out of 12 tested environmental variables only Total N was significant ($p<0.01$) while soil Carbonate, Total P, pH, C to P ratio, and N to P ratio were close to significance. After Bonferroni correction for multiple testing only Total N remained slightly significant ($p=0.0500$).

Hierarchical clustering analysis showed that geologically younger dunes (QY, QM, QO) grouped separately to the three older dunes, confirming that the community composition of *Phytophthora* species is correlated to the spatio-temporal variables of the sampling area. *P. multivora* and *P. cinnamomi* were the most frequent species and had an almost uniform distribution within the chronosequence series, suggesting indifference of these two generalist and invasive pathogens to local ecosystems differentiation.

Pathogenicity trial of *P. multivora* and *P. cinnamomi*

On the average, after inoculation the health condition of the roots had greatly deteriorated in Proteaceae, less markedly in Fabaceae and only slightly in Myrtaceae compared to control plants. Symptoms were more severe by *P. cinnamomi* in Proteaceae; in Myrtaceae there were no major differences between *Phytophthora* species; and in Fabaceae isolate b of *P. multivora* was as pathogenic as *P. cinnamomi*, whereas isolate a caused almost no symptoms.

Infection caused a reduction of root dry weight in Proteaceae, which was large by *P. cinnamomi* and moderate by *P. multivora*. In Myrtaceae root dry weight was slightly reduced by *P. cinnamomi* only. In Fabaceae the deterioration of root health after inoculation was not paralleled by a reduction in root weight except for a weak effect by isolate b of *P. multivora*.

Discussions

The efficacy of the qPCR assay developed in this thesis was largely demonstrated by the successful outcome of quantification data from three experiments (**I**, **II**, **III**).

The employment of such an effective diagnostic tool was of primary importance to demonstrate that nursery potted plants, already known as the most important inoculum reservoir of soil born pathogen such as *Phytophthora* (Parke and Grünwald, 2012), can remain asymptomatic even when infected. *Phytophthora* was detected in tissues or soil of all plant species sampled from two large nurseries which trade with European and non-European countries. In addition, both recycled water and the compost produced in the nursery tested positive. Our results confirm previous concerns about contamination of potted plants from nurseries (Themann *et al*., 2002; Chastagner *et al.,* 2009), and present a solid proof that the commercial trade in live plants for cultivation is an important pathway for soil born *Phytophthora* species (Santini *et al*., 2013; Martin *et al*., 2012; Goss *et al*., 2009). The qPCR approach, by reiterating the issue about inefficacy of European visual inspection to intercept infected potted plants in imported consignments, is proposed as a suitable diagnostic tool to prevent the trade of contaminated nursery material.

The efficacy of the proposed TaqMan chemistry was measured in experiment **II** where, rather than checking the minimum amount of detectable inoculum (during experiment **I** the qPCR assay demonstrated a very high sensitivity, since the lowest quantity of *Phytophthora* DNA measured was 0.02 pg DNA /µg DNA extracted (environmental sample)), the aim was to verify whether DNA from dead *Phytophthora* in soil/sand samples was detectable and how long time would be necessary for it to be degraded and no longer amplified. In samples containing treated (dead) mycelium, the DNA of *Phytophthora* disappeared two weeks after treatment and in these samples no positive amplifications occurred after this period. This result confirmed the method as a valid early detection tool in soil, because it seems to be sufficient to incubate the samples for a 2-week period for detecting only the pathogen that was initially alive, drastically diminishing the number of false positives. However, results were obtained *in vitro* and their validity should be further verified in field experiments. In our opinion this is a critical point: many efforts in the management of *Phytophthora* species are currently into force. Eradication of *P. ramorum* in West US forests and of *P. cinnamomi* (experimental investigations) in Australia are the most relevant and discussed actions in the forest environment (Kanaskie *et al.,* 2011; Crone *et al.*, 2014), while cultivation practices aimed at controlling the spread of these pathogens are constantly carried out in nurseries worldwide. The study reported in Paper II demonstrated that the risk of diagnostic errors caused by qPCR detection of dead DNA in environmental samples could be reduced by applying a sound lab protocol, a result that justifies the recommendation of this method for management of Phytophthoras. The TaqMan assay developed in this thesis is a valid and safe diagnostic tool, more efficient than the traditional isolation techniques (as demonstrated in Paper I) whose suitability for eradication purposes has been already questioned (Dunstan *et al.*, 2010).

After a first part of this thesis during which *Phytophthora* was studied in soil and root tissues, qPCR was employed on aerial trap content sampled at ca. 30 metres height (**III**). Wind dispersed inoculums was collected in Pistoia (Tuscany) that is one of the biggest plant nursery poles in Europe. The seasonal variation of *Phytophthora* DNA in the air was partly explained by meteorological variables, and in particular the peaks of concentration were associated with previous abundant rainfall and high relative humidity, while temperature (high and low, respectively) was a strong limiting factor in early summer and late autumn. When relative humidity rose up because of diminishing air temperature and sporadic rain events, sporangia were not released (or even produced), suggesting that aerial sporulation of *Phytophthora* is driven by repeated rainfalls when temperature remains within a suitable range. Interestingly, our data are in good agreement with the results recently obtained in a similar investigation performed in a Mediterranean environment in Spain (Manzano *et al.*, 2015). However, while the TaqMan probe tested in this thesis was designed to detect only the *Phytophthora* genus, Manzano and co-workers (2015) made a taxonomic analysis of the aerial inoculum by visual screening of microscopic characters of Phytophthora-like

propagules, a technique that do not enable distinguishing between related taxa of Oomycetes. Based on the review of the literature published to date, this thesis is the first unequivocal report about the seasonal variation in air dispersed *Phytophthora* species, since it is based on a specific and reliable molecular assay. Ecological considerations are easily generated by these results: a few species of *Phytophthora* are thought to be aerial as they cause necrosis of above ground plant organs (Oßwald *et al.*, 2014), but limited is the knowledge of how and when sporangia release occurs and of how far these propagules can be transported, which is an important issue. The seasonal variation of the quantity of *Phytophthora* DNA collected in our traps provides a first general idea of the phenomenon at the genus level. The presence of a peak late in the autumn might induce to exclude that the detected Phytophthoras belonged only to species that attack agricultural crops such as the late blight agent *P. infestans*. Considering the high concentration of ornamental plants in the area, the idea that the detected inoculum is from nursery pathogens is reasonable. The absence of aerial inoculum of *P. ramorum* and *P. lateralis,* two serious and potentially invasive pathogens that are recommended as quarantine organisms by EPPO, was demonstrated by the specific qPCR MGB TaqMan probe developed in this thesis.

The study of *Phytophthora* taxonomy in Kwongan, a hyper diverse shrub land in Western Australia (**IV**), was carried out as a part of an extended survey aimed to understand the ecological links between particular components of this ecosystem, i.e. soil nutrients and plant taxonomy, which largely depend on geological age, and soil pathogen diversity (Laliberté *et al.*, 2015). The final aim of this research is to verify the hypothesis that soil born *Phytophthora* species act as a co-factor in determining the extremely high plant species diversity found in this ecosystem. The work is still in progress and the results obtained so far are not sufficient to proof the hypothesis yet. However, new and interesting knowledge about *Phytophthora* in this ecosystem was collected. A surprising result is that *Phytophthora* species are widespread in Kwongan to such a degree that was not expected. Similarly unexpected is the presence/abundance of some taxa, for instance the species belonging to Clade VI, which are typical of water-rich terrestrial habitats. In the glass house trial, the pathogenicity of *P. cinnamomi* and *P. multivora*, two generalist species frequently detected in Kwongan, resulted high on plant species that are common in Kwongan, a finding that may support the hypothesis of an active influence by at least this two pathogens in determining plant species composition in this ecosystem.

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BIODIVERSITY RESEARCH

The potential of symptomless potted plants for carrying invasive soilborne plant pathogens

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ABSTRACT

Aim The most common pathway for the movement of plant pests across borders is the horticultural trade in live plants, especially potted ornamentals. Soilborne pathogens, possibly alien and potentially invasive, have a higher possibility of surviving transportation and becoming established at their destination if they are carried in potted plants. The European Union (EU) has an open-door phytosanitary system, under which any plant that is not specifically regulated can be imported. Inspections are focussed on a small number of economically important plant pests and even then limited to visual examinations of the aerial parts of the plant. Inspections fail to detect regulated pests or others internal to the tissues, or in the soil, if plants appear asymptomatic, or if incipient symptoms are limited to the roots. *Phytophthora*, a soilborne pathogen universally infamous for its ruinous outbreaks, but poorly regulated in Europe, was chosen to illustrate the risk inherent in the nursery pathway. The aim of this study was to demonstrate the level of infestation by *Phytophthora* in ornamental plants largely traded to, from and within Europe.

Location European Union.

Methods As *Phytophthora* species are not easily isolated, a real-time PCR assay was developed, based on a genus-specific TaqMan MGB probe, to detect the pathogens in plant tissues or soil even when present at low concentrations, and before symptoms occurred. *Phytophthora* species were identified by isolation and sequencing of the ITS (internal transcribed spacer) region.

Results *Phytophthora* was detected by qPCR in 87% of the tested pots and in 70% of the asymptomatic potted plants. Potted plants in soil carried several *Phytophthora* species without showing any external symptoms.

Main conclusions The results of this study strongly support the case for more rigorous European legislation on the trade of live plants in pots. As eradication of soilborne organisms is difficult, if not impossible, an embargo on plant movements into the EU and between member states is the only advisable measure against the spread of these pathogens.

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INTRODUCTION

International trade has increased exponentially during the past centuries, and the establishment of non-native species has generally followed the same trend (Hulme, 2009; Aukema *et al.*, 2010; Liebhold *et al.*, 2012). International trade of woody plants, especially live plants for horticulture, is recognized as a major pathway of unintentional

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Diversity and Distributions

introductions of plant pests, both herbivores and micro-organisms, as hitchhikers and contaminants (Kenis *et al.*, 2007; Smith *et al.*, 2007; Roques, 2010; Santini *et al.*, 2013).

The trade in ornamental plants is characterized by huge volumes and rapid changes in the species and their origins, which render this pathway difficult to regulate, and increases the risk of introducing alien and unknown pests (Bradley *et al.*, 2012; EPPO 2012 and references therein). Non-native organisms have a higher risk of establishment when they are carried with their hosts. In fact, they can survive, and possibly grow or multiply, on the host's tissues or in the soil during transit, moving to other individuals of the same plant species, or other hosts, once at their destination (Levine & D'Antonio, 2003; Aukema *et al.*, 2010).

Soil as an invasion pathway *per se* is particularly insidious, as it is an enormous reservoir of living micro-organisms which are mostly unknown and not easily detected. One gram of soil is estimated to contain more than 1000 prokaryotes (Curtis & Sloan, 2005) and approximately 1000 gigabase pairs of microbial genome sequences (Vogel *et al.*, 2009). Fungi and fungal-like organisms are a major and largely unknown component of the soil's living communities (Hawksworth, 2001; Jeffery *et al.*, 2010). One-gram soil samples from aeroplane passenger footwear contained eight genera of plant pathogenic fungi regulated by the New Zealand Ministry of Agriculture and Forestry, demonstrating just how easily living pathogens can be accidentally transported across borders globally (McNeill *et al.*, 2011). For these reasons, the importation of soil, on its own or as a growing medium around plant roots, is prohibited by several countries with advanced biosecurity regulation, such as Australia, New Zealand, Canada and the USA.

While Europe prohibits the import of soil and growing media containing soil or organic matter from the majority of non-European countries, the importation of plants rooted in soil from outside the EU is permitted provided they have been officially declared free from harmful organisms (phytosanitary certificate or plant passport within the EU) and show no sign of infestation or disease (Annex IV in EU 2000).

This regulation is based on pest risk analysis (PRA) of putative dangerous species, that is a detailed evaluation of a species' ecological characteristics and the potential effects of its introduction. Currently, the EU regulates ca. 250 plant pests and pathogens not present, or not widely distributed in the EU, and whose introduction into and spread within all member states is banned (Annex Ia and IIa in EU 2000). The number of EU-regulated pests is small, especially when compared with the much longer lists of quarantine organisms that EPPO recommends to member governments (EPPO 2013). Inspections are concentrated on well-known pests and pests of economically important plants. Where instances are considered low risk, inspections are reduced. Moreover, the time available for the inspection of individual consignments often limits the ability to find pests (Liebhold *et al.*, 2012). In the case of live plants, inspections are generally limited to visual examination of aerial parts of the plants; destructive sampling is only practiced in exceptional cases. Ordinary inspections may fail to detect regulated and non-regulated/unknown pests, especially if these are small, or internal to the tissues, or in soil, if plants are asymptomatic, or if incipient symptoms are limited to the roots.

Many fungal pathogens can survive as saprophytes in the soil on dead organic matter, or as quiescent spores that may be resistant to harsh environmental conditions, such as dehydration or heat (Shippers & Gam, 1979). In the past two decades, the number of virulent infectious diseases caused by fungi and fungal-like organisms has increased by up to 13-fold, in both animals and plants (Fisher *et al.*, 2012). Fungal diseases threaten both agriculture production and wildlife conservation. Among examples that have recently emerged are the most lethal pathogens ever witnessed in native forests, such as sudden oak death syndrome (Rizzo & Garbelotto, 2003) and jarrah dieback (Dell & Malajczuk, 1989). Recent outbreaks of fungal diseases are mostly related to new introductions or host shifts in both plants (Brasier, 1991; Engelbrecht *et al.*, 2004) and animals (Martel *et al.*, 2014; Blehert, 2012; Warnecke *et al.*, 2012; Vicente *et al.*, 2012; James *et al.*, 2009).

The genus *Phytophthora* comprises oomycete microbes, many of which are potentially invasive and lethal soilborne pathogens (Brasier, 1999). It is widely known for having caused some of the most destructive crop and forest epidemics ever documented, such as the Irish potato famine in which more than one million people died, or sudden oak death in the USA, and larch death in the UK (Goheen *et al.*, 2002; Brasier & Webber, 2010). Many species of the genus are strictly linked to soil for dispersal and well adapted to live in water and spread from plant to plant via motile zoospores. Many *Phytophthora* species are able to survive in the soil for long periods in unfavourable conditions, in the form of resting chlamydospores (Hwang & Ko, 1977; Fichtner *et al.*, 2005; Shishkoff, 2007). Several researchers have demonstrated that the diffusion of *Phytophthora* is linked to the vicinity of nurseries, a common factor in many areas of the world (Themann *et al.*, 2002; Jung *et al.*, 2009; Moralejo *et al.*, 2009). Among plant pathogens in the genus, only *Phytophthora fragariae* Hickmanthe is specifically listed and regulated by European phytosanitary legislation (EU 2000). In addition, emergency measures have been specified and are currently in place for *Phytophthora ramorum* and *Phytophthora kernoviae* (EU 2002, 2004, 2007).

The objective of this study was to investigate the level of infestation by *Phytophthora* species both in tissues and growing media of potted ornamental plants. Special interest was taken in verifying whether symptoms in the aerial parts of potted plants could be considered a reliable means of detecting infested plants. As it may be difficult to isolate *Phytophthora* species using traditional techniques, a real-time quantitative PCR (qPCR) assay based on a genus-specific dual-labelled fluorescent TaqMan probe was developed and optimized. This is a sensitive and highly specific molecular diagnostic technique allowing rapid pathogen quantification, even from asymptomatic tissues (Luchi *et al.*, 2005, 2006,

2013) or water samples (Aw & Rose, 2012), and is especially useful in the case of pathogens that are difficult to culture (Kerby *et al.*, 2013). The qPCR assay was applied to analyse the presence of *Phytophthora* species in tissues and soil of potted ornamental plants from retail nurseries.

METHODS

Material sampling

The study was carried out at two large European retail nurseries which, besides propagating native ornamentals, also import a large number of potted plants from non-EU nurseries for propagation and resale to European and extra-European buyers. A total of 72 potted plants (4–6 plants per species) belonging to 17 woody ornamental species were collected: *Arbutus unedo* L., *Buxus sempervirens* L., *Ceanothus thyrsiflorus* E., *Crataegus monogyna* Jacq., × *Cupressocyparis leylandii* (Dallim. & A.B. Jacks.) Dallim., *Cupressus sempervirens* L., *Elaeagnus* sp., *Euonymus fortunei* (Turcz.) Hand.-Maz., *Hibiscus* sp., *Laurus nobilis* L., *Myrtus communis* L., *Nerium oleander* L., *Pittosporum tobira* (Thunb.) W.T. Aiton, *Prunus laurocerasus* L., *Prunus lusitanica* L., *Thuja occidentalis* L., and *Viburnum tinus* L. The material (root tissues and soil from the pot of each individual plant) for isolation and molecular analyses was collected both from plants showing the symptoms of root rot in the crown (i.e. wilting, yellowing of the leaves, fading/greying in the colour of the foliage on conifers, defoliation, or dieback of leaves or shoots) (hereafter 'symptomatic') and from plants with healthy crowns (hereafter 'asymptomatic'). The two classes included an equal number of plants. As above-ground symptoms of infection by *Phytophthora* species may not appear until root decay is advanced, the roots of asymptomatic plants were carefully examined to detect incipient symptoms (i.e. rotten fine and feeder roots; major roots showing reddish-brown lesions, internal brown or black tissues). The compost produced in the nursery was also tested for the presence of *Phytophthora*, as was irrigation water from ponds and drainage systems. Samples for DNA extraction were collected in 1.5-ml Eppendorf tubes and stored at −20 °C.

Isolation of *Phytophthora*

Phytophthora species were isolated from roots, soil, compost and irrigation water. Soil samples and compost were processed using apple baits, while irrigation water was processed using 1-month-old leaves of *Quercus robur* as bait (Erwin & Ribeiro, 1996). Isolation from baiting was carried out by placing small apple/leaf fragments (c.a. 0.5 × 0.5 cm) on PARPNH medium (Erwin & Ribeiro, 1996). Roots were washed under running water, and small root fragments (c.a. 1 cm long) were directly placed in PARNPH medium. All plates were incubated in the dark at 20 °C for 7 days. After a 5- to 6-day incubation period, mycelia resembling that of

Phytophthora species were transferred to new 1.5% PDA Petri dishes to obtain pure cultures of each isolate. *Phytophthora* isolates were grown on 90-mm Petri dishes covered by cellophane discs (Celsa, Varese, Italy) of the same diameter. After an additional ten days' growth, the mycelium was scraped from the surface of cellophane and placed in 1.5 ml Eppendorf tubes for DNA extraction.

DNA extraction

Mycelium and root samples (ca. 100 mg fresh weight) were transferred to 2-ml microfuge tubes with two tungsten beads (3 mm) (Qiagen, Venlo, Netherlands) and 0.4 ml lysis buffer [EZNA Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA)] and ground with a Mixer Mill 300 (Qiagen) (2 min; 20 Hz). DNA was extracted from all samples using the EZNA Plant DNA Kit (Omega Bio-tek), following the manufacturer's protocol.

Water samples (40 ml each) were placed in 50-ml tubes (Sarstedt, Verona, Italy) and centrifuged for 10 min at 10,000 rpm. After that, 1 ml of water containing the pellet was transferred to a new 2-ml microtube (Sarstedt) and centrifuged at 16,000 g for a further 10 min. The supernatant was discarded and the pellet used for DNA extraction with the EZNA Plant DNA Kit (Omega Bio-tek).

From compost and soil (c.a. 80 mg for each sample), DNA was extracted with the EZNA Soil DNA Kit (Omega Bio-tek).

Real-time qPCR detection of *Phytophthora* species

Primers and TaqMan® MGB probe to amplify *Phytophthora* DNA were designed using PRIMER EXPRESS® Software 3.0 (Applied Biosystems, Forster City, CA, USA) using the ITS2 (internal transcribed spacer) region of *Phytophthora palmivora* (isolate Ph32SA from the culture collection of the Institute for Sustainable Plant Protection IPSP-CNR, Italy. GenBank accession number KT148922, sequence CNRpal32SA in TableS1)). The upstream and downstream primers were PhyF (5′-TCGGCTGTGAGTCCTTTGAA-3′ forward primer) and PhyR (5′-GCCACGCTTTTGGAGCAA-3′ reverse primer). The TaqMan® MGB probe (PhyPr: 5′-ACTGAACTGTACTTCTC-3′) was labelled with 6-carboxy-fluorescein (FAM) at the 5′ end, and a non-fluorescent quencher (NFQ) with minor groove binder (MGB) ligands, at the 3′ end.

Homology of the *P. palmivora* amplicon sequence with the sequences of other species (35 *Phytophthora* species, Table 1) was performed using standard nucleotide–nucleotide BLAST (BLASTN) in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The qPCR assay was tested on DNA from axenic cultures of 13 *Phytophthora* species belonging to six genetic clades (Table 1) and commonly isolated from nurseries (Kroon *et al.*, 2012) (*P. cambivora*, *P. cinnamomi*, *P. citrophthora*, *P. cactorum*, *P. citricola*, *P. cryptogea*, *P. gonapodyides*, *P. lateralis*, *P. nicotianae*, *P. palmivora*, *P. quercina*, *P. ramorum* and *P. syringae*). Multiple isolates per species were

Table 1 Homology between the designed amplicon sequence (sequence CNRpal32SA in TableS1) and the sequences of 35 *Phytophthora* species, from genetic clades 1–10, deposited in GenBank.

Species	Clade	Amplicon BLAST identity (%)
*P. cactorum**	1	95
*P. nicotianae**	1	93
P. infestans	1	98
P. hedraindra	1	93
P. pseudotsugae	1	93
*P. citricola**	2	95
*P. citrophthora**	2	96
P. capsici	2	96
P. pini	2	96
P. multivora	2	95
P. ilicis	3	98
P. pseudosyringae	3	98
P. nemorosa	3	98
*P. palmivora**	4	100
P. arenaria	4	98
*P. quercina**	4	93
P. heveae	5	95
P. katsurae	5	93
*P. gonapodyides**	6	96
P. megasperma	6	97
P. humicola	6	96
P. pinifolia	6	96
*P. cambivora**	7	95
*P. cinnamomi**	7	96
P. alni	7	94
P. europaea	7	94
P. cryptogea	8	95
P. drechsleri	8	96
*P. lateralis**	8	93
*P. ramorum**	8	95
*P. syringae**	8	98
P. polonica†	9	83
P. fallax†	9	82
P. kernoviae†	10	87
P. gallica†	10	88

*Positive DNA testing with the qPCR MGB TaqMan assay.
†Less than 15 sequences in the GenBank sequence database.

tested. The possible cross-reaction of the qPCR assay with DNA of closely related (*Pythium* spp.) and unrelated (*Fusarium* sp., *Mortierella* sp.) species, which are common in nurseries, was also tested. All isolates tested belong to the culture collection of the IPSP-CNR.

DNA extracts from roots, soil, compost, irrigation water, and mycelium as control, were assayed in MicroAmp Fast 96-well Reaction Plates (0.1 mL) closed with optical adhesive, and using the StepOnePlusTM Real-Time PCR System (Applied Biosystems, Life Science, Foster City, CA, USA). PCRs were performed in a 25 µl final volume containing 12.5 µl TaqMan Universal master mix (Applied Biosystems), 300 nM forward primer (Eurofins MWG Operon, Ebersberg, Germany), 300 nM reverse primer (Eurofins MWG Operon,

USA), and 200 nM TaqMan MGB probe (Applied Biosystems), 20 ng per tube genomic DNA. Each DNA sample was assayed in two replicates. Two wells, each containing 5 µl of sterile water, were used as the no-template control (NTC). The PCR protocol was 50 °C (2 min), 95 °C (10 min), 50 cycles of 95 °C (30 s), and 60 °C (1 min).

Results were analysed using an SDS 1.9 sequence detection system (Applied Biosystems) after manual adjustment of the baseline and fluorescence threshold. Quantification of *Phytophthora* DNA in unknown samples was made by interpolation from a standard curve generated with a *P. palmivora* DNA standard (sample Ph32SA) that was amplified in the same PCR run. The standard curve was generated from eight fivefold serial dilutions (ranging from 20×10^4 to 0.25 pg tube^{-1}) of a known concentration of *P. palmivora* DNA and analysed in triplicate. Reproducibility of the qPCR assay was assessed by computing the coefficient of variation (CV) among the mean values in eight independent assays. PCR efficiency was calculated on the slope of the standard curve ($Eff = 10^{-1/slope} - 1$) (Bustin *et al.*, 2009), from eight independent experiments.

Identification of *Phytophthora* species

To determine the range of *Phytophthora* species present in the samples, pure cultures of *Phytophthora* were obtained and identified through amplification of the internal transcribed spacer (ITS, including both ITS1 and ITS2) of the ribosomal DNA with the ITS6/ITS4 primer pair (White *et al.* 1990). The PCRs were performed in a 25 µl final volume containing 1x PCR buffer with MgCl$_2$ (Genespin, , Milan, Italy), 5 µM of each primer, 0.2 mM dNTPs (Genespin), 0.1 U µl^{-1} of Taq DNA polymerase (Genespin) and 2 µl of DNA template. The reactions were incubated at 95 °C for 5 min followed by 35 cycles each consisting of 94 °C for 90 s, 56 °C for 1 min, 72 °C for 2 min, with a final cycle of 72 °C for 10 min. Amplification products were separated by electrophoresis on gels containing 1% (w/v) of agarose LE (Genespin). The approximate length (bp) of the amplification products was determined using the 100-bp DNA ladder Ready to Load (Genespin). Amplification products were purified with a mi-PCR Purification Kit (Metabion International, Planegg, Germany) and sequenced at Macrogen (Seoul, South Korea). ITS sequences were blasted in the NCBI database to identify the most similar available sequences. All ITS sequences obtained in this work have been placed in GenBank. Accession numbers are reported in Table S1.

Data analyses

Chi-square tests were applied to identify the significant differences between the frequencies of detection of *Phytophthora* species (by isolation on selective medium or by qPCR) in symptomatic vs. asymptomatic plants, and in different substrates (soil, roots, compost, water). Kruskal–Wallis test was applied to identify significant differences in *Phytophthora*

DNA quantity estimated by qPCR, between symptomatic and asymptomatic plants, and between substrates. The variation in frequency of isolation of *Phytophthora* species between substrates (roots vs. soil), pots carrying plants with or without external symptoms (symptomatic vs. asymptomatic leaves), and between plant species, was illustrated by heatmaps accompanied by dendrograms obtained through hierarchical clustering. Statistical tests were performed in R (R Development Core Team 2013) and STATISTICA 6.0 (StatSoft Inc., Tulsa, OK, USA). Heatmaps, bar plots and boxplots were produced with the GPLOTS package in R (http://cran.r-project.org/web/packages/gplots/gplots.pdf).

RESULTS

Isolation of *Phytophthora* on selective medium

Phytophthora was isolated on selective medium from nearly half of the potted plants, while the pathogen was not isolated from water or compost (Fig. 1a). The frequency of isolation did not differ significantly between symptomatic and asymptomatic plants (54 vs. 46% $\chi^2_{(df1)} = 0.05$, ns) (Fig. 2a), nor did it differ between soil and roots (39 vs. 44% $\chi^2_{(df1)} = 0.54$, ns) (Fig. 3a).

Real-time qPCR assay for *Phytophthora* species

BLAST searches in NCBI showed 95–100% homology between the designed amplicon sequence and the sequences of 31 *Phytophthora* species from genetic clades 1 to 8, deposited in Gen-Bank. BLAST identity of the amplicon sequences of four species from clades 9 and 10 was 82–88%. It should be noticed, however, that less than 15 sequences were available in NCBI for species from clades 9 and 10 (Table 1). No homology was found with sequences of *Pythium* or fungal species tested. The qPCR assay was able to amplify DNA from all tested *Phytophthora* isolates, while DNA from isolates of other fungal species, tested as controls for specificity, such as *Pythium* spp., *Fusarium* sp. and *Mortierella* sp., was never amplified.

(a) Potted plants with symptomatic leaves / Potted plants with asymptomatic leaves
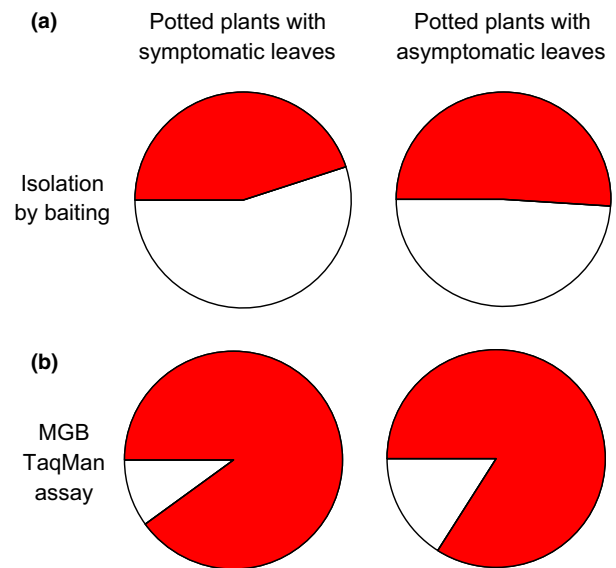
Isolation by baiting

(b) MGB TaqMan assay

Figure 2 Detection of *Phytophthora* species [coloured = positive, white = negative (%)] in potted plants with or without visible disease symptoms to the crown, by classical isolation on selective medium (a) or by qPCR with a specific MGB TaqMan probe (b).

A standard curve was produced by measuring eight five-fold dilutions of isolate Ph32SA in the range 20×10^4–0.25 pg tube^{-1}. The standard curve had slope of -3.37, correlation coefficient of 0.99 and Y-intercept of 33.4. Efficiency of the PCRs was 0.98 ± 0.02 (SE). Reproducibility of the standard curve points performed on six curves was high (CV varied from 1.04 to 2.4%).

Detection of *Phytophthora* in potted plants, water and compost through qPCR

Real-time PCR was in general much more efficient than isolation for detecting *Phytophthora* species, especially in soil and environmental samples. *Phytophthora* was detected in 87% of the pots tested through qPCR with the specific

(a) Potted plants / Compost / Water

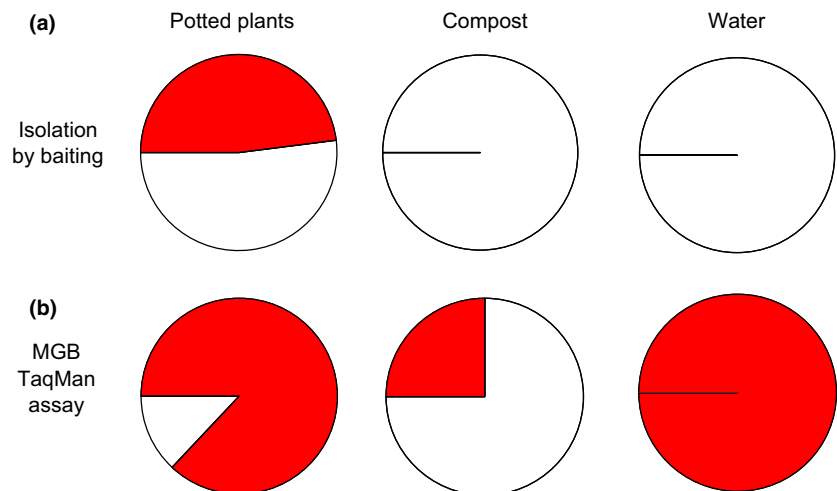Isolation by baiting

(b) MGB TaqMan assay

Figure 1 Detection of *Phytophthora* species [coloured = positive, white = negative (%)] in potted plants, compost produced in the nurseries and water from the drainage systems, by classical isolation on selective medium (a) or by qPCR with a specific MGB TaqMan probe (b).
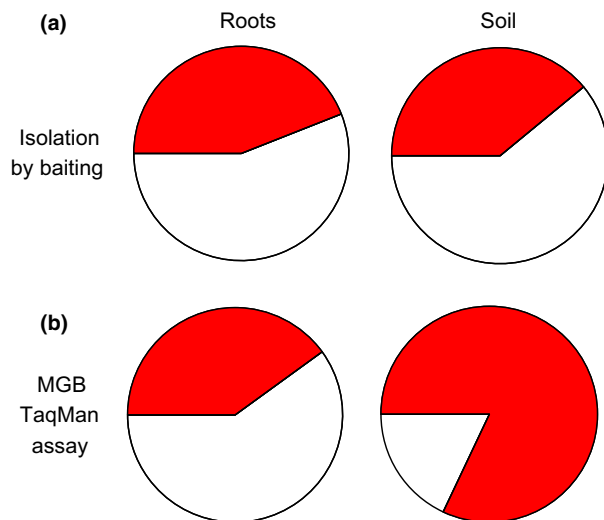
(a)

Roots   Soil

Isolation
by baiting

(b)

MGB
TaqMan
assay

Figure 3 Detection of *Phytophthora* species [coloured = positive, white = negative (%)] in roots or soil samples from all potted plants (with or without visible disease symptoms to the crown), by classical isolation on selective medium (a) or by qPCR with a specific MGB TaqMan probe (b).

TaqMan MGB probe. All samples from irrigation water and a quarter of the compost samples were positive to the assay (Fig. 1b). The difference in detection frequency between pots containing symptomatic plants and pots containing asymptomatic plants (90 vs. 84% positive samples, Fig. 2b) was non-significant ($\chi^2_{(df1)} = 0.08$). The frequency of detection of *Phytophthora* by qPCR in soil was twice as high as the frequency in root tissues (82 vs. 40% $\chi^2_{(df1)} = 14.27$ $P < 0.005$; Fig. 3b).

More than half of the plants with asymptomatic leaves had symptomatic roots upon visual inspection (Fig. 4a). All plants with asymptomatic leaves and damaged roots were positive to the *Phytophthora*-specific TaqMan assay (Fig. 4c), and in all the pots containing a plant of this kind, the pathogen was detected both in roots and in soil (Fig. 4c). Also positive to the qPCR assay were the large majority (70%) of the asymptomatic potted plants with roots that seemed healthy to visual inspection (Fig 4c). In nearly half of the plants with both asymptomatic leaves and healthy roots, *Phytophthora* was detected in root tissues (Fig 4c), and from about half of those plants, *Phytophthora* was also isolated on selective medium (Fig. 4b).

Phytophthora DNA was quantified through qPCR in root tissues (13.41 ± 5.34 pg DNA/μg DNA extracted), soil (5.99 ± 1.58 pg DNA/μg DNA extracted), compost (5.87 ± 1.63 pg DNA/μg DNA extracted) and irrigation water (251.93 ± 160.92 pg DNA/μg DNA extracted) (Fig. 5a). The minimum quantity of *Phytophthora* DNA measured by qPCR in environmental samples was 0.02 pg DNA/μg DNA extracted. The difference in DNA quantity was non-significant between pots containing symptomatic plants and pots containing asymptomatic plants (Kruskal–Wal-

lis$_{(1,89)} = 0.11$, $P = 0.74$; Fig. 5b), and between root and soil samples (Kruskal–Wallis$_{(1,89)} = 0.01$, $P = 0.92$; Fig. 5c).

Phytophthora DNA was detected and quantified by qPCR in the soil of all plant species tested. It was also found in the roots of all plant species except *Ceanothus thyrsiflorus*, *Hibiscus* sp., *Nerium oleander* and *Prunus laurocerasus* (Fig. 6a).

Identification of *Phytophthora* species through ITS analysis

Phytophthora isolates (59 in total) were assigned to eight species by means of ITS analysis (Table 2, Fig. 6). All *Phytophthora* species, except *P. citricola*, were isolated from soil irrespective of the symptoms shown by the plant contained in the pot (Fig. 6a,c). Four species (*P. cinnamomi*, *P. palmivora*, *P. nicotianae* and *P. citrophthora*) were isolated with higher frequency (6–15%) both from roots of various plants and from soil of pots containing different plants (Fig. 6a,d). Among the other four species, which were less frequent (< 5%), *P. cactorum* was isolated both from roots and from soil of *Viburnum*; *P. citricola* was isolated only from roots of *Viburnum*; while *P. cryptogea* and *P. syringae* were isolated only from soil (Table 2, Fig. 6a,d)

The species of *Phytophthora* that were most frequently isolated in this study are characterized by higher growth temperatures, ability to produce chlamydospores and wider host ranges (Table 2).

DISCUSSION

To illustrate the risk of introducing harmful microbes through the commercial trade in live potted plants, the presence of *Phytophthora* in various ornamental plant species from nurseries was analysed.

Diagnostic tests currently employed to detect *Phytophthora* are not always reliable. Baiting, besides being time-consuming, presents a number of technical problems and may fail to detect the pathogens, as shown in the present study, especially when they are in the environment (e.g. in soil) in the form of resting chlamydospores, a state that is commonly induced under unfavourable conditions (Erwin & Ribeiro, 1996). ELISA immune-detection tests, recommended by EPPO for screening of plants suspected of being infected by *Phytophthora*, are subject to false negatives depending on *Phytophthora* species and/or type and quality of tested plant tissue (Martin *et al.*, 2012). PCR-based diagnostic methods, including those more recently developed, detect only single species and/or are not sensitive enough to reveal low inoculum concentrations.

Consequently, a *Phytophthora*-specific TaqMan MGB probe assay that fulfils these requirements was developed. The qPCR assay amplified all tested *Phytophthora* isolates, belonging to 13 species from six genetic clades, and detected *Phytophthora* DNA in environmental samples in quantities as low as 0.02 pg μg^{-1} of total DNA extracted.
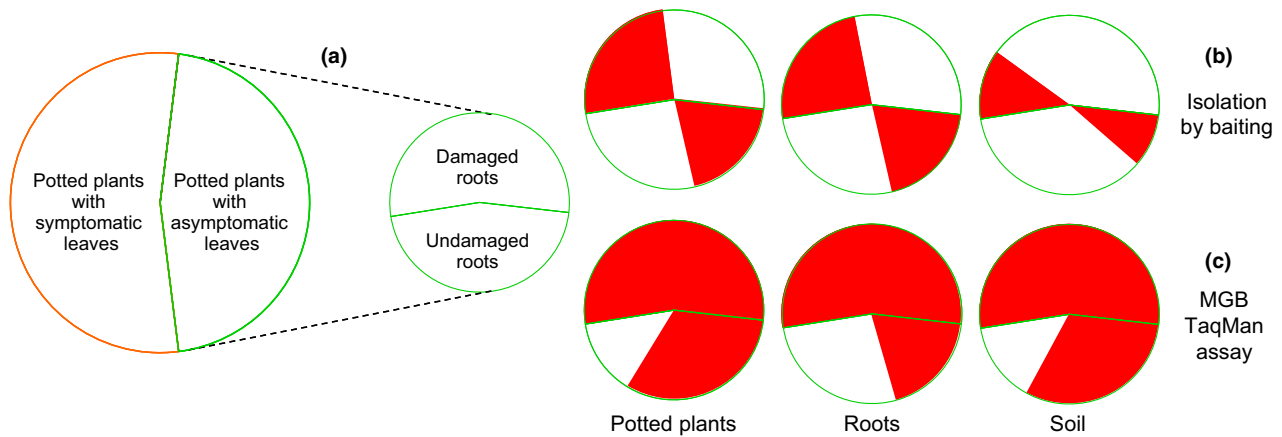
Figure 4 Percentage of asymptomatic plants (i.e. plants without visible disease symptoms to the leaves) that had (damaged roots) or had not (undamaged roots) symptoms to the roots (a). Percentage of samples, from pots containing asymptomatic plants, with either damaged or undamaged roots, which tested positive to *Phytophthora* species by isolation of the fungus on selective medium (b) or by qPCR with a specific MGB TaqMan probe (c).
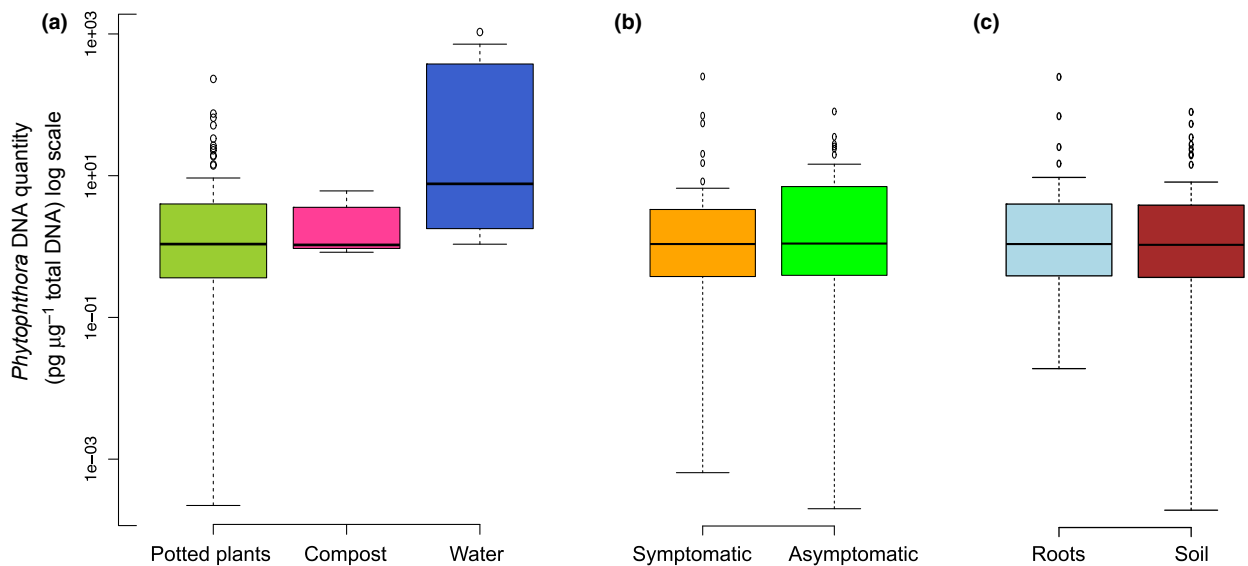

Figure 5 *Phytophthora* DNA quantities (horizontal line = median, box = 1st–3rd quartiles, whisker = min.-max., dots = outliers and extremes; logarithmic scale), estimated by qPCR with a specific MGB TaqMan probe, in tested substrates. Differences between symptomatic and asymptomatic plants (b), and between root and soil samples (c) were non-significant according to Kruskal–Wallis statistic.

It has long been known that nurseries are potentially ideal inoculum reservoirs for soilborne pathogens such as *Phytophthora* species (Hardy & Sivasithamparam, 1988; Parke & Grünwald, 2012). In this study, *Phytophthora* was detected in the tissues or in the soil of all plant species sampled from two large European retail nurseries which trade with European and non-European countries. In addition, both recycled water and the compost produced in the nursery tested positive for *Phytophthora*. These results corroborate previous concerns about contamination of potted plants from nurseries (Ferguson & Jeffers, 1999; Themann *et al.*, 2002; Chastagner *et al.*, 2009) and confirm that the commercial trade in live plants for cultivation is an important pathway for alien

soilborne pathogens such as *Phytophthora* species (Frankel, 2008; Goss *et al.*, 2009, 2011; Martin *et al.*, 2012; Santini *et al.*, 2013).

Many of the *Phytophthora* species isolated in this study are not confined to nurseries and have the potential to invade natural ecosystems. A striking example is *P. cinnamomi*, one of the '100 of the world's worst invasive alien species' (Global Invasive Species Database, http://www.issg.org/database/welcome/). In this study, *P. cinnamomi* was detected at high frequency in soil and roots of both symptomatic and asymptomatic plants belonging to several ornamental species, and especially species from the Mediterranean region. This evidence is of primary concern as this pathogen is particularly
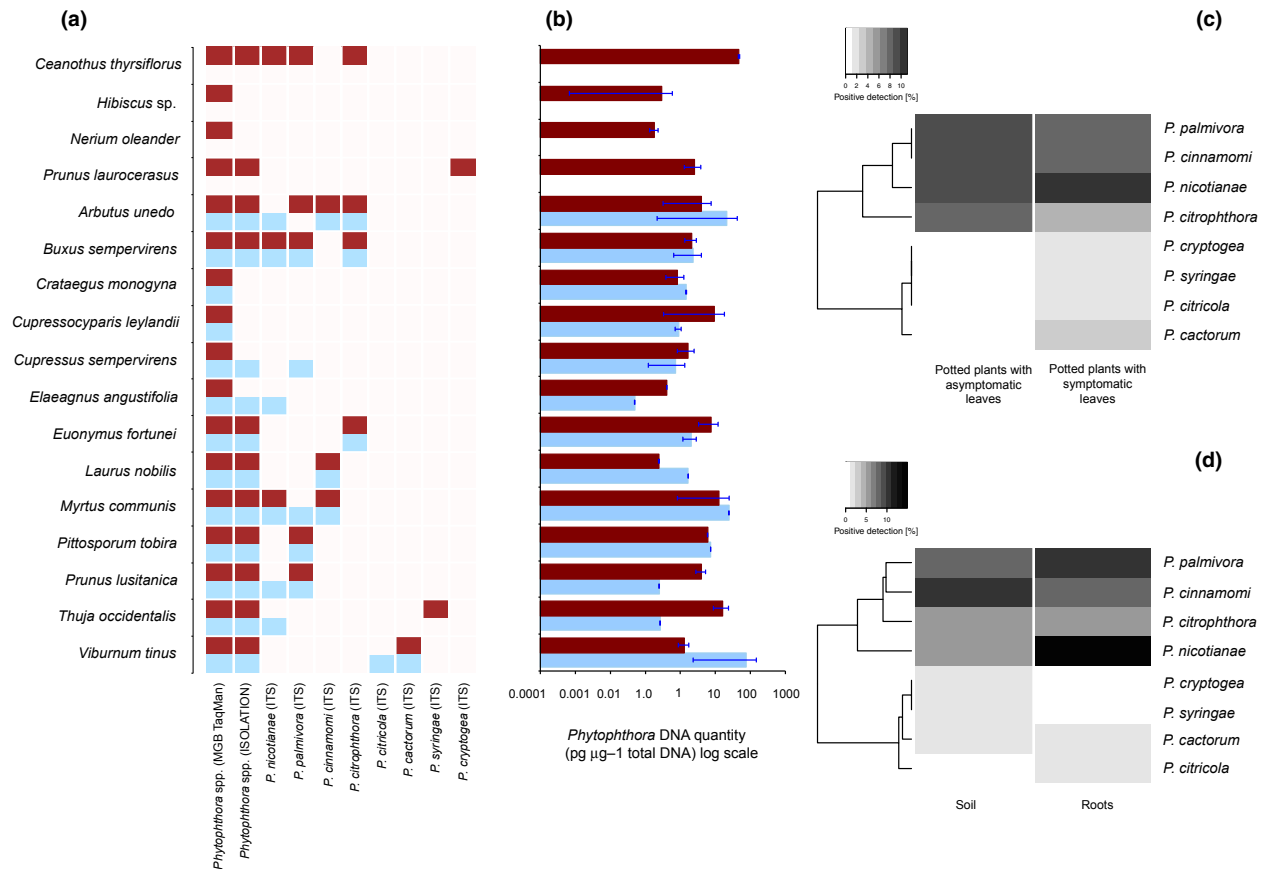
Figure 6 Summary of detection of *Phytophthora* species by isolation on selective medium and ITS analysis, and by qPCR with a specific MGB TaqMan probe. (a) Presence–absence of *Phytophthora* species, and (b) average quantity (±SE, logarithmic scale) of *Phytophthora* DNA estimated by qPCR, in roots (light colour) or soil (dark colour) of potted plants belonging to 17 woody ornamental species. Clustering of *Phytophthora* species based on isolation frequencies in symptomatic and asymptomatic plants (c), and in root and soil samples (d).

adapted to, and harmful in, these environments, as shown by epidemic outbreaks in the jarrah forests of Western Australia (Dell & Malajczuk, 1989) and on oaks in the Mediterranean region (Brasier *et al.*, 1993). Moreover, those *Phytophthora* species that were detected at lower frequency in this study can also be extremely dangerous. For instance, *P. cryptogea*, a species adapted to more temperate conditions, and able to attack many widespread broadleaved trees (Vettraino *et al.*, 2002, 2008; Perlerou *et al.*, 2010), was isolated from *Prunus laurocerasus*. In temperate Europe, *P. laurocerasus* is one of the most commonly planted ornamental shrubs in gardens and parks, naturalized, and even invasive, in some areas (EPPO, 2014).

The species of *Phytophthora* most frequently isolated in this study share some biological traits, such as wide host ranges, abundant production of resistant spores (chlamydospores) and high growth temperatures, which characterize pioneer micro-organisms and increase the possibility of successfully colonizing new environments. The hazard represented by these *Phytophthora* species is evident as they were found both in symptomatic and asymptomatic plants, roots and soil, and on a wide range of taxonomically unrelated hosts.

The ability to detect soilborne pathogens, including *Phytophthora* species, in commercial consignments, and not only in plant tissues but also in potting media, is a critical element in the management of these organisms, which may spread through movement of infested soil (O'Brien *et al.*, 2009). The 'plants-for-planting' pathway is difficult to control for various reasons. Horticulture is a major European industry; in 2013, 84,500 tonnes of live plants were imported, while exports reached 400,000 tonnes (Eurostat Comext http://epp.eurostat.ec.europa.eu/newxtweb/). Faced with such huge quantities, only a small percentage of plants can realistically be inspected. Moreover, the market in live plants, especially ornamentals, is quite mutable. Traded species and geographical origins can change rapidly, and this exacerbates the risk of introducing new pests from different locations.

On entry into Europe, international plant stocks are visually inspected only if they are recognized as potential hosts of regulated harmful organisms, but the process of such regulation is too slow for such a dynamic international trade. Additional analyses may be applied if plants show signs or symptoms of infection, however, even in this case, inspections may still fail to identify contaminated consignments if

Table 2 Main features of the *Phytophthora* species isolated from roots and soil of potted plants belonging to 17 woody ornamental species collected in two European retail nurseries.

Species	Clade	Isolation frequency (%)	Growth temperature (°C)†			Chlamydospores†	Known host range (no.)*		
			Min.	Optimum	Max.		Species	Genera	Status‡
Phytophthora cinnamomi Rands	7	High	5/6	24/28 (26/32)§	32/34 (36/37)§	Frequent and abundant	266	90	A
Phytophthora palmivora E.J. Butler	4	High	11	27.5/30	35	Frequent and usually abundant	138	> 90	A
Phytophthora nicotianae Breda de Haan	1	High	5/7	27/32	37	Frequent	255	90	C
Phytophthora citrophthora (R.E. Sm. & E.H. Sm.) Leonian	2	High	< 5	24/28	32/33	Medium	88	51	C
Phytophthora cactorum (Lebert& Cohn) J. Schröt	1	Medium	2	25	31	Medium (produced by some but not all isolates)	154	54	C
Phytophthora citricola Sawada	2	Low	3	25/28	31	Rare	75	38	C
Phytophthora cryptogea Pethybr. &Laff	8	Low	< 1	22/25	31/33	Rare	141	49	C
Phytophthora syringae Kleb	8	Low	< 5	15/20	23/25	Rare	29	14	C

A, Alien to Europe; C, cryptogenic (unknown origin, most likely alien to Europe).

*http://nt.ars-grin.gov/fungaldatabases/

†From Erwin & Ribeiro 1996

‡From Santini *et al*. 2013.

§According to the USDA *Phytophthora* database.

infected plants are asymptomatic owing to invisible or systemic infection, as demonstrated in this study. This is a common occurence in early-stage infections by root pathogens, or if fungistatic chemicals have been used that temporarily suppress the disease, or when potting media are infested with pathogens in a latent phase.

Policy measures against entry and spread of alien soilborne pathogens through the trade of potted plants should not rely on visual inspections of aerial and/or subterranean plant organs alone. The present study confirms that tissues of many plants species may be colonized by *Phytophthora* while still remaining fully asymptomatic to the naked eye (Shishkoff, 2007; Olson & Benson, 2013). *Phytophthora* was detected by qPCR at the same high frequency, and in equal inoculum quantities, both in plants with symptomatic and asymptomatic leaves, as well as in most plants with apparently healthy roots. The results of this study strongly suggest the introduction of much more stringent European legislation on the trade in potted plants.

Phytophthora is a striking example of the risk of spreading harmful pathogens, even those well-known, through the nursery trade, yet only three *Phytophthora* species are currently proscribed in European regulations. Among the host plants included in the present study, only two, *Prunus* and *Crataegus,* of 17, are regulated as potential carriers of harmful organisms of relevance other than *Phytophthora* (EU, 2000), and only three, *Arbutus unedo, Laurus nobilis* and *Viburnum* spp. are regulated for *Phytophthora ramorum* (EU, 2007).

The European and Mediterranean Plant Protection Organisation (EPPO) has detailed the phytosanitary risk inherent in the nursery/soil pathway in a specific study (EPPO, 2012). The relative importance of soil as a pathway for the introduction of pathogens to Europe has dramatically increased in the past 30 years (Santini *et al.*, 2013). Yet the new European regulation on the prevention and management of the introduction and spread of invasive alien species (EU, 2014), already the subject of criticism by the scientific community (e.g. Hulme, 2015), fails to identify soil as a potential pathway.

There is presently a blatant contradiction in the prohibition of soil imports into Europe and the legal import of live plants in soil. Even assiduous inspection will not completely prevent the introduction of new pathogens because, as shown here, they can remain latent in healthy plant tissues. However, restricting trade to bare-rooted plants alone could significantly reduce the inoculum potential of the latent microbe communities present in the soil of potted plants, putting European phytosanitary legislation in line with that of other countries such as the USA, China, Australia and New Zealand.

ACKNOWLEDGEMENTS

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BIOSKETCH

The authors focus towards dynamics of arrival and spread of forest pathogens in Europe. Their research encompasses pathogens early detection and diagnosis for preventing entry of invasive alien pathogens.

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A simple lab procedure for selective qPCR detection of viable *Phytophthora* inoculum in soil

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Summary

River sand and forest soil were inoculated with either viable (untreated) or non-viable (treated) inoculum of three *Phytophthora* species in order to develop a lab procedure to selectively detect viable *Phytophthora* inoculum by means of a genus-specific qPCR MGB TaqMan probe, reducing the number of false positives due to dead cells in soil samples.

An *in vitro* experiment was designed including all four combinations between treated/untreated matrix and treated/untreated inoculum. The treatment applied to matrices was autoclaving (121°C for 20 minutes) repeated 3 times at 24h intervals. A single autoclave cycle was used to treat the inoculum. *Phytophthora* DNA was quantified in all samples by qPCR one, two, three and four weeks after treatment.

In samples with viable mycelium the quantity of *Phytophthora* DNA remained high and stable during the experiment. On the contrary, the quantity of *Phytophthora* DNA in samples where the inoculum was killed by heat had diminished a week after treatment. Samples where both the matrix and the pathogen were treated became negative from two weeks on. There was no significant difference between sand and soil.

Results demonstrate that non-viable mycelium in sand/soil is rapidly degraded in the conditions of this procedure and become undetectable by qPCR after two weeks of incubation. Instead, in soil containing viable mycelium the qPCR signal is stable or increases in the same time period. Therefore, the TaqMan assay here challenged can be used to selectively detect viable *Phytophthora* in sand/soil samples after a 2-week incubation in the conditions employed in this study.

Keywords

Phytophthora, environmental DNA, qPCR, viable cell detection, phytosanitary inspections, eradication

Introduction

Eradication protocols against *Phytophthora* diseases have been applied at the regional scale in Western USA and Western Australia. These operations are very expensive but justified by the need of reducing the risk of huge environmental loss posed by *Phytophthora*s. Davis *et al*. (2012) estimated in 10 million AUD the 20-year total cost of *Phytophthora cinnamomi* management in the Fitzgerald River National Park (330.000 ha) in Western Australia, mainly due to eradication and prevention. Considerable effort is being spent controlling *Phytophthora ramorum*, the agent of sudden oak death (SOD), in the west coast of the USA. Management of SOD in South Western Oregon from 2001 to 2009, which involved disease eradication and host removal in approximately 1200 ha of forests, cost 4.5 million US$, a price that reached 6000 US$ per hectare in the period 2008-2009. Management procedures consisted of early detection (aerial and ground surveys, stream baiting) and destruction (herbicide treatment, felling and burning) of infected and nearby host plants in a 15-100 m radius (Kanaskie *et al.,* 2011). A protocol including host destruction, fungicides, fumigation and physical root barriers was successfully applied for eradicating *P. cinnamomi* from spot infestations in Western Australia and Tasmania (Dunstan *et al*., 2010), where no viable pathogen was detected 6 to 9 months after the end of treatments. Crone *et al*. (2014) in a similar environment in Western Australia after destroying trees and herbaceous plants by herbicide applications, observed a temporary

reduction in *P. cinnamomi* detection followed by a progressive re-emergence and re-establishment of the pathogen during the following two years.

In nurseries, *Phytophthora* management is easier than in forests since the system is simplified, conditions can be better controlled, and the entire volume of contaminated material may be treated repeatedly (Parke & Grunwald 2012; Jung & Blaschke, 2004; Osterbauer *et al.*, 2004). Nevertheless, Phytophthoras in nurseries are widespread and the level of infestation in potted plants for commercial trade is alarming (Jung *et al.*, 2015; Migliorini *et al.*, 2015).

Whichever the means used to remove the Phytophthoras from contaminated nurseries or forest areas, crucial is the technique employed to verify the presence of the pathogens during and after the treatment, to understand how fast the inoculum is lowered, and for how long time the plants or the area remain free from disease. The studies cited above about *P. cinnamomi* employed, with only minor differences, the same diagnostic method, i.e. extensive sampling of plants and soil followed by baiting and isolation of the pathogen on selective media, and taxonomic assignment by microscopy and/or by sequencing of the internal transcriber spacer (ITS) DNA region. Considering the huge effort and economic cost required for *Phytophthora* management and ensure the success of eradication campaigns, development of dependable methods to detect viable inoculum of the pathogens is of a primary importance.

Phytophthora species produce propagules inside cortex cells of roots and promote formation of surrounding layers of lignified cells by infected plants, which assure long-term storage of the pathogens below ground (Fichtner *et al.*, 2011; Shishkoff, 2007). Resting/survival structures such as thick-walled dormant oospores or dense stromata-like aggregates of hyphae, protected against microbial decomposition or drought by thick host cell walls, papillae or lignotubers, are produced both in fine and wooden roots of trees infected by various *Phytophthora* species as a strategy for long-term persistence in the environment (Jung *et al.*, 2013 and references therein). Soil is an important inoculum reservoir in the SOD disease cycle (Fichtner *et al.*, 2007). Therefore, because of self-produced or host-provided defense barriers, propagules of Phytophthoras may escape the poisoning effect of chemical treatments and remain temporarily inactive but viable in soil.

In the absence of active mycelial growth and zoospores from sporangia, recovery of Phytophthoras from soil by baiting depends on germination of oospores and/or chlamydospores (Dunstan *et al.*, 2010). However, the conditions of culture in the laboratory are not necessarily efficient to induce mycelial growth from resting structures and thus enable detection of the pathogens by baiting.

To monitor the efficacy of eradication actions and control nursery plant commodities, especially plants in soil, real time PCR (qPCR) would be a profitable tool since it enables detection of small quantities of the pathogen hidden in plant structures or in the soil, thus minimizing the risk of false negatives (Herder *et al.*, 2014). Real time PCR in fact amplifies much shorter products than classical PCR, increasing detection efficiency and preventing inhibition of reaction (Schena *et al.*, 2013). Diagnostic methods based on qPCR have been successfully employed for detecting many *Phytophthora* species in plant tissues and environmental samples (Hayden *et al.*, 2006; Schena *et al.*, 2006; Lees *et al.*, 2012). Migliorini and colleagues (2015) showed that qPCR is far more efficient than baiting for detecting *Phytophthora* species in plant tissues, water and soil.

However, qPCR has some limitations for detection of soil borne pathogens, as reviewed by Sanzani *et al*. (2014). The main defect is that nucleic acids from dead cells may lead to positive PCR signals, i.e. false positives. Nucleases are common in the environment and can degrade the DNA of dead microorganisms, but the rate of degradation strongly depends on the environment. Indeed, quantitative studies of DNA degradation kinetics performed by using qPCR have shown that the rate of degradation of bacterial DNA after cell death is variable as a function of the capacity of the substrate to bind DNA (Wolffs *et al.*, 2005). Schena & Ippolito (2003) found that DNA of *Rosellinia necatrix*, a rot agent in trees, was rapidly degraded once inoculated in soil. RNA-based diagnostic methods, which target the active microbial community, would avoid detection of non-viable material, but fast decay of RNA strongly limits their use in soil samples

(Wagner *et al.*, 2015). Detection based on mRNA is more difficult and costly because of the reverse-transcription phase, and strongly depends on expression levels of the target gene and on extraction protocols (Livak & Schmittgen, 2001).

The aim of the present study was to develop a simple lab protocol to selectively detect viable inoculum of *Phytophthora* species in soil samples by means of a genus-specific qPCR TaqMan MGB probe, reducing the risk of false positives due the presence of DNA from dead cells.

Materials and methods

Inoculum preparation
Three *Phytophthor*a species, *P. citrophthora* (Genebank accession number: KT148897, IPSP-CNR, Italy), *P. nicotianae* (Genebank accession number KT148933, IPSP-CNR, Italy) and *P. ramorum* (Isolate PramG44, IPSP-CNR collection), were used. Strains were grown on V8 Agar for ten days at 20°C. Eight flasks containing 400 ml of vermiculite, 4 g of millet, 240 ml of V8 broth, 0,8 g CaCO3 (melted in V8 broth) each were autoclaved at 121°C for 20 minutes. Each *Phytophthora* species (a 9 cm dim. Petri dish V8Agar previously colonized by the isolate) was inoculated into two flasks and incubated for five weeks at 20 °C in dark conditions. Two mock-inoculated flasks containing the soil/sand matrix and a 9 cm diameter . Petri dish pure were included as negative controls. The flasks were handy shaken periodically in order to uniformly mix the growing *Phytophthora* mycelium with the medium.

Matrix inoculation
Inoculation was carried out in two matrices, i.e. river sand and forest organic soil. River sand was collected at 10-15 cm depth on a dry and nude bank of the river Carzola (Vaglia, Florence, Italy), while organic soil was collected at the same depth under the litter of a mixed pine-oak forest on Monte Morello (Sesto fiorentino, Florence, Italy). The matrices were stored for 24 h at 5°C in the darkness.
The following day, five theses per matrix were prepared: untreated matrix with untreated inoculum (uu), untreated matrix with treated inoculum (ut), treated matrix with untreated inoculum (tu), treated matrix with treated inoculum (tt), and untreated matrix with mock-inoculum (ctrl) (Table 1). The treatment applied to matrices was autoclaving (121°C for 20 minutes) repeated 3 times at 24h intervals. A single autoclave cycle (121°C for 20 minutes) was used to treat the inoculum from flasks.
Four glass Petri dishes (12 cm diameter) per *Phytophthora* species plus 3 control dishes were prepared with each matrix. Each Petri dish was filled with 100 ml of matrix (either treated or untreated) to which 25 ml of inoculum (either treated or untreated) were added. The inoculum was gently blended to the matrix to obtain a uniform mixture. Dishes were incubated in the dark at 20°C for 4 weeks.
At the same time, the vitality of *Phytophthora* was tested by plating a small quantity of matrix from each dish containing viable inoculum on *Phytophthora*-selective PARPH media (Erwin & Ribeiro, 1996)

Sample collection, DNA extraction and *Phytophthora* DNA quantification
For each thesis 0.4 g of matrix were collected once per week for 4 weeks from ten spots within the plate by using a sterile spatula, and placed in 2.0ml microtubes (SARSTEDT) at -80°C. Matrix samples were ground in 2ml microfuge tubes with two tungsten beads (3 mm) (Qiagen) and 0.4-ml lysis buffer [EZNA Soil DNA Kit (Omega Bio-tek)] by using a Mixer Mill 300 (Qiagen) (2 min; 20 Hz). DNA extraction was carried out using the EZNA Soil DNA Kit (Omega Bio-tek), following the manufacturer's protocol. The total extracted DNA was quantified with the NanoDrop ND-1000 spectrophotometer. Quantitation of *Phytophthora* DNA was carried out by using the real-time TaqMan® MGB assay described in Migliorini *et al.* (2015).

In addition, to make certain that the treatment applied to kill the inoculum did not prevent nor reduced the capacity of the qPCR TaqMan assay to amplify the target DNA, pure mycelia of *P. citrophthora*, *P. nicotianae* and *P. ramorum* (0.2 grams per species) grown on Cellophane-PDA media for one week, were placed in 2ml microtubes (SARSTEDT). DNA was extracted from samples and quantified as described above. Samples were treated as the inoculum used in the experiment (autoclaving at 121°C for 20 min) and processed directly.

Statistical analyses

Kruskal–Wallis test was applied to identify significant differences in Phytophthora DNA quantity estimated by qPCR, between experimental theses. Statistical tests were performed in R (R Core Team 2015). Boxplots were produced with the GPLOTS package in R (http://cran.r- project.org/web/packages/gplots/gplots.pdf).

Results

There were no significant differences in *Phytophthora* DNA quantity between sand and soil samples for any thesis and at any time point during the experiment (Kruskal-Wallis test; p-value > 0.05). Therefore, the results presented below were obtained analyzing all data irrespective of the matrix (Fig. 1).

Detection of DNA in treated samples assayed directly after autoclaving (t0) was comparable (Kruskal-Wallis chi-squared = 0.06, df = 2, p-value = 0.97) to detection in the theses with untreated (viable) *Phytophthora* inoculum (uu and tu), where the quantity of target DNA remained stable until the end of the experiment (Kruskal-Wallis test, p-value>0.75).
For the duration of the experiment, the quantity of *Phytophthora* DNA in untreated matrices inoculated with mock- (ctrl) or non-viable (ut) inoculum did not differ significantly from the quantity detected in environmental samples directly after collection (env0), as expected, and remained stable throughout the experiment (Kruskal-Wallis test, p-value > 0.39).

In treated samples where the matrix was cleaned by repeated autoclaving and the pathogen was killed by heat (tt), *Phytophthora* DNA was only detected a week post inoculation in a lower quantity than in the other untreated theses, possibly because of initial DNA degradation during the week pasted since inoculation. All tt samples became negative from two weeks on.

Discussion and conclusions

The viable (untreated) inoculum of *Phytophthora* species survived in soil/sand matrices in the experimental conditions and was detected in similar quantity up to 4 weeks after inoculation. Similarly, as shown by control samples, the viable inoculum contained naturally in the untreated matrix was detected until the end of the experiment. This result indicates that incubation of soil/sand samples collected outside in the conditions of this protocol does not increase the number of false negatives if a sample of statistically correct size is analyzed up to 4 weeks after collection.

The aim of this study was to verify for how long time DNA from non-viable *Phytophthora* in soil/sand samples was detectable by a genus-specific qPCR assay previously described (Migliorini *et al.,* 2015) before being degraded and no longer amplified in experimental conditions still suitable for survival of the viable inoculum. DNA starts to degrade soon after cell death owing to the action of endogenous nucleases, water, UV radiation, and microorganisms in the environment (Shapiro, 2008). Once the cell structure ruptures, DNA and cellular fluids are released into the environment. Microorganisms, if present, grow on these substrates and their exogenous DNases further degrade the DNA (Hebsgaard *et al.,* 2005; Willerslev & Cooper, 2005).
Although in terrestrial soils a low proportion of DNA, adsorbed to organic or inorganic particles that protect it from degradation agents, can persist for long periods of time in cold and dry climates (Parducci *et al.,* 2012), warm and wet conditions facilitate rapid degradation (Willerslev & Cooper 2005). Moreover, the persistence of DNA depends on the nature of the sediment, e.g., in loamy sediment the persistence time may be similar to the persistence in water (Deere *et al.,* 1996). The persistence of DNA in water kept at constant room temperature under no direct sunlight ranges from one week (Piaggio *et al.,* 2014; Thomsen *et al.,* 2012) to nearly one month (Dejean *et al.,* 2011). Our results are consistent with the above-cited literature. In moist soil/sand samples containing treated (non-viable) mycelium that were kept in warm conditions, the DNA of non-viable *Phytophthora* disappeared two weeks after treatment and no positive

amplifications occurred after this period. In conclusion, these results confirm that the qPCR assay hereby tested can be employed as a valid early detection tool in soil/sand samples, because it is sufficient to incubate the samples for a 2-week period (and up to 4 weeks) in the conditions of this procedure for detecting only the pathogen that was initially alive, drastically diminishing the number of false positives.

The method used to treat inoculum, i.e. autoclaving at 121°C for 20 minutes, is a common lab practice to ensure sterilization without reducing PCR template activity of DNA in a significant manner. PCR amplification of DNA contained in human saliva still occurred after 60 minutes of autoclaving with three different commercial amplification kits (Gefrides *et al.*, 2010). The treatment might still have accelerated cell rupture and hence reduced the time of degradation. Fumigation or treatment with fungicides, which are used in field management of Phytophthoras, could not been employed in this experiment instead, because mixed treated-untreated theses would have undergone cross contamination. Resistant stages of the life cycle might survive short autoclaving (as well as fungicide treatment), but this instance seems not to have occurred in our experiment. The inoculum used in this study probably did not contain any resistant spore. It should be notices however that non-viable resistant structures contained in soil samples might resist degradation for longer time and still lead to positive detection after 4 weeks of incubation. Adaptation of this protocol to non-viable resistance forms of the pathogens would require a specific experiment.

Many efforts in the management of *Phytophthora* species are currently into force. Eradication of *P. ramorum* in West US forests and experimental investigations for eradicating *P. cinnamomi* in Australia are the most relevant and discussed actions in the forest environment (Kanaskie *et al.*, 2011; Crone *et al.*, 2014), while cultivation practices aimed at controlling the spread of these pathogens are constantly carried out in nurseries worldwide. The study reported hereby demonstrated that the risk of diagnostic errors (false positives) caused by detection of dead DNA by qPCR in soil/sand samples can be reduced by applying a sound lab protocol, a result that justifies the recommendation of this method for detection of Phytophthoras in commercial plant stocks from nurseries and in areas managed for *Phytophthora* eradication. The TaqMan assay developed in this thesis is a valid and safe diagnostic tool more efficient (i.e. faster and less subject to false negatives) than traditional isolation techniques, whose suitability for inspection of plant consignments and control of eradication measures has been already questioned (Migliorini *et al.*, 2015; Dunstan *et al.*, 2010).

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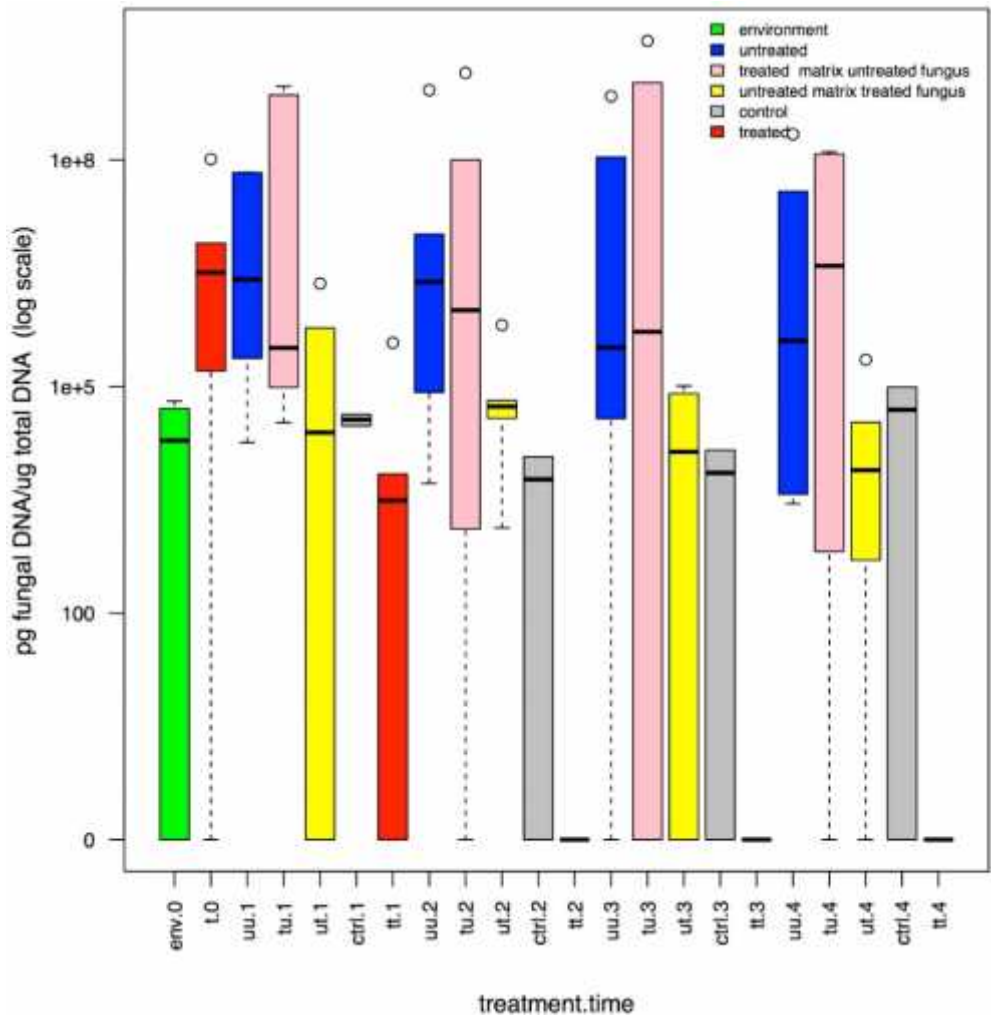
Phytophthora DNA quantities (pg of *Phytophthora* DNA on µg of total DNA; horizontal line = median, box = 1st–3rd quartiles, whisker = min-max, dots = outliers and extremes), estimated by qPCR with a specific MGB TaqMan probe in the tested theses. The four theses are illustrated by codes in the X-axis: env0 refers to sampling at time zero and shows the assay of soil short after collection from the field; uu (untreated matrix/untreated inoculum), tu (treated matrix/untreated inoculum), ut (untreated matrix/treated inoculum) and tt (treated matrix/treated inoculum) are the four theses plus the control (ctrl, mock-inoculated untreated matrices). Numbers following the treatment codes on the x-axis refer to the week (1 to 4) after treatment when the samples were assayed by qPCR. t0 indicates *Phytophthora* mycelia directly assayed after heat treatment.

Tables
Table1

Thesis	Matrix	Inoculum	Species		
uu	untreated	untreated	*P. citrophthora* *P. nicotianae* *P. ramorum*		
ut		treated			
tu	treated	untreated			
tt		treated			
ctrl	untreated	mock-inoculum			

Experimental design. Three *Phytophthora* species (*P. nicotianae*, *P. citrophthora*, *P. ramorum*) were inoculated in two matrices, river sand and forest organic soil. Treated (non viable) and untreated (viable) matrices were inoculated with either treated (non viable) or untreated (viable) *Phytophthora* inoculum. Controls (ctrl) consisted of mock-inoculated untreated matrices.

Real Time PCR detection of airborne *Phytophthora* species in a plant nursery area

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Summary

The presence of airborne *Phytophthora* propagula was studied by mean of a specific qPCR assay in samples collected weekly from May to December 2012 with a Hirst-type spore trap placed at 35 m above ground level in one of the largest poles of woody ornamental nursery in Europe. Airborne DNA was amplified by two MGB qPCR TaqMan assays: a generic probe, previously developed to detect *Phytophthora* spp. in environmental samples, and a second probe designed to specifically detect *P. ramorum* and *P. lateralis,* two invasive pathogens threatening natural and artificial ecosystems in the Northern Hemisphere.
P. ramorum and *P. lateralis* were not detected in the analyzed samples, while all the samples tested positive to the general TaqMan® MGB assay for the genus *Phytophthora*.
Presence and quantity of *Phytophthora* DNA was correlated with meteorological data. *Phytophthora* showed two main peaks of sporulation, the first in late spring and the second in autumn. Occurrence of extremely high quantities of *Phytophthora* DNA in the air was preceded by rain in both in spring and in autumn. In both cases, the 10-day period preceding the highest values was characterized by relative humidity above 75%, and mean temperature around 13°C.
From mid-May to the end of August, the daily mean quantity of *Phytophthora* detected in the air increased with relative humidity, while it decreased with increasing mean temperature. The effect of rain in the same period was positive and close to significance. From the end of August to December there was no significant correlation between meteorological variables and aerial DNA quantity of *Phytophthora* species.
Our results are in agreement with those obtained by other Authors with different diagnostic methods, but the approach here used enables much faster and more precise detection and quantification of the target organisms. Our results also suggest that TaqMan® MGB assays could be applied to study the phenology of spore release in many airborne plant pathogens of woody plants and agricultural crops, thus implementing disease management strategies.

Introduction

Propagules of fungi and fungal-like organisms are commonly dispersed in the air causing infections and allergic reactions (Lacey, 1996). Aerial sampling of airborne spores in conjunction with molecular diagnostic techniques can be used for early and fast detection of plant pathogens. However, aerial sampling has been rarely applied to forest pathogens, although the method would deserve attention and development especially for early detection of microorganisms that may cause huge economic losses, such as *Phytophthora* species.

The genus *Phytophthora* counts many serious and invasive plant pathogens (Erwin and Ribeiro, 1996; Agrios, 2005) whose worldwide spread in the past few centuries provoked severe epidemical outbreaks in

agricultural crops (Andrivon, 1996; Bourke, 1964;) and forest trees (Dell and Malajczuk, 1989; Rizzo and Garbelotto, 2003). The long-distance dispersal of these fungal-like organisms is largely human mediated and linked to the trade of plants for planting (Migliorini *et al.*, 2015; Goss *et al.*, 2011; Moralejo *et al.*, 2009). Their presence in commercial nurseries is widespread (Jung *et al.*, 2015; Pérez Sierra and Jung, 2013).

For natural dispersal, *Phytophthora* species that infect roots mainly depend on transport of free-swimming zoospores in water and are considered soil-borne pathogens. However, some soil-borne *Phytophthora* species, such as *P. ramorum*, *P. kernoviae*, *P. palmivora*, *P. lateralis*, and *P. austrocedrae,* may colonize aboveground organs, form sporangia on the surface and release inoculum to the air, directly or through water splashes. In *P. ramorum*, caducous sporangia on infected leaves or twigs are locally splash-dispersed or spread by wind and wind-driven rain, even over long distances depending upon environmental conditions (Davidson *et al.*, 2005; Moralejo *et al.*, 2006). Sporangia of *P. kernoviae* are caducous and may be locally dispersed in the air, as shown by occurrence of new infections on trap plants in a diseased *Rhodondendron* stand (Denman *et al.*, 2009), and by disease transmission from infected *Rhodondendron* leaves onto nearby stems of *Fagus sylvatica* (Brasier *et al.*, 2004). *P. kernoviae* has also been recovered from symptomatic needles of *P. radiata* (Dick *et al.*, 2014). *P. palmivora*, a formidable pathogen in the tropics with more than 150 known plant hosts (Farr and Rossman, 2015), which is commonly detected as a root-rot agent on nursery plants in Europe, may form caducous sporangia on fruits, leaves and stems and release inoculum to the air through rain splash or wind-blown rain (Hunter and Kunimoto, 1974). *P. lateralis*, which is known as a root pathogen forming noncaducous sporangia, was recently reported to cause crown infections on *Chamaecyparis lawsoniana* in the UK and France (Robin *et al.*, 2011; Green *et al.,* 2013) and on *C. obtusa* in Taiwan (Webber *et al.,* 2012). Similarly, *P. austrocedrae*, a species of unknown origin that attacks roots and forms phloem lesions into the lower stem causing widespread mortality of *Austrocedrus chilensis* in Patagonia (Greslebin *et al.*, 2007), has been observed to causes lesions on branches of *Juniperus communis* with no connection to the base of the tree up to 5 m height in UK (Green *et al.*, 2012, 2014).

Other *Phytophthora* species, such as *P. pluvialis* (the agent of red needle cast disease) (Dick *et al.*, 2014) and *P. pinifolia* (the agent of a disease referred to as 'Daño Foliar del Pino' (DFP) (Ahumada *et al.*, 2013) on *Pinus radiata,* typically infect aerial plant parts and are considered air-borne pathogens. Sporangia of *P. pinifolia* are mainly produced during the growing season but may remain on the foliage throughout the year (Durán *et al.*, 2008). Intense aerial sporulation from needles is the primary cause of diseases symptoms in plants infected by these *Phytophthora* species.

Studies on air dispersal of *Phytophthora* diseases of woody plants are limited and have mainly focused on efficacy of spread from infected leaves to neighboring plants and the effect of environmental factors on dispersal (for instance Denman *et al.*, 2009; Davidson *et al.*, 2005). Air dispersion and related aspects of the life cycle, e.g. haustoria development from airborne propagules landed on the leaf surface and leaf penetration (Hohol and Suter, 1976), have been studied in detail in *Phytophthora infestans*, a worldwide pathogen of important crops which typically causes foliar necrotic lesions surrounded by sporangia. In *P. infestans*, release and viability of sporangia are associated to climate and weather conditions. Sporangia are massively formed in lesions in overcast or rainy weather, while subsequent sunny and windy weather triggers spore liberation (Nielsen *et al.*, 2007). Dispersal efficiency is limited by direct exposure to sunlight, which drastically reduce viability and germination of sporangia within minutes (Mizubuti *et al.*, 2000; Sunseri *et al.*, 2002). Sporangia are released in the early morning (Aylor *et al.*, 2001), may be picked up in the atmosphere by turbulent airflow and transported, still viable, to distant locations and great height above ground level (Skelsey *et al.*, 2009; Techy *et al.*, 2010, Aylor *et al.*, 2011).

Aerial dispersal of Phytophthoras has been generally studied by collecting aerial parts from host plants, naturally growing or placed (plant baits), in the neighborhoods of symptomatic plants, (Denmann *et al.*, 2009), or by placing plates, filters or spore samplers in the canopy drip of infected plants or in the open air (Manzano *et al.*, 2015; Reeser *et al.*, 2011; Aylor *et al.*, 2001). Taxonomical assignment and quantification of

air-borne *Phytophthora* inoculum, which is crucial to estimate dispersal intensity, were based on microscopic identification, counts of sporangia and assessment of mycelial growth. These methods are time consuming and demand extensive competence in classical taxonomy.

Many new diagnostic tools are becoming available to detect plant pathogens before host infection (Fang and Ramasamy, 2015; Martinelli *et al.*, 2015; Sails and Tang, 2015). An attractive means to detect and quantify the target air-borne pathogen in a precise and faster way is the use of an active spore-trap combined with a real-time quantitative PCR (qPCR) assay designed on specific regions of DNA. Quantitative PCR protocols are commonly employed in aerobiology instead of time-demanding isolation methods. In recent years, qPCR assays have been developed and efficiently applied to detection and quantification of airborne inoculum of crop pathogens, such as, for instance, *Mycosphaerella graminicola* (Carisse *et al.*, 2009; Duvivier *et al*., 2013), *Sclerotinia sclerotiorum* (Roger *et al.,* 2009; Almquist and Wallenhammar, 2015), *Peronospora* spp. (Klosterman *et al.,* 2014), *Rhynchosporium secalis* (Fountaine *et al.,* 2010), *Botrytis squamosa* (Duvivier *et.al.,* 2010), and of a few fungal diseases of trees like *Fusarium circinatum* (Garbelotto *et al.*, 2008), *Ceratocystis platani* (Luchi *et al.,* 2013) and *Venturia inaequalis* (Meitz-Hopkins *et al.,* 2014).

Phytophthoras are typically generalist pathogens that may potentially spread from plants in nurseries to trees and shrubs in the natural environment (Vettraino *et al.*, 2002, 2008; Brasier, 2004; Perlerou *et al.,* 2010). The aim of this study was to use qPCR diagnostics to assess occurrence and seasonal variation of air-dispersed inoculum of *Phytophthora* species in one of the most important ornamental nursery poles in Europe.
Besides employing a genus-specific qPCR assay recently described (Migliorini *et al.*, 2015), we developed and used a new assay designed to uniquely detect air borne inoculum of *P. lateralis* and *P. ramorum*. Both species are potentially invasive and recommended for regulation as quarantine pests by the European and Mediterranean Plant Protection Organization (EPPO Reporting Service 2011, 2013). *P. ramorum*, which has been repeatedly detected in nurseries in Italy (Gullino *et al.*, 2003; Ginetti *et al.*, 2014), is the object of emergency measures taken by EU Member States to prevent spread of the pathogen in Europe (EU 2002, 2004, 2007).

Materials and methods

Sample collection and preparation
Aerobiological samples were collected from May to December 2012 with a Hirst-type spore trap (VPPS, 2000; Lanzoni, Bologna, Italy) placed on top of a building 35 m above ground level in Pistoia (Italy), in the middle of an area that is one of the biggest European poles of production and commerce of ornamental nursery plants. This sampler type enables continuous monitoring of airborne spore concentration. Air samples are collected in a vacuum pump (air flux set to 10 l/min) and fungal spores, as well as other particles, are deposited by impaction on a polyester Melinex tape coated with silicon. A tape 345 mm long (48 mm/day x 7 + 9 mm for mounting the tape) corresponds to seven days. Every week the tape was removed from the instrument and transported to the laboratory. Each tape was cut in a sterile laminar flow in pieces corresponding each to 24-hour harvesting of air-dispersed organisms.

DNA extraction
DNA was extracted by placing each piece of tape in 10 ml sterile falcons (Sarstedt, Verona, Italy) and vortexing for 5 min with 220 µl of NONIDET (0,1%) plus 0,2 gr of Glass beads acid-washed (Sigma-Aldrich, St. Louis, MO, USA) and following the EZNA Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA) manufacture's short protocol.

Real-time PCR assay designed to detect *P. ramorum* and *P. lateralis*

A qPCR assay based on a TaqMan MGB probe specific to *Phytophthora ramorum* and *P. lateralis* DNA was designed with PRIMER EXPRESS Software 3.0 (Applied Biosystems, Forster City, CA, USA) on the internal transcribed spacer 2 (ITS2) region of *Phytophthora ramorum* (KC473522). The upstream and downstream primers were PramF (5'-GCAGGGCTTGGCTTTTGA-3' forward primer) and PramR (5'-GCCGAACCGCCACTCTACT-3 ' reverse primer). The TaqMan[TM] MGB probe (Pram_PR: 5'-TCGACGGTGTTGTGCG-3') was labelled with 6-carboxy-fluorescein (FAM) at the 5' end and a non-fluorescent quencher (NFQ) with minor groove binder (MGB) ligands, at the 3' end. Homology of the *P. ramorum* amplicon sequence with the sequences of other species in the NCBI database was performed using standard nucleotide–nucleotide BLAST (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The qPCR assay was tested on DNA from axenic cultures of 15 *Phytophthora* species belonging to six genetic clades (Kroon *et al.*, 2012) (*P. alni, P. cambivora, P. cinnamomi, P. citrophthora, P. cactorum, P. citricola, P. cryptogea, P. europaea, P. gonapodyides, P. lateralis, P. nicotianae, P. palmivora, P. quercina, P. ramorum* and *P. syringae*). Multiple isolates per species were tested. The possible cross-reaction of the qPCR assay with DNA of closely related (*Pythium* spp.) and unrelated (*Fusarium* sp., *Mortierella* sp.) species, which are common in nurseries, was also tested. All isolates tested belong to the culture collection of the IPSP-CNR (Sesto Fiorentino, Italy).

Quantitative real time PCR detection of *Phytophthora* species
Aerial DNA of *Phytophthora* species was detected and quantified by the real time PCR (qPCR) TaqMan® MGB assay specifically developed in this paper for *P. ramorum* and *P. lateralis* and by the TaqMan® MGB assay for the genus *Phytophthora* described in Migliorini *et al.* (2015).
Groups of 24-hour DNA samples were pooled together to obtain twenty 10-day DNA samples (May - December) (Tab.1). In periods of time when *Phytophthora* DNA was especially abundant, 24-hour samples were processed separately. All samples were assayed in MicroAmp Fast 96-well Reaction Plate (0.1mL) closed with Optical Adhesive by means of the StepOnePlus[TM] Real Time PCR System (Applied Biosystems, Life Science, Foster City, CA). PCR reactions were performed in 12.5 µl final volumes containing: 6.25 µl TaqMan universal master mix (Applied Biosystems), 300 nM forward primer (Eurofins MWG Operon, Ebersberg, Germany), 300 nM reverse primer (Eurofins MWG Operon), and 200 nM TaqMan MGB probe (Applied Biosystems), 2.5 µl template genomic DNA per tube. Each DNA sample was assayed in two replicates. Two wells containing 2.5µl of sterile water each were used as the no-template control (NTC). The PCR protocol was 50°C (2 min), 95°C (10 min), 50 cycles of 95°C (30 s), and 60°C (1 min). Results were analyzed using the StepOne[TM] Software (Applied Biosystems) after manual adjustment of the baseline and fluorescence threshold. *Phytophthora* DNA in unknown samples was quantified by using TaqMan MGB probe as reported in Migliorini *et al.* (2015), while quantification of *P. ramorum* and *P. lateralis* was made with the new and specific TaqMan MGB probe. The standard curve was generated with a *P. ramorum* DNA standard (sample Pram1 from IPSP-CNR collection) from seven five-fold serial dilutions (ranging from 20 to1.28 x10^{-3} ng tube^{-1}) of a known concentration of *P. ramorum* DNA and analyzed in triplicate. Reproducibility of the qPCR assay was assessed by computing the coefficient of variation (CV) among the mean values in eight independent assays. PCR efficiency was calculated on the slope of the standard curve (Eff = $10^{-1/slope}$-1) (Bustin et al., 2009), from eight independent experiments.
Statistical analyses
Kruskal–Wallis test was applied to identify significant differences in *Phytophthora* DNA quantity estimated by qPCR, between 10-days periods. Pearson correlation analysis was performed between mean daily values of meteorological variables and mean daily *Phytophthora* DNA quantities in 10-days periods. Meteorological data [rain (mm), maximum, minimum and average temperature (°C), and relative humidity (RH %)] were provided by the Experimental Centre for Nursery Gardening (CeSpeVi, http://www.cespevi.it).
Statistical analyses were performed in R (R Core Team 2015). Boxplots were produced with the GPLOTS package in R (http://cran.r- project.org/web/packages/gplots/gplots.pdf).

Results

Real-time PCR assay designed to detect *P. ramorum* and *P. lateralis*

BLAST searches in NCBI showed 100% homology between the designed amplicon sequence and the sequences of *P. ramorum* and *P. lateralis* deposited in GenBank. No homology was found with sequences of Pythium or fungal species in GenBank. The qPCR assay amplified DNA from isolates of *P. ramorum* and *P. lateralis*, while it did not amplified DNA from isolates of other *Phytophthora* or fungal (Pythium spp. Fusarium spp., Mortierella spp.) species tested as controls for specificity.

The standard curve had slope of 3.48, square correlation coefficient (R^2) of 0.998, and Y-intercept of 29.8. Efficiency of the PCR reactions was 0.94 ± 0.07 (SE). Reproducibility of the standard curve was evaluated for each standard dilution on the basis of Ct values. The CV obtained from seven different standard curves ranged from 0.7 to 1.6%.

Detection and quantification of *Phytophthora* species in air samples by qPCR

All samples tested negative to the TaqMan® MGB assay designed to selectively detect *P. ramorum* and *P. lateralis*, while they all tested positive to the general TaqMan® MGB assay for the genus *Phytophthora*. The daily quantity of DNA of *Phytophthora* species in the air varied with a seasonal pattern (Fig. 1) and there were significant differences between 10-day time periods (Kruskal-Wallis chi-squared = 26.81, df = 7, p-value = 0.00036). In spring, from May to mid June, the daily mean quantities of *Phytophthora* DNA were above the median value, which is a robust estimate of the overall central tendency value. The concentration of *Phytophthora* DNA in the air decreased significantly in the end of June (Kruskal-Wallis chi-squared = 19.133, df = 1, p-value = 1.219e-05), further diminished later on until September, and slightly increased in autumn. Day-to-day variations were generally large, especially in the peak period in spring, with extreme values up to 30 times higher than the average. In addition, an extremely high value (20-fold the average of the period) occurred in the first half of November. After that, the aerial inoculum of *Phytophthora* species dropped to values similar to those detected in August.

Effect of climatic variables on *Phytophthora* inoculum in the air

Occurrence of extremely high quantities of Phytophthora DNA in the air was preceded by rain (Fig. 2A) both in spring (about 50 mm two days before the highest peak) and in autumn (about 40 mm two days before the peak). In both cases, the 10-day period preceding the highest values was characterized by relative humidity above 75% (Fig. 2B), and mean temperature around 13°C (±1.03°C and ±2.04 °C SD, respectively) (Fig. 2). Average maximum (around 18.5°C) and minimum (around 8°C) temperatures were also very similar. Excluding the extreme values of DNA, there were significant correlations between climatic variables and aerial concentration of *Phytophthora* DNA. From mid-May to the end of August, the daily mean quantity of *Phytophthora* detected in the air increased with relative humidity (r=0.72, p=0.038), while it decreased with increasing mean temperature (r=0.92, p=0.001). The effect of rain in the same period was positive and close to significance (r=0.61, p=0.092).

From the end of August to December there was no significant correlation between meteorological variables and aerial DNA quantity of *Phytophthora* species. After the peak in November, the aerial inoculum quickly diminished and remained as low as it was in summer despite abundant rain and high relative humidity (Fig. 2A, 2B), when maximum temperatures fell below 10° and minimum temperatures reached below zero values (Fig. 2C, 2D).

Discussion and Conclusions

In the present study, applying a qPCR TaqMan assay to samples collected by a Hirst-type volumetric spore trap, we observed long-distance vertical and horizontal air transport of *Phytophthora* species in an area intensively cultivated with ornamental woody plants. Up to date, air dispersal of *Phytophthora* species in field conditions/outdoors has been quantified with classical detection methods based on counts of sporangia (Lima *et al.*, 2009; Manzano *et al.*, 2015), a technique that is time consuming, requires competence in morphological taxonomy, and is limited by the uncertainty of classifying spores visually if not supplemented by growth of colonies. Among the few examples in the literature, aerial traps combined with molecular methods were applied to detect and quantify the aerial inoculum of *Fusarium circinatum*

(Garbelotto *et al.*, 2008) and *Ceratocystis platani* (Luchi et al. 2013). Very recently, Fall and collaborators (2015) have developed a qPCR TaqMan assay for detecting *P. infestans*, which was tested in lab conditions but not yet in the field.

P. ramorum, the agent of Sudden Oak Death (SOD) disease, is signaled in 14 European countries, mainly as pathogen of nurseries and ornamentals and has recently out-broken in plantations of Japanese larch and of many tree species in Britain and Northern Ireland and It has been isolated in Norwegian natural Bilberry plants, (Brasier and Webber, 2010; Webber *et al.*, 2010; Herrero *et al.*, 2011; Santini *et al.*, 2013). Similarly, less incisive (and less studied) *P. lateralis* was the cause of crown infections on *Chamaecyparis lawsoniana* in the UK and France (Robin *et al.*, 2011; Green *et al.*, 2013). Epidemiological traits of the two pathogens had been investigated by the description of infection strategies, including the attitude to cause aerial infections, but no evidence of potential over canopy aerial diffusion mechanisms in the infected area has been well screened. In this paper, *P. ramorum* and *P. lateralis* were not detected in the air by the specific TaqMan probe here presented. The sensitivity of this assay is high enough to affirm that none of these pathogens was present in the air flux intercepted by the trap since the assay is able to detect extremely small quantities of DNA of both species (the lower detection limit of the standard curve was 0.512 pg μl^{-1}). In Italy, *P. ramorum* has been seldom reported in nurseries (Gullino *et al.*, 2003; Ginetti *et al.*, 2014). Ginetti *et al.* (2014) detected *P. ramorum* during spring 2013, in the same nursery area of our experiment and only one year before. Our findings enrich the results of these Authors by excluding that inoculum of *P. ramorum* was present at a greater height above ground level, suggesting that the diffusion of this pathogen previously observed was not due to wind-dispersed sporangia. The TaqMan probe quantification method presented in this paper may be considered for future investigations in the instance of new local outbreaks of *P. ramorum* in Italy or other European countries.

On the other hand, the genus-specific TaqMan assay applied in this study detected propagules of *Phytophthora spp.* in the air in the whole sampling period that covered a large part of the year, i.e. from May to December. The trap was located in an area where nurseries of ornamental plants occupy most of the agricultural land and are largely surrounded by forests and wood plantations. Consequently, it would be unlikely that the detected *Phytophthora* DNA has originated from agricultural crops. Based on these considerations, our paper is the first published report on aerial sampling of *Phytophthora* species that were not released from typical host crops cultivated in the surroundings. To the best of our knowledge, the literature reports aerial detection of wind-transported sporangia of *Phytophthora* even at 90 meters AGL, but only from source fields of infected agricultural crops located in the proximity of the sampling sites (Techy *et al.,* 2010; Aylor *et al.*, 2011; Manzano *et al.*, 2015).

Based on sporangia standard curves generated by testing serial dilutions of sporangia DNA extractions with a TaqMan assay for *P. infestans* (Fall *et al.*, 2015), the quantities of *Phytophthora* DNA detected in the air in the present study could be roughly converted in airborne sporangia concentrations. The daily number of sporangia of *Phytophthora* species detected in the air at 35 m AGL in our survey was on average below 0.1 m^{-3}, and greater than 1 m^{-3} only on the two days of maximum concentration, i.e. in the first week of May (~7.0 sporangia m^{-3}) and in the second week of November (~1.2 sporangia m^{-3}). In their recent study in Spain, Manzano and collaborators (2015) counted on average 0.3-0.9 sporangia m^{-3} belonging to *Phytophthora* species or other Peronosporales with similar propagules, in air samples collected with a Hirst-type spore trap at 1.5 m above ground. In the same study, peak concentrations ranged from 7.2 to 23.4 sporangia m^{-3}. Higher concentrations than in our study are obviously explained both by inclusion of sporangia of *Phytophthora*-like organisms and sampling in proximity of the ground. Negative vertical gradients in the concentration of airborne spores have been long known for fungal species (Gregory and Hirst, 1957; Lacey 1996). In the case of *P. infestans*, sporangia concentrations measured with UAVs in the atmosphere up to 90 m above and at downwind distances up to 500 m from an infected potato field ranged between 0.01 to 9.84 sporangia m^{-3} (Aylor *et al.*, 2011).

As shown for *P. infestans* on potato, disease severity increases linearly as the atmospheric content of sporangia increases. The concentrations of *Phytophthora* sporangia inferred in our study on the peak days may be sufficient for causing disease with significant severity on foliage. In a lab experiment including multiple *P. infestans* lineages, a single sporangium m^{-3} was sufficient to cause a severity of 1%, and 6 sporangia m^{-3} were sufficient to reach 10% leaf area diseased in potato (Fall *et al.*, 2015).

The aerial inoculum of *P*hytophthoras greatly increased in days that closely followed significant raining events, suggesting that sporangia dispersion was linked to weather trend in the short (days) period. High humidity and high oxygen and low carbon dioxide concentrations are also required for sporulation in Phytophthora (Ribeiro, 1983). Such requirements favor dispersal by encouraging sporulation in conditions that retard desiccation.

After a peak of detection in November, the pathogens quickly dropped to mid-summer levels, opposite to the trend of rainfall. The corresponding decrease of temperatures was reasonably the cause. During summer, high temperatures stopped the growth of the pathogens. Among the air-dispersed species mentioned above in this paper, only *P. palmivora* is able to grow over 30 °C (Table 1). Hence, the long period with maximum temperatures above this threshold might have been the cause of the observed rapid decrease of inoculum from early June to mid-September.

Phytophthora inoculum decreased in early summer with relative humidity, once this meteorological variable was decreased both by disappearing of rain and rising of air temperature. Differently in September, with a progressive increase of relative humidity due to both increasing rainfall and, possibly to a greater extent, decrease of air temperature, *Phytophthora* DNA was not detected with corresponding quantities, i.e. at similar values of relative humidity between early and late summer corresponded different quantities of *Phytophthora* species DNA. Thus the variable is only a partial predictor of sporulation and interesting, even if its value increased because of diminishing air temperatures and occurrence of occasional raining events, sporangia were not released (or even produced), suggesting that the aerial sporulation phase in the life cycle of *Phytophthora* species is driven by repeated rainfalls, provided that temperature remains in a precise range. In accordance with our results, Manzano *et al.* (2015) found that airborne Phytophthora-type sporangia exhibited strong dependence on rain and that this correlation is valid within a short temporal range (weekly). Fall *et al.* (2014) did not find the same correlation for *P. infestans* as their study was conducted in New Brunswick where rainfall was substantially uniform during the whole summer.

Temperature effects and time variation in sporangia production/sporulation on host tissues have been reviewed in Tooley *et al.* (2015). On potato, *P. infestans* was found to produce sporangia within an optimum temperature range of 18-22°C (Crosier, 1934), while 23°C was the optimum for sporulation on Petunia (Becktell *et al.,* 2005). Sporangia appeared after 6-8 hours of favorable conditions. The optimum temperature for sporulation was 24°C both for *P. palmivora* (Timmer *et al.* 2010) and *P. citrophthora* (Gerlach *et al.*, 1976). For *P. cactorum* the optimum range was 12.5-27.5°C (Grove *et al.*, 1985). As regard to the effect of water, in *P. palmivora* and *P. nicotianae* maximum production of sporangia was observed after 72 hours of wetness conditions on fruit surface (Timmer *et al.*, 2000; Timmer *et al.*, 2010). Goth and Wester (1963) observed that sporangia production occurred within 24 h at 30°C and 90–100% relative humidity when lima beans were inoculated with corn seeds infected with *P. phaseoli*. Trujillo (1965) reported that *P. colocasiae* could produce sporangia on taro with a wetting period as short as 2–3h. In *P. ramorum,* the best temperatures for formation of sporangia on rhododendron 'Cunningham's White' leaves artificially infected were 15°C and 20 °C, and sporangia were formed significantly earlier (by 3.24 and 1.49 days) when leaves where prior placed at the optimum temperature for 24 and 72 h, respectively. This mechanism was confirmed for other tested suboptimal temperatures, i.e. 4, 10, 25 and 30°C, demonstrating the influence of a pre-incubation period on production of sporangia (Tooley *et al.*, 2015).

In some *Phytophthora* species, as shown by the literature, production, release and wind-dispersal of sporangia is a phenomenon influenced by several variables but, as reported by Erwin and Ribeiro in 1996, it

is the balance between these environmental factors that appears to govern whether or not high numbers of sporangia are released. By using an *in lab* approach, the cited Authors could demonstrate just the partial effect of individual factors, and consequently the description of a general mechanism can be only hypothesized. Our results are in agreement with those obtained by other Authors with different diagnostic methods, but the approach here suggested enables a much faster and more precise detection and quantification of the target organisms. Our results also suggest that TaqMan® MGB assays might be applied to monitor and investigate the spore release phenology of many airborne plant pathogens of woody plants and agricultural crops, thus implementing disease management strategies.

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Figure captions
Figure 1
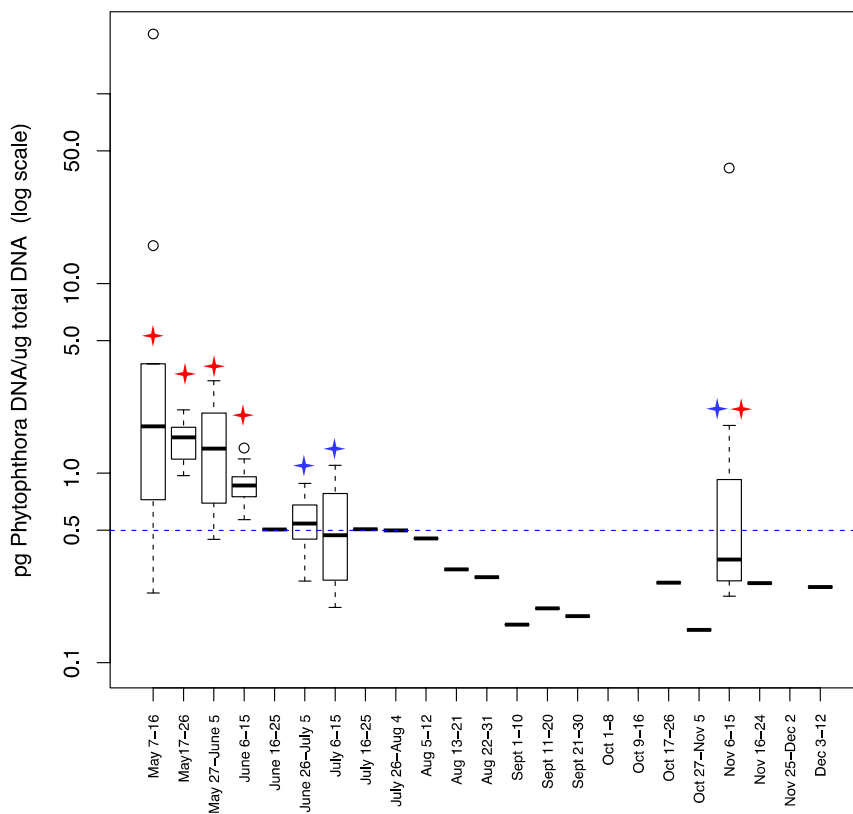
Phytophthora DNA quantities (horizontal line = median, box = 1st–3rd quartiles, whisker = min.-max., dots = outliers and extremes; logarithmic scale), estimated by *Phytophthora* genus specific MGB TaqMan probe during the period May-December 2012. Similar symbols indicate statistically non-different values (see M&M and Results for details).

Figure 2
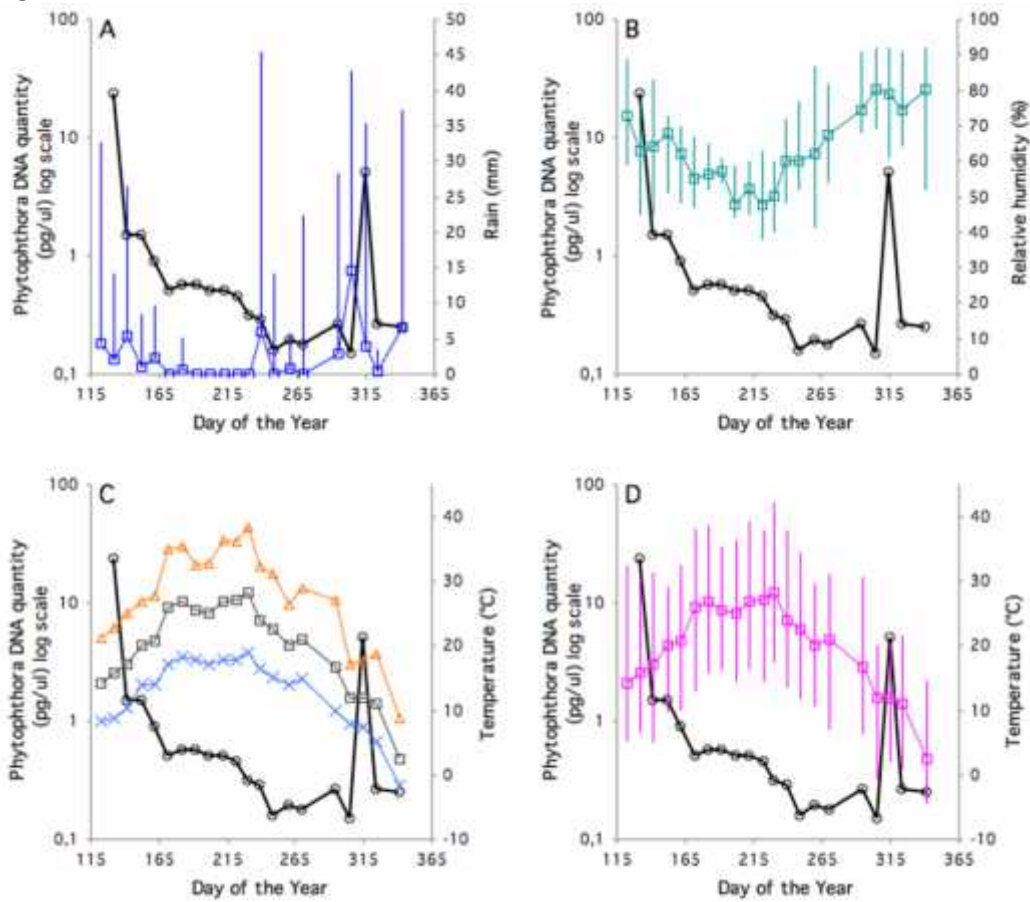
Seasonal variation in the DNA quantity (pg/µl) of *Phytophthora* species shown with meteorological variables, i.e Rain (mm, A); relative humidity (B); maximum, minimum and mean air temperature (C); and extremes of air temperature (D).

Tables

Table 1

Species	Min. temp.(°C)	Opt. temp.(°C)	Max. temp.(°C)	Reference
P. palmivora	11	27.5-30	35	Erwin and Ribeiro (1996)
P. pinifolia	10	25	30	Hansen (2012)
P. ramorum	2	20	26-30	Parke and Rizzo (2011)
P. infestans	4	20	26	Erwin and Ribeiro (1996)
P. kernoviae	-	18	26	Dick and Parke (2012)
P. lateralis	<10	20	ca. 25	Hansen (2011)
P. ilicis	5	20	25	Erwin and Ribeiro (1996)
P. pluvialis	<5	15-20	25	Reeser *et al.* (2013)
P. austrocedrae	10	17.5	20	Greslebin *et al.* (2007)

Minimum, maximum and optimal growing temperatures of the *Phytophthora* species mentioned in this study, which are characterized by an aerial dispersion phase or are considered aerial species due to a strong attitude to cause lesions in above ground organs of host plants.

Phytophthora distribution, taxonomical diversity and pathogenicity in a hyper diverse bushland hotspot in a dune system in the South West region of Western Australia

Summary

This work is part of a wide study project about the ecology of bushland biodiversity hotspots in the South West region of Western Australia (SWWA), coordinated by several research groups from different Universities. The aim is to analyze factors involved in determining the extremely high number of plant species stably present in these natural environments. The scientific bases for the investigations have been well explained by Laliberté *et al.* (2015) who reviewed the current knowledge on this argument, proposed a few explanatory hypotheses, and defined the guidelines for future investigations. This introduction is based on that article and the works cited therein. Concepts reported hereafter with no references were derived from scientific works available in the review's bibliography. Here I have extracted and summarized some aspects in order to introduce the lab activity carried out at the University of Western Australia in Perth during the second year of my PhD. My work consisted in understanding the role of soil borne pathogenic *Phytophthora* species in influencing/determining the high botanical diversity observed in a local natural environment. The study comprises two investigations: a) soil samples from a dune system that represents a geological chronosequence were analyzed by 454 sequencing in order to describe *Phytophthora* species diversity across a natural environmental gradient; b) the pathogenicity of two important pathogens with wide host range, *P. multivora* and *P. cinnamomi*, on woody plant species common in SWWA was evaluated by means of a glasshouse trial.

A link between geological age of the dunes and *Phytophthora* species diversity was suggested by the results of the sequencing experiment (a). The results of the greenhouse experiment (b) besides confirming that *P. cinnamomi* is a more severe pathogen than *P. multivora*, as observed in field conditions, also showed a different degree of susceptibility between plant families and within plant genera.

Introduction

A plant biodiversity hotspot is a community of trees and woody shrubs with a high degree of taxonomical diversification.

The object of the present study is a natural formation, located in southwestern Australia, known as "kwongan", and characterized by up to 40 shrub species as mature individuals in 0.01 ha. This ecosystem type has worldwide distribution and is recorded in lowland tropical rainforests and seasonally-dry shrubland ecosystems, its homologous in South Africa is "fynbos".

The investigations included in this project are oriented to analyze mainly dry shrublands with priority to kwongan as more studies have been conducted on it, but many elements from lowland tropical rainforest are often evaluated and compared. A great interest is to clarify which mechanisms are at the origin of such a hyperdiverse community that includes several climax plants and, considering the similar final effect, how they can operate in two different kinds of climatic environments. Apparently the two ecosystems have little in common but a few key aspects of similarity are crucial for the hypothesis developed within the cited review.

The soil of both ecosystems is old and very humid. Precipitations in shrublands, even if concentrated in the few months of the rainy season, are abundant. This brings to nutrient impoverishment, in particular to a low phosphorus (P) availability due to depletion from soil profiles, and, as a consequence to limit plant productivity. Kwongan appears to be one of the most extreme cases of P-impoverished ecosystems in Australia and, possibly, the world. On the contrary, regions with a Mediterranean-like climate with a larger temporal distribution of annual rains such as California, Chile and Mediterranean Basin have more fertile

soils and support a less diverse Mediterranean shrublands. Nitrogen (N) and Phosphorus are the most common limiting or co-limiting plant growth nutrients. While Nitrogen is generally considered to be the key limiting nutrient in temperate, boreal and arctic ecosystems, phosphorus is the nutrient thought to most strongly limit plant growth in lowland tropical rainforests and many seasonally dry shrublands. In an overview of nutritive elements in a two million years old dune chronosequence in SWWA, where kwongan has a relevant extension, phosphorus presence and availability was screened throughout the geological formation in the coast area (Jurien Bay dune chronosequence, northern part of the Swan Coastal Plain marine / dune systems, south of Western Australia (Lalibertè *et al.*, 2012)), which includes six well-defined age stages, by chemical analysis of soil samples and leaves of native woody plants growing in site. Total phosphorus (P) declined continuously along the chronosequence to extremely low levels after 2 million years of pedogenesis, eventually representing some of the poorest soils at global level in phosphorus content. In contrast Organic carbon and nitrogen (N) accumulated rapidly to maximum amounts in intermediate-aged Holocene (<6,500 years old) dunes and then declined as soils age. Ratios of soil organic carbon to P and N to P increased continuously along the chronosequence, consistent with a shift from N limitation on young soils to extreme P limitation on old soils. Phosphorus fractionation by sequential extraction revealed a rapid decline in primary and non-occluded phosphate and an increase in organic and occluded P as soils age. Concentrations of extractable (bioavailable) N and P, as well as exchangeable cations, were greatest in Holocene dunes and declined to low levels in Pleistocene dunes (ca 120,000–2,000,000 years old) (Turner *et al.*, 2015). Working on plant nutrient uptake in the same sampling sites, Hayes *et al.* (2014), in solid relation with results by Tuner *et al.* (2015), described the community-wide variation in leaf nutrient concentrations and resorption that was consistent with a shift from N to P limitation during the long-term ecosystem development. The range in leaf P for individual species along the chronosequence was exceptionally large for both green and senesced leaves. From the youngest to the oldest soil, cover-weighted mean leaf P declined while P-resorption efficiency increased from 0% to 79%. All species converged towards a highly conservative P-use strategy on the oldest soils. Data are summarized in Table 1.

It is logical to suppose that plant communities in kwongan needed to develop adaptation to variable availability of nutrients especially to front the poor mobility of P. Experimental and field observations on plants growing in soil with P limitations proved how root morphological and architectural traits are important for the acquisition of this macro-element.

Hakea prostrata (*Proteaceae*), a common species in P-impoverished kwongan shrublands, in glasshouse experiments produced very short-living (<3 wk), dense clusters of fine roots that are specialized for efficient P acquisition. Some *Cyperaceae* produce specialized short-lived (<10 d) dauciform roots with very long and dense root hairs. Other species from these ecosystems are expected to function similarly, producing ephemeral, but physiologically active, fine roots to 'mine' P. Short lifespan cluster roots are produced by *Embothriumcoccineum* (*Proteaceae*) from soils high in total P, but low in 'available' P, in southern South America, despite relatively low temperatures (Image 1). *Proteaceae*, essentially Southern Hemisphere, is the most representative cluster roots plant family. All species examined in this family (1600 species in 73 genera, 23 of which with rainforest habitats) produce these root clusters. Almost identical structures have been identified in the pea legume, *Viminaria juncea* and with a much lower density and number of rootlets in *Lupinus* (pea), *Acacia* (Mimosaceae), Casuarinaceae, Myricaceae, Elaeagnaceae, Betulaceae, *Ficus* (Moraceae) and Curcurbitaceae (Lamont, 2003).

Fine roots studied in tropical rain lowforests (more literature is available compared to shrublands), beside the particular anatomy, are characterized by a faster turn over. The diffusion of P is very slow in dry soils and in low pH conditions (older knowgan soils (Hayes *et al.*, 2014) and strongly weathered tropical soil). Consequently, the high diffusion shells (i.e. nutrient-depleted cylinders of soil around roots) have very small (<1 mm) radii: the strategy used consists in enhancing P acquisition by the increase of root (hair) length and thus the volume of soil explored. Because respiration costs for root maintenance can be high relative to the cost of making new fine roots, there would be little advantage in maintaining fine roots for a long period if they rapidly deplete P in their rhizosphere. By contrast, in ecosystems in which N limits productivity,

investing C into more durable, better defended fine roots might be a viable strategy, because mass flow can make a significant contribution to N acquisition. Cluster roots represent an adaptation for *in situ* mining of available P. They are more present in the higher part of root apparatus, where soil P is much concentrated and they are typical for several dry shrubland species, for instance the entire group of Proteaceae. Mycorrhizal symbioses are excluded because their function is substituted by an analogous iper-branched system of fine roots and because the short life of cluster roots is presumably not compatible with penetration by biotrophic fungi. Not many studies are conducted yet on shrublands, but it seems reasonable to suppose that in those P-deficient ecosystems a correlation may exist between plant hyper diversity and cluster roots-no mycorrhizal plant species.

In order to explain the ecological dynamics driving to such a high concentration of many species in restricted areas, soil biology and plant anatomy here discussed are supposed to play a primary role. The aim of this work is to link both aspects in a general hypothesis and, including them within the theory of Negative Density Dependence, proof that some group of soil pathogens are involved in this phenomenon.

The Negative Density Dependence (NDD) is a process by which, as the presence of conspecific neighbor increases, plants show a decrease in performances, promoting coexistence of more species in the same area. More specifically to our topic, the Janzen-Connell is a variant of this general hypothesis in which seed dispersal limits potential recruitment in areas of low adult density far from seed-bearing plants, whereas natural enemies limit realized recruitment in areas of high adult density close to seed-bearing plants. Because of several affinities with the ecological dynamics described for the ecosystems analyzed in this work, a general hypothesis to link this process to the aspects previously reported can be formulated.

NDD mechanisms are variable: intraspecific resource competition, host-specific enemies whose impact increases with the density of the host and temporal variation in environmental attributes that affect regeneration, coupled with a long-lived stage that survives through good and bad times; the final result, in any case, is the occurring of persistence climax plants co vegetation described for lowland raining forest, kwongan and fynbos. Fire effect in kwongan was studied as NDD factor on *Banksia* and *Hakea*, both Proteaceae genera. Seeds recruitment after fire occurs by the over canopy and soil seed bank and germination is limited due of competition with neighbor seeds and re-sprout of parents. Germination is less active on litter than in exposed bared sand, however, host specific pests and pathogens were also proposed as NDD regulator of plants diversity with the role of limiters of seedlings recruitment in areas of high adult density close to seed-bearing plants (Image 2a). Janzen-Connell effect is active in case that plant pathogens (or enemies as insect, vertebrate seed predators and herbivores) have a limited capacity of dispersion and relevant degree of host specialization. Fungal interference is proved to be effective for NDD (Bagchi *et al.*, 2014) but little is known about the contribution of Oomycetes.

Following a significant pathogen-host taxonomical affinity, Oomycete soil borne pathogens in tropical rain forest and, although less investigated, in temperate and subtropical forests, may be the cause of strong density-dependent seedling recruitment failure: soil borne pathogens Janzen-Connell NDD effect (J-C NDD).

If we consider that in many other plant ecosystems like temperate and boreal forests, a host-specific ECM network provides efficient defenses against soil pathogens leading to a positive feedback that promotes mature monodominat stands (Image 2b), Oomycetes figure in a good position to be considered as primary factors in driving the extraordinary high plants diversity found in dry shrublands: their penetration through cluster roots, placed in the higher part of soil where more available P but also the majority of pathogens are located, is not contrasted by mycorrhizae, and a growing number of species within the two main genera *Phytophthora* and *Pythium* are known as dieback or damping-off agents in kwongan host plants.

Two species, *Phytophthora arenaria* and *Phytophthora constricta,* were isolated and described by Rea *et al.* (2011) as putative native species in dry shrublands of SWWA. Both are associated to dead and dying *Banksia* species (Proteaceae) and may be found in the rhizosphere soil associated with such plants. Due to their narrow host range and the dependence of pathogenicity incidence on strong rain events (both confirmed by glass house trials) such species fit really well within the prerequisites for hypothesizing that

Oomycetes have a driving role in the soil pathogen mediated NDD theory. Numerous species in the genus have strong pathogenic attitude but, even considering only those with root infection strategies, host specificity can vary from limited to extended to really broad plant groups (Scott *et al.*, 2013). New native species from Clade 6 were isolated and described in SWWA by Jung et al. (2011b): *Phytophthora gibbosa, P. gregata, P. litoralis* and *P. thermophila* have been recovered from rhizosphere soil of dying plants from four native species (*Banksia grandis, Eucalyptus marginata, Xanthorrhoea gracilis* and *X. preissii*), from *Acacia pycnantha* (endemic to Eastern Australia), from several unidentified species of the genera *Banksia, Eucalyptus, Grevillea, Hakea* and, *Patersonia*, and also from the exotic species *Pinus radiata* used for timber plantations. Disease expression was often low impact or only associated with plant death of scattered individuals. Additional studies in SWWA kwongan elucidated how *P. cinnamomi* and *P. multivora*, introduced the former and putative endemic the latter, interfering in stand mortality, would be capable to drive the taxonomical composition of the plant community. It was estimated that 40% of native plant species in SWWA is susceptible to *P. cinnamomi* (Shearer *et al.*, 2004) and many management efforts are conducted for eradication in natural ecosystems (Dunstan *et al.,* 2010). *P. multivora* was well described for the first time in 2009 (Burgess *et al.*, 2009) reporting the attitude to attack many tree plants (Scott *et al.*, 2009; Scott *et al.*, 2012).

Such generalist pathogens, due to a wide spreading capacity on numerous host species of the same areas, should be supposed to influence plant diversity with a reverse mechanism respect to the J-C NDD effect, triggering exactly opposite dynamics to those which are supposed to act in determining the taxonomical diversification of the plant host community driven by narrow host range Phytophthoras. This other effect from generalist pathogens would trigger instead the loss of diversity in plant communities as a final result. But this effect has not been observed.

P. cinnamomi for instance arrived into, native plant communities since the early 1900s, impacted with irreversible effects on plant ecosystems but considerable variation of susceptibility among and within families of threatened flora show how classification within family and genus are poor predictors of species susceptibility. Within apparently susceptible plant species, individuals are resistant to *P. cinnamomi* infection (Shearer *et al.*, 2007). In accordance to this case, Sedio and Ostling (2013) confirmed that the J-C NDD effect is not strictly determined by host specific pathogens.

Considering the natural variation existing in susceptibility, a second scenario can be conceived: the different susceptibility of the hosts may be the origin of a differentiate response to generalist pathogens and the selection operated consequently would play the same effect proposed for host specialized Phytophthoras, with analogous reaction by ecosystem where plant decline is limited to small spots and species distribution is enriched to extremely high levels.

A sub-aim of this study is illustrated as a concrete development of the first, more general, that was reported above. The review of Laliberté *et al.* (2015) was oriented to discuss root traits that enable efficient acquisition of P in P-impoverished soils, and hypothesize a trade-off between efficient P acquisition and resistance to soil borne pathogens. Such a mechanism would tend to equalize differences in competitive ability among co-occurring species, thereby enhancing opportunities for coexistence and increasing local species diversity.

In order to test whether the J-C NDD hypothesis also apply to the soil pathogen *Phytophthora* as a decisive mechanism, two experiments were carried out:
   a) Deep sequencing of the genus *Phytophthora* in soil samples from Jurien Bay dune system with the aim of associating pathogen species variation along the dune chronosequence and soil variables;
   b) A glass house screening of *P. multivora* and *P. cinnamomi* pathogenicity behavior on kwongan host plant species.

Diversity of *Phytophthora* species in the sandy soils of a natural dune system forming a geological chronosequence in Western Australia.

Material and Methods

Sixty soil samples were collected from six dunes (ten samples per dune) of the Jurien Bay dune system located North to Perth City in Western Australia. Each sample (70 grams of sand), corresponding to a single plot, was obtained by mixing 10 grams from seven different sand cores (one per subplot of 2 m X 2 m). The exact position of plots was described by Laliberté et al. (2012) and Hayes *et al.* (2014). DNA extraction from soil samples was performed by the use of MO BIO Laboratories, Inc. PowerSoil DNA Isolation Kit.

454-Library Preparation

Each one of the 60 samples, previously divided in two different sub-samples during the extraction step, was amplified in six different wells, three per sub sample. One tag per soil sample was used for a total of sixty. Negative (water) controls were included in all PCR and qPCR reactions. Following the methodology reported by Català *et al*. (2015), amplicon libraries were generated using a nested PCR approach. The first amplification's round was done in a 25 µl final volume containing 1x PCR buffer with 2.5 Mm MgCl$_2$ (Promega), 0.2 µM of 18Ph2F and 5.8S-1R *Phytophthora* specific primers (Scibetta *et al.* 2012), 0.6 mM dNTP (Promega), 0.4 mg/ml of BSA (Fisher), 0.025 U/µl of Taq DNA polymerase (Promega), 2µl of template DNA. The reagents were incubated at 94°C for 2 min followed by 35 cycles each consisting of 95°C for 20 sec., 60°C for 25 sec, 72°C for 1 min. and a final cycle of 72°C for 7 min. Amplification products were re-amplified by qPCR (Rotor Gene Q, Qiagen) at the following condition: 25 µl final volume containing 1x PCR buffer with 2.5 Mm MgCl$_2$ (Promega), 0.2 µM of both forward and reverse fusion primers, 0.6 mM dNTP (Promega), 0.4 mg/ml of BSA (Fisher), 0.012 µl/ml of syber green, 0.025 U/µl of Taq DNA polymerase (Promega), 2µl of DNA template. Fusion primers were designed following the unidirectional sequencing protocol for library construction (Lib-L chemistry for emulsion PCR, emPCR, 'One-Way Reads'; GS Junior System Guidelines for Amplicon Experimental Design, 2010). The template-specific sequence of the forward fusion primer was the universal ITS6 primer (5'-B-KEY-ITS6–3') (Cooke *et al.* 2000), while that of the reverse fusion primer was the same reverse primer used in the first PCR round (5'-A-KEY-MID-5.8S-1R-3'). A and B represent the pyrosequencing adaptors and multiplex identifier (MID) is provided for post sequencing sample identification. The reactions were incubated at 94°C for 2 min followed by 25 cycles each consisting of 95°C for 20 sec., 60°C for 25 sec, 72°C for 1 min. and a final cycle of 72°C for 7 min. In order to estimate the amount of amplified DNA, qPCR products were separated by electrophoresis on gels containing 1% (w/v) of Agarose LE (Genespin). The approximate base pair (bp) lengths of the amplification products were determined using the 100 bp DNA ladder Ready to Load (Genespin). According to band density, within each group of six wells that contained the same sample, a variable volume per amplification well was supplied and mixed in a 0.2 ml micro centrifuge tube in order to have a total of sixty samples with a volume between 20 and 50µl of qPCR products in each. Supplied variable volume (µl) was written on the all gel images. In Image 3 an example is reported. Pooled DNA material was separated by electrophoresis on gel containing 1% (w/v) of Agarose LE (Genespin). The approximate bp lengths of amplification products were determined using the 100 bp DNA ladder Ready to Load (Genespin) (Image 4a and 4b). According to similarity in band intensity, the samples were arranged in 13 units by pooling each one in a micro centrifuge tube. Samples were purified with AgencourtAMPure XP Bead PCR Purification, and their DNA quality was checked by electrophoresis on 1% (w/v) Agarose LE gel (Genespin) (Image 5). A different aliquot of each (the stronger the signal, the smaller the µls amount) was pooled in a unique micro centrifuge tube and processed as follows: dilution at 1/100; dilution at 1/500; purification with AgencourtAMPure XP Bead PCR Purification; dilution at 1/25000; /100000; /50000; /500000; /5000000.

Sequencing

EmPCR of pooled amplicons (dilution 1/25000) was performed using the GS Junior Titanium emPCR Kit (Lib-L) according to the GS Junior emPCR Amplification Method manual – lib-L, (March 2012) except that the amplification primer in the emPCR was reduced from 20uL to 5uL. This was done in order to limit the brightness of the signals due to the short length of the amplicons, which was found to decrease the number of wells removed by the mixed read and low quality filters, and increase the number of reads passing filter more than five-fold for the same % enrichment.

Sequencing was realized according to GS Junior Titanium Sequencing Kit; the method is described in the GS Junior Sequencing Method Manual March 2012. Sequences manual adjustment, GeneBank database identification and *Phytophthora* species classification and counting were performed with the software Geneious version 7.1.9.

Multivariate analysis of Phytophthora species communities: ordination analysis and hierarchical clustering

The observed variation in *Phytophthora* species composition and abundance in the dune system was first analyzed through non-metric multidimensional scaling (NMDS), an ordination method based on an indirect gradient analysis approach, which shows the continuous variation in community structure among sites (Buttigieg and Ramette, 2014). NMDS was performed using the 'metaMDS' function in the 'VEGAN' package (Oksanen *et al.*, 2013) in R (R Core Team, 2015). The Bray-Curtis dissimilarity calculation method was used in 'metaMDS'. The analysis output is a projection of the relative position of sample points into a low dimensional ordination space (two or three axes). Multiple regression of environmental variables (soil age, available N, available P, Carbonate, Organic C, Total N, Total P, pH, C to N ratio, C to P ratio, N to P ratio) with ordination axes (environmental variable is used as dependent and selected ordination axes as explanatory variables) was performed by using the 'envfit ' function on a reduced number of samples for which environmental data were available. Significance was tested by permutation test. Vectors (for continual variables) and centroids (for categorical variables) were projected onto ordination diagram using the plot function.

In order to discretize the continuous variation observed along the dune chronosequence, aiding structure detection and hypothesis generation, hierarchical cluster analysis was also applied (Buttigieg and Ramette, 2014). Cluster analysis may help to identify (relatively) distinct regions along a gradient, which may correspond to an ecologically meaningful grouping. The grouping was illustrated by heatmaps accompanied by dendrograms obtained through hierarchical clustering with the function 'heatmap.2' in the 'GPLOTS v2.8.0' package (Warnes, 2010) in R. Default parameters were used in 'heatmap.2'.

Results and discussions

A total of 141882 good quality reads were considered for the analysis. There were 34 *Phytophthora* species included in Clades 1, 2, 3, 4, 5, 6, 7 and 8 (Tab. 3) from the 60 soil samples of the dune chronosequence.
The best solution reached after 20 tries in NMDS analysis with three (k=3) and two (k=2) dimensions are illustrated in Figure 1a and 1b respectively. The two-dimension plot shows both the communities ("sites", open circles) and species (red labels). Reading NMDS plots is quite straightforward: objects that are ordinated closer to one another are likely to be more similar than those further apart. However, the scale of the axes is arbitrary as is the orientation of the plot. Solutions with higher stress values (usually above 0.20) should be interpreted with caution and those with stress above 0.30 are highly suspect. The same figure also shows the respective representation of the goodness of fit (Fig. 1c, d), and the respective Shepard plot

(Fig. 1e, f), which shows scatter around the regression between the inter-point distances in the final configuration (i.e., the distances between each pair of communities) against their original dissimilarities. In this study, narrow scatter around the line suggests that original dissimilarities are well preserved in the reduced number of dimensions both for k=3 and for k=2.

In Figure 2 convex hulls connecting the vertices of the points made by the communities (sampling sites) located in the same dune were drawn on the three- (Fig. 2a) and two-dimension (Fig. 2b) plots. This is an intuitive representation to help understanding how *Phytophthora* communities and species cluster based on the chronosequence stages of the dune system.

The results of NMDS and multiple regression analysis of environmental variables with ordination axes for a reduced number of samples are shown in Figure 3, where vectors of the continual variables with significant or close-to-significance effects (Tab. 2) were projected onto the ordination diagram. Out of 12 tested environmental variables only Total N was significant (p<0.01) while soil Carbonate, Total P, pH, C to P ratio, and N to P ratio were close to significance. After Bonferroni correction for multiple testing only Total N remained slightly significant (p=0.0500).

A hierarchical clustering analysis was performed in order to relate the frequency values of *Phytophthora* species to the chronosequence stages of the six dunes (Fig. 4). Geologically younger dunes (QY, QM, QO) grouped separately to the three older dunes, confirming that the community composition of *Phytophthora* species is correlated to the spatio-temporal variables of the sampling area. *P. multivora* and *P. cinnamomi*, putatively native the first and introduced from New Guinea-SE Asia the second, were the most frequent species and had an almost uniform distribution within the chronosequence series, suggesting indifference of these two generalist and invasive pathogens to local ecosystems differentiation.

Clade 1, 3, 4, 5 and 8 were represented in the analysis by a low taxonomical differentiation. Within these clades, *P. nicotianae* (Clade 1), *P. quercina/ohioensis* (Clade 3), *P. arenria/alticola* (Clade 4) and *P. cryptogea/kelmania* (Clade 8) were the most common species. All these species have multiple hosts, have been isolated on a large group of plant genera in nursery, urban and forest plants both in Europe and Australia (Barber *et al.*, 2013, Moralejo *et al.*, 2009) except for *P. arenaria/alticola* that have narrow host range and are responsible of damages on few plant species. *P. arenaria* is a pathogen on *Banksia* spp. in Australia, while *P. alticola* attacks *Eucaliptus spp.* in South Africa (Rea *et al.*, 2011; Maseko *et al.*, 2007; Kroon *et al.*, 2012). As reported in Català *et al.* (2015) the limitations of the ITS1 region for *Phytophthora* taxonomic identification are particularly evident and resolution must often stop at a group of two-three closely related species. The correct classification, in some cases, was conjectural. *P. ohioensis* for instance was possibly the species to be inferred when *P. quercina* was found, because *P. ohioensis* has been officially isolated in Australia while to the host range of *P. quercina is* restricted to *Quercus* species in Europe (Jung *et al.*, 1999). Similarly but more dubiously, *P. arenaria*, isolated and described as a soil born pathogen on *Banksia* spp in kwongan, is a more correct determination than *P. alticola*, recorded in South Africa. The most frequent species found within Clade 2 are a large group of taxonomically close species, i.e. *P. multivora*, *P. acerina/pini/plurivora*, *P. capensis/citricola,* distinguished by molecular analysis only recently and isolated in anthropic and natural environments worldwide (Jung and Burgess, 2009; Scott et al., 2009; Rytkönen, 2011; Kroon et al., 2012). *P. multivora* is the most frequently sequenced species and also distributed in all dunes, as showed in cluster analysis (Fig. 4). These results suggest that, exactly like in the case of the invasive *P. cinnamomi*, *P. multivora* might has been introduced into the studied environment and to further investigate the role of this pathogen in the kwongan vegetation may be of crucial importance. Less frequently were isolated two soil born pathogens signaled in Australia and South Africa, i.e. *P. elongata*, found in *Eucalyptus marginata* forests (Rea *et al.*, 2010), and *P. frigida*, that causes collar and root rot disease outbreaks in cold tolerant *Eucalyptus* and *Acacia* species in South Africa (Rea *et al.*, 2011). Among the sequenced species, those included in Clade 7 are generalist pathogens with a worldwide distribution with the exception of *P. fragariae*, a *species associated to* Rosaceae that has never been signaled in nature (Erwin and Ribeiro, 1996; Kroon et al. 2012, Jung *et al.*, 2015). The species recorded in Clade 6, even if at low frequency, were the largest taxonomical group. *P. litoralis* and *P. thermophila* are sister species that, together with *P. paludosa,*

have been recorded in South West Australia as common waterways agents of scattered mortality within the native vegetation, although pathogenicity and host range are not defined (Jung et al 2011b). Similarly *P. amnicola* and *P. fluvialis* (Burgess, 2012; Jung *et al.*, 2011a) share the waterway habitat and ecology. *P. bilorbang*, morphologically similar to several species within Clade 6, could be responsible for the decline syndrome of blackberry along the river in the south-west of Western Australia (Aghighi *et al.*, 2012). *P. stagnum* (*P. x stagnum*), a hybrid between *P. chlamydospora* and *P. mississippiae* has been recovered from irrigation water in nurseries (Yang *et al.*, 2014; Burgess et al., 2015). *P. taxon pgchlamydo*, *P. lacustris* (Nechwatal *et al.*, 2013), *P. riparia* (Hansen *et al.*, 2012) and *Phytophthora inundata* (Brasier *et al*., 2003) are collected worldwide, mainly signaled in nurseries (*P. inundata/humicola*, indivisible in our work, are extremely close species (Jung *et al.*, 2011b) while *P. rosacearum* (Hansen *et al.*, 2009), rare in nature, was recorded in ornamental woody plants in Europe (Jung *et al.*, 2015). Major characters of Phytophthoras in Clade 6 are the frequent hybridization and the association to water related environment. The first is an ecological adaptation to the second: in wet environments, where water stream mediated diffusion is a condition favorable for continuous asexual multiplication (Jung *et al.*, 2011b), species hybridization is, as underlined in Burgess (2015), the only available gene recombination strategy in substitution to mating type breeding. According to this, cited articles about sampling to Clade 6 species in Australia are basically the result surveys in the rivers more reach southern area of West Australia. The results of our study suggest that the same pathogens are established also in dry lands and their survival strategies (and pathogenicity characters) in a more severe condition climatic areas should be investigated.

Pathogenicity of *Phytophthora multivora* and *Phytophthora cinnamomi* on woody plant species native to Western Australia in a glass house experiment.

Material and methods

River sand for potting was filled in vegetal fibers bags and sterilized by a heater-vapor system at 65 °C for two hours. Sterilized sand was put in 1.8 dm^3 volume plastic pots after a layer of fly web was set in the pot bottom in order to limit sand loss. Twenty forest and bush plants species (22-41 individuals per species) native to Western Australia were included in the experiment (Tab. 4).

Irrigation before *Phytophthora* inoculation was carried out three times a day (3 minutes in the morning, mid-day, and early afternoon). A fertilization with "Osmocotescotts - Native gardens + trace elements (Scotts, Australia)" was performed a month after planting. Twenty-four flasks containing the following media were autoclaved three times at 24 hours distance at 121°C for 20 minutes: 1l vermiculite; 10 g millet; 600 ml V8 (Broth); 2 g CaCo3. Each flask contained 400 ml of vermiculite, 4g of millet, 240 ml of V8 broth (CaCo3 previously added). Two isolates of *P. multivora* (TRH4B2, TRH7B2; in the following reported as '*P. multivora* a' and '*P. multivora* b') and one isolate of *P. cinnamomi* (MP 94-48) (isolated from WA material; collection of CPSM, Murdoch University, Perth) were inoculated in six flasks each while six flasks were left as control. The inoculum added to each flask consisted of Carrot agar media (CA) from a single 9 cm diameter Petri dish previously colonized by the isolate. Non-inoculated CA Petri dishes were used for control flasks. The substrate from the Petri dishes was cut in irregular square pieces under laminar flow and transferred to flasks. Two weeks after inoculation, flasks were handy shaken in order to mix the growing *Phytophthora* hyphae with the entire media. Two PVC irrigation tubes 1.5 cm large and 15 cm long were planted in each pot at 5-8 cm deep at a distance of 10 cm from each other. The space internal to the tube was maintained free from sand, creating an air cylindrical volume free of roots for subsequent deposition of inoculum. Four weeks after flasks preparation, PVC tubes were removed from the pots and 5 grams of inoculum was placed inside each one of the two tubes (ten grams per pot) with sterilized spoons. Irrigation was changed to once per day using distilled water only. Pathogen diffusion was induced by fluting each pot in a single 2-liter plastic container for 24 hours in distilled water every two weeks (12 weeks in total; 6 flutings for the entire experiment). Plant height was registered every two weeks. Measurements were taken from the collar level

to the apical bud or, according to difficulties for defining an apical part, to the highest leaf. Dead samples were also registered. After the first fluting and for the remaining part of the experiment, a randomized complete blocks design was applied. Five weeks after inoculation, pot contents were plated on NARPH media (Nystatin, Ampicillin, Rifamicin, Pentachloronitrobenzene, Hymexazol, Cornmeal agar) in order to check inoculum viability. One month after the first fertilization, a second fertilization was applied with "Thrive, Yakes" (half of recommended dose). After the last fluting, plant roots were washed under running water and weighted. A visual classification of the health condition of below ground organs was realized: from 0 = dead to 4 = no damage.

Results and discussions

Lower roots DW and worst health state mean values were found in Proteaceae while a less pathogenic incidence was revealed in Fabaceae and Myrtaceae (Fig. 5). At the family level, with the exception of Fabaceae, when pathogenicity was estimated through reduction in DW only, *P. cinnamomi* resulted more pathogenic than *P. multivora* (Fig. 6). Data about both the health state of roots (Fig. 7A and C) and the dry weight (DW) (Fig. 7B and D) revealed different behaviors between *Phytophthora* species and between the two *P. multivora* isolates. Results are reported for the three main family screened: Proteaceae, Fabaceae, Myrtaceae. Both methods used to evaluate pathogenicity (Rating and DW) showed that *P. cinnamomi* was more aggressive than *P. multivora* isolates in Proteaceae while in Myrtaceae and Fabaceae (where Rating and DW have inverted results) damages are lower. *P. multivora* follow the same trend although there was a strong interspecific variation and the gap between the two isolates was evident in all plant families and both evaluation methods. At the plant species level there was a wide variation. *P. cinnamomi* and *P. multivora* were active with different severity. *Banksia* species were the most damaged (Figure 8A and B). Banksia is a highly representative genus in Proteaceae and in general within dry shrublands in SWWA.

In line with the aim of this work, the different effect of the two *Phytophthora* species and of the two isolates of *P. multivora* supports the "Soil born *Phytophthora* J-C NDD mediated effect hypothesis" described in the Introduction of this work, according to which plant hyperdiversity in kwongan may be partially driven by generalist soil pathogens. The glass house trial carried out on a limited group of plants showed not only that plant families and species within families have different susceptibility to soil borne Phytophthoras, but also showed that in *P. multivora* pathogenicity strongly depended on isolate in all host plants tested. Due to the widespread distribution of *P. cinnamomi* and *P. multivora* in all chronosequence stages found in the 454 experiment, this result may suggest that plant species composition and richness in kwongan is influenced by the degree of resistance to soil borne *Phytophthora* species.

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Images

Image 1
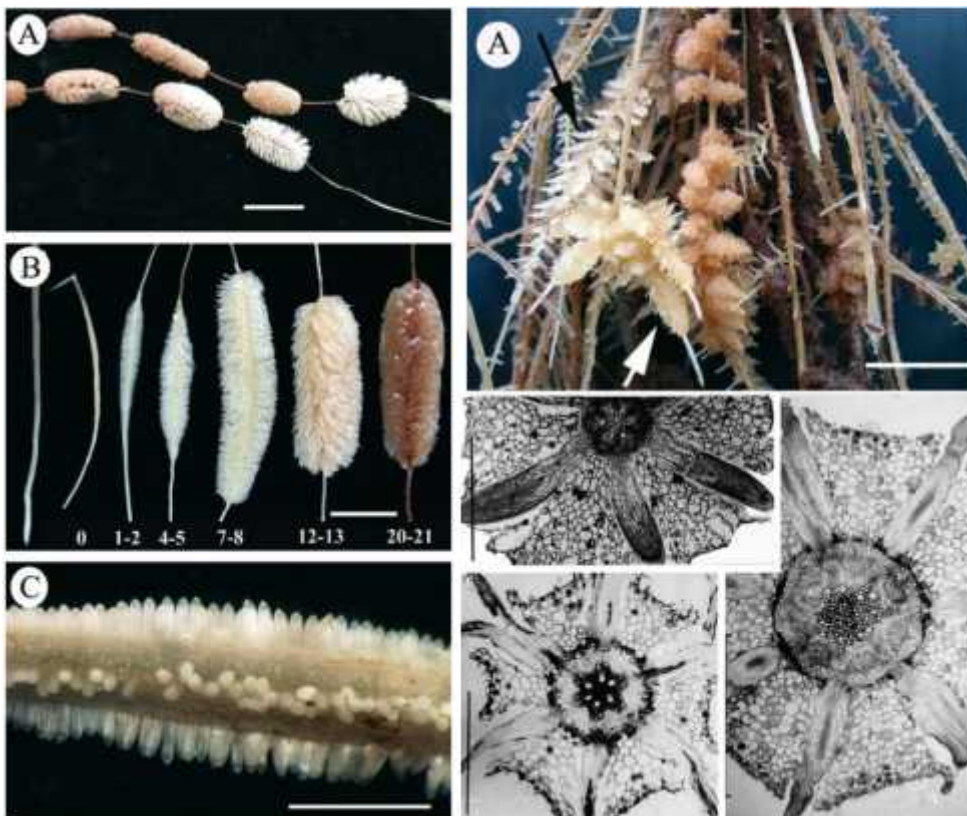
(A, B, C) Cluster roots of *Banksia grandis* and *Hakea prostrata*. White bar is 5 mm in C and 20 mm in all other cases (Shane and Lambers, 2005; modified). In black and white: transections of *H. prostrata and Viminaria juncea*, a legume with identical structures (Lamont, 2003; modified).
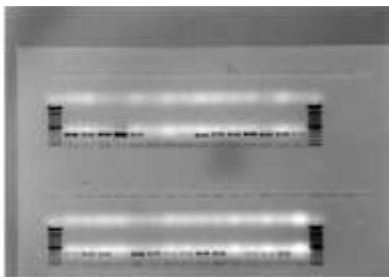
Image 2
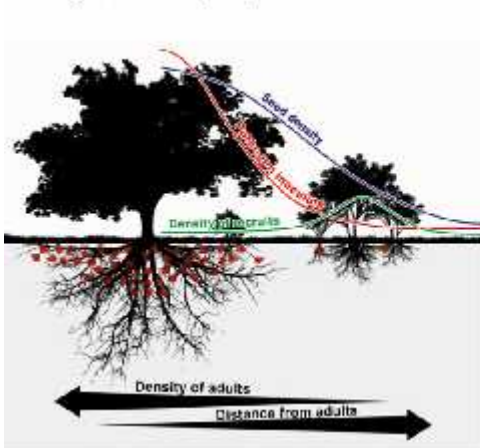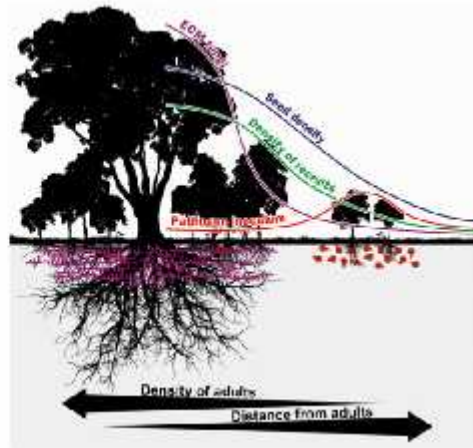
Negative density dependence-Janzen-Connell effect (a) and monodominance (b). Soil pathogens build up in roots of adult plants reduce survival and growth of conspecific seedlings, which perform better far from conspecific mature plants, where the inoculum of soil pathogens is lower. In (b), seedlings of an ectomycorrhizal (ECM) plant species show higher survival and growth (and reach higher densities) near a conspecific mature plant because they recruit into an established ECM fungal network that provides resistance against soil-borne pathogens, whilst also enhancing phosphorus acquisition. Moreover, ECM fungi can directly suppress the activity of soil-borne pathogens. This can eventually lead to monodominance by the ECM species (Laliberté *et al.,* 2015).

Image 3
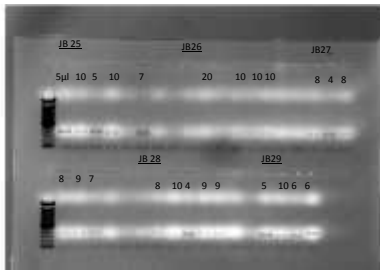
Gel electrophoresis of qPCR-amplified samples 25, 26, 27, 28 and part of 29 with reported the variable volume (μl) to be supplied.
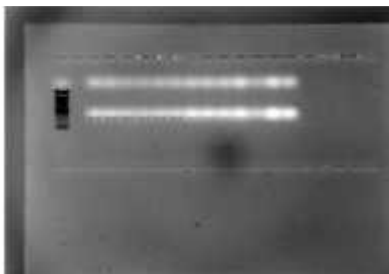
Image 4a

Gel electrophoresis of pooled amplified DNA

Image4b
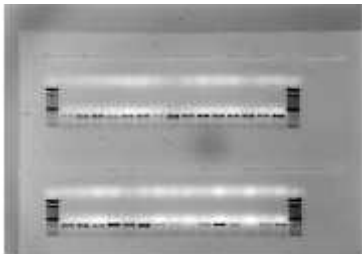
Gel electrophoresis of pooled amplified DNA

Image 5

Gel electrophoresis of the purified samples pooled in thirteen units.

Tables

Table 1

Dune system	Estimated soil age (ka)	Most likely (co-) limiting nutrient(s)	Carbonate (%)	Organic C (g m^{-2})	Total N (g m^{-2})	Total P (g m^{-2})	pH (CaCl$_2$)	Cation Exchange Capacity (cmol$_c$/kg)	C:N	C:P	N:P
Quindalup (young)	0,01 - 0,05 (Holocene)	N	82	8,9	495,5	384,3	8,2	12,93	18	23,2	1,3
Quindalup (medium)	1 (Holocene)	N, P	66	14,678	763,3	346,4	8,1	5,52	19,2	42,4	2,2
Quindalup (old)	6,5 (Holocene)	N, P	25	17,72	362,2	194,8	8	5,01	48,9	90,9	1,9
Spearwood (west)	125 (Middle Pleistocene)	P	0	3,864	166,5	28,8	6,1	1,94	23,2	134	5,8
Spearwood (old)	480 (Middle Pleistocene)	P	0	2,917	119,2	12,7	5,7	0,96	24,5	229	9,4
Bassendean	sibly late Pleistocene)	P	0	4,063	117,8	6,4	4,4	0,98	34,5	639	18,5

Geological age and soil element content in the sampling sites of the Jurien Bay dune system from Patrick *et al.* (2014) and Turner *et al.* (2015).

Table 2

Environmental Variable	NMDS1	NMDS2	r2	Pr(>r)
SoilAge	-0.69692	0.71715	0.70	0.111
Available N	-0.21040	0.97762	0.73	0.200
Available P	-0.68164	-0.73169	0.40	0.667
Carbonate	-0.11540	-0.99332	0.84	0.092°
Organic C	0.50813	-0.86128	0.63	0.260
Total N	**0.12456**	**-0.99221**	**0.96**	**0.004**
Total P	-0.00191	-1.00000	0.87	0.072°
pH	0.37419	-0.92735	0.82	0.078°
Cation Exchange Capacity (CEC)	-0.24368	-0.96985	0.57	0.285
C to N ratio	0.52691	0.84992	0.24	0.671
C to P ratio	-0.58295	0.81251	0.73	0.074°
N to P ratio	-0.52623	0.85034	0.77	0.094°

Variables with significant effect are reported in bold.
° Indicates variables with effect close to significance.

Results of multiple regression analysis of NMDS with environmental variables. NMDS1 and NMDS2 indicate the relationship of the environmental variable with the first and second NMDS axis. These values are not correlation coefficients, but coordinates of the vector head on given ordination axes. r2 indicates the variation explained by the multiple regression model; square-root of this value is used to scale lengths of vectors (arrows) in the ordination diagrams (Fig. 3, variables with higher sqrt (r2) are represented by longer arrows). Pr(>r) is the significance of the regression, calculated by permutation test with given number of permutations, in this case 720, indicates whether the variable is related to ordination axes more than would be randomly generated one.

Table 3

Species	Clade	Number of Reads
P. nicotianae	1	15130
P. sp. nov. 1A	1	40
P. acerina/pini/plurivora	2	9720
P. capensis/citricola	2	2613
P. elongata	2	3601
P. frigida	2	725
P. meadii/colocasiae	2	1082
P. multivora	2	28650
P. citrophthora	2	88
P. sp. nov. 2A	2	20
P. quercina/ohioensis	3	6640
P. sp. nov. 3A	3	30
P. arenaria/alticola	4	12781
P. katsurae/agathis	5	6
P. amnicola	6	328
P. bilorbang	6	29
P. fluvialis	6	6477
P. paludosa	6	65
P. lacustris	6	460
P. inundata/humicola	6	407
P. litoralis	6	1018
P. moojotj	6	612
P. pg chlamydo	6	285
P. riparia	6	224
P. rosacearum	6	885
P. stagnum	6	3939
P. thermophila	6	2469
P. cambivora	7	3654
P. cinnamomi	7	19122
P. fragariae	7	78
P. neiderhauserii	7	301
P. parvispora	7	960
P. cryptogea/kelmania	8	19408
P. sansomeana	8	35

Thirty-four *Phytophthora* species sequenced with 454 and belonging to Clades from 1 to 8 are reported with the correspondent number of reads. In eight cases more than one species is listed in the same row because they cannot be distinguished on the basis of the ITS1 sequence.

Table 4

Species	Potted plants number
Banksia menziesii	37
Eucalyptus gonphocephala	40
Casuarina obesa	40
Banksia attenuata	40
Agonis flexuosa	35
Gastrolobium spinosum	38
Banksia littoralis	40
Banksia seminuda	41
Hakea marginata	31
Acacia rostellifera	40
Banksia speciosa	41
Acacia dentifera	40
Hakea undulata	34
Banksia media	23
Banksia occidentalis	41
Petersonia occidentalis	40
Corymbia calophylla	25
Xanthorrhoea gracilis	29
Eucalyptus kochii	22
Melaleuca brevifolia	41

Bush and tree species native to Western Australia included in the study.

Figure 1
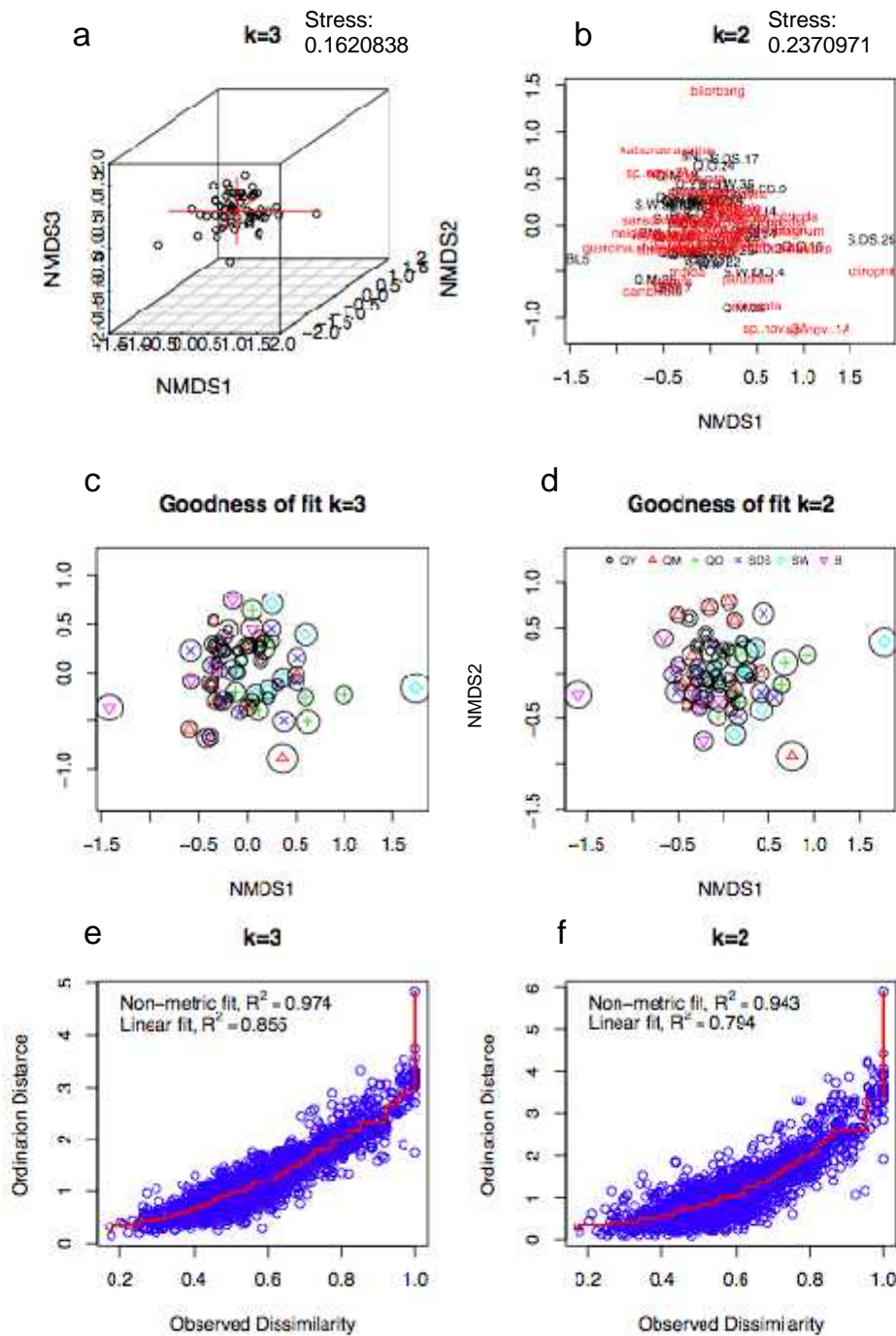
Three (a) and two-dimension (b) NMDS analysis of *Phytophthora* communities in the Jurien Bay dune chronosequence (Western Australia) based on relative abundance of 454-sequences from soil samples. Plots show the communities ("sites", open circles, a and b) and species (red labels, b). In the plots of goodness of fit for three- (c) and two-dimension (d) analyses, points with bigger diameter indicate worse fit. The Shepard plots (ordination diagrams) for three- (e) and two-dimension (f) analyses show scatter around the regression between the inter-point distances in the final configuration (i.e., the distances between each pair of communities) against their original dissimilarities. QY=Quindalup young age; QM=Quindalup medium age; QO=Quindalup old age; SW=Spearwood west (young); SDS=Spearwood old; B=Bassendean

Figure 2
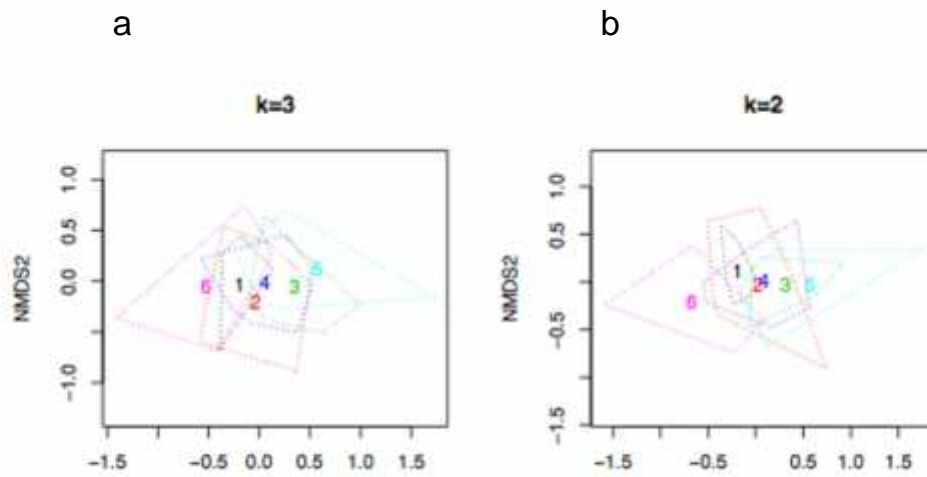
Representation of the results of three- (a) and two-dimension (b) NMDS analyses where convex hulls connect the vertices of the points made by the communities (sampling sites) located in the same chronosequence stage of the dune system (1=Quindalup young age; 2=Quindalup medium age; 3=Quindalup old age; 4=Spearwood west; 5=Spearwood old; 6=Bassendean).
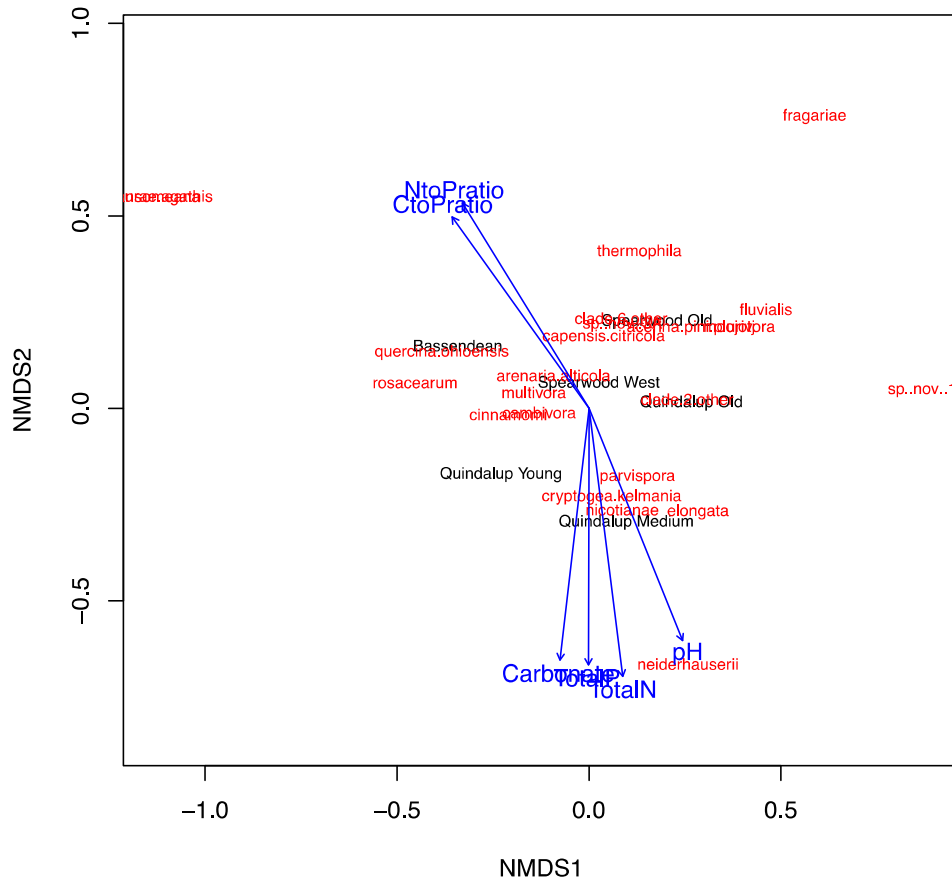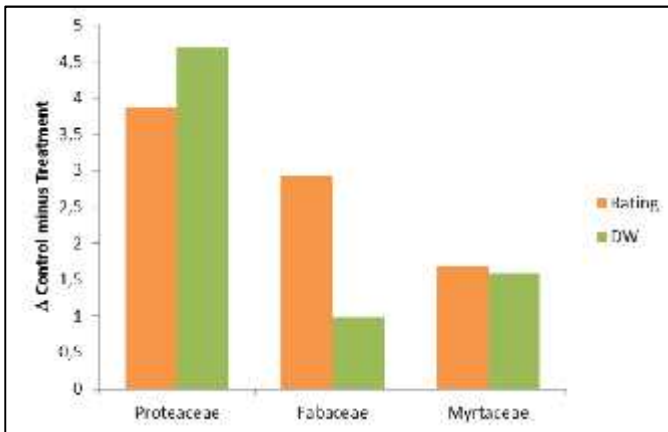
Figure 3

Results of multiple regression analysis of environmental variables with ordination axes for the sampling sites for which environmental data were available. Vectors of the continual variables with significant or close-to-significance effects (Tab. 2) were projected onto the ordination diagram. The square root of the variation explained by the multiple regression model (r2 in Tab. 2) was used to scale lengths of vectors (arrows) in the ordination diagrams (variables with higher sqrt (r2) are represented by longer arrows).

Figure 4
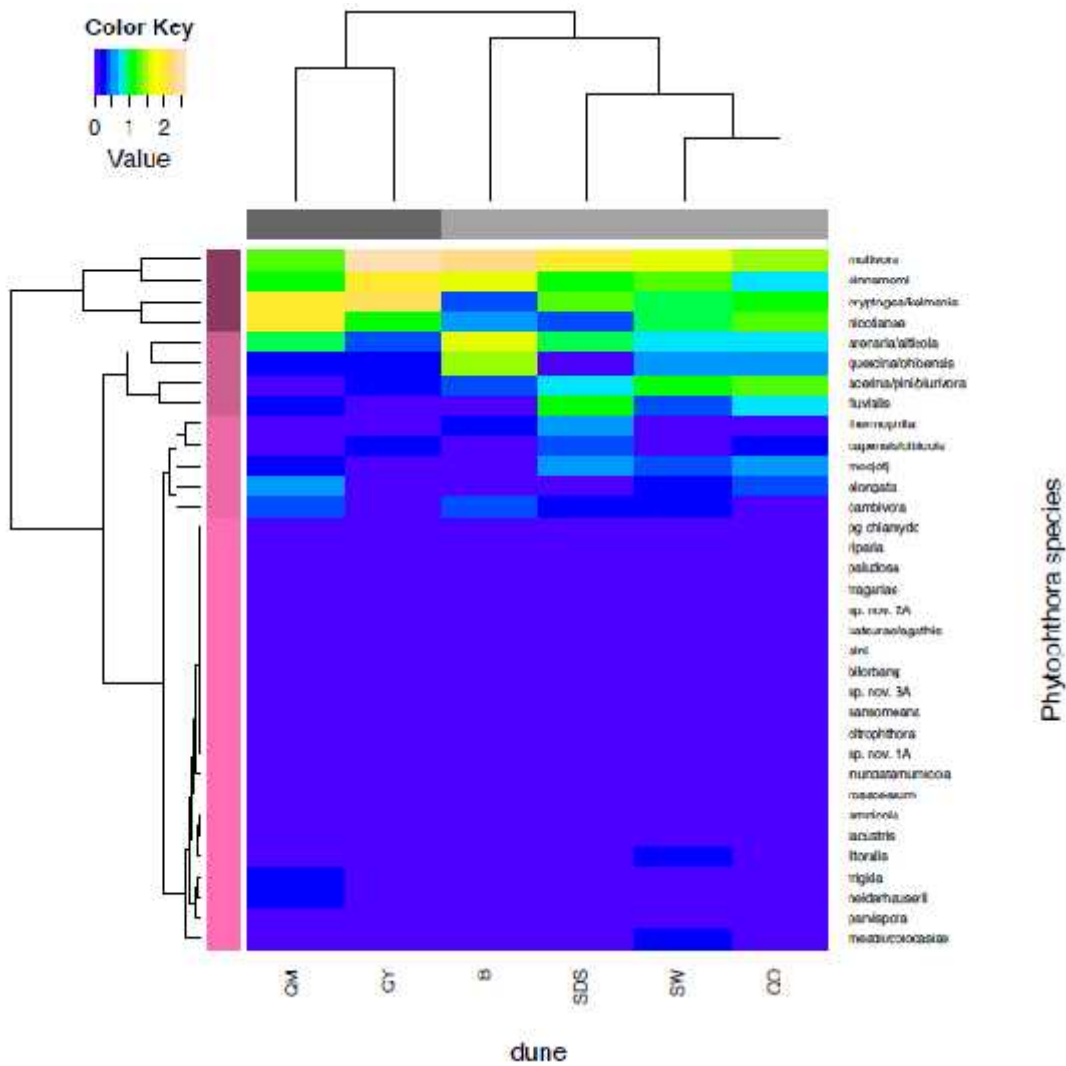
Hierarchical clustering of *Phytophthora* species (Y axis) and dunes in a geological chronosequence (X axis) based on the relative frequencies of reads assigned to different species in a 454 sequencing of soil samples QY=Quindalup young age; QM=Quindalup medium age; QO=Quindalup old age; SW=Spearwood west; SDS=Spearwood old; B=Bassendean. Further details about the dunes are reported in Table 1.

Figure 5

Plant samples susceptibility illustrated by the difference between control value and the respective treated plants. Mean values per family of both Rating and DW. *Phytophthora* was more aggressive on Proteaceae.

Figure 6
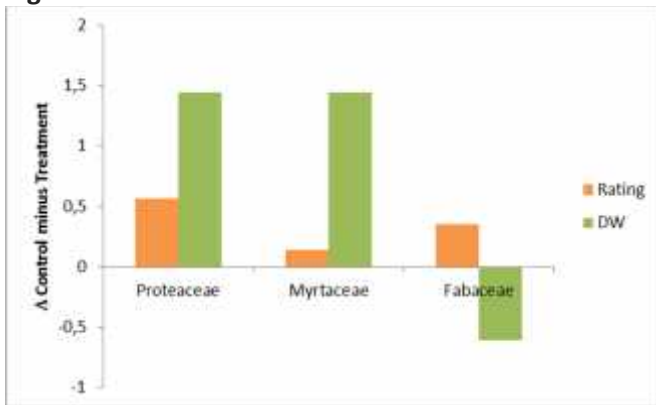
Comparison between pathogenicity of *P. cinnamomi* and *P. multivora* illustrated through health Rating and DW measures. Histograms show the difference between *P. cinnamomi* and *P. multivora* (average between the two isolates) values. Larger positive values indicate higher pathogenicity by *P. cinnamomi,* which resulted more pathogenic than *P. multivora* with the only exception of the effect on DW observed in Fabaceae.

Figure 7
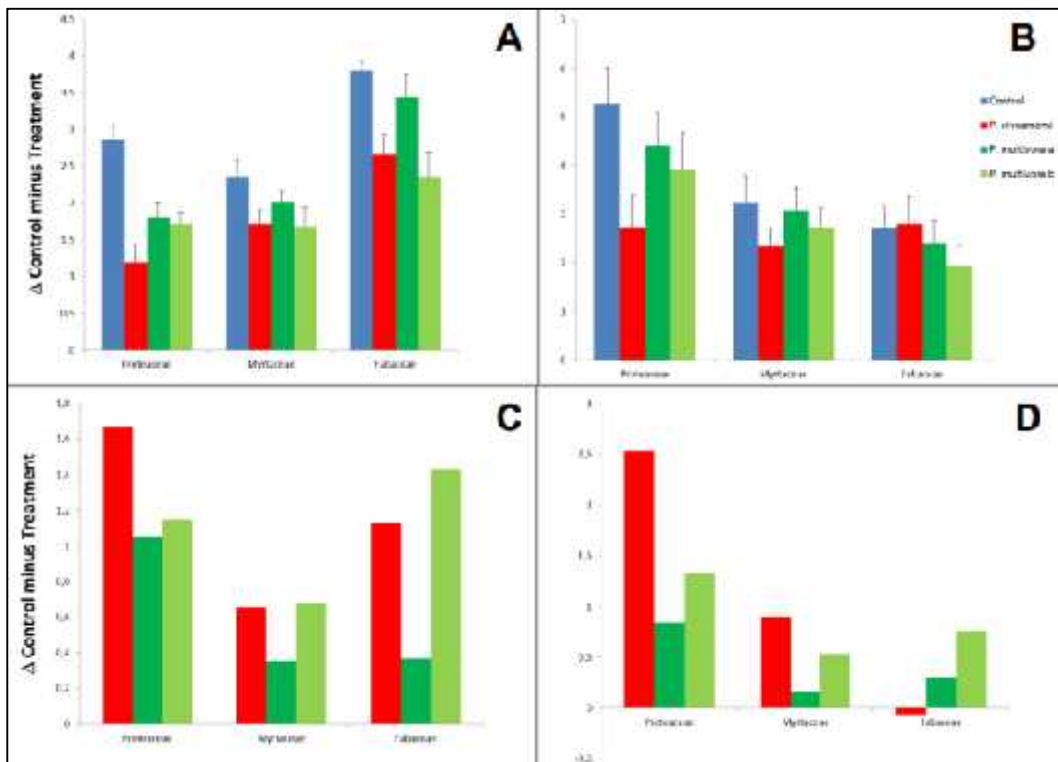
Average values of root health visual rating (A) (0 = dead - 4 = healthy) and root dry weight (g) (DW) (B) for the three main families. Standard deviation is reported in the histogram. *Phytophthora* caused damages in all families, although with different intensity depending on species and isolate. Based on DW, Fabaceae were the only group where no significant weight loss was observed after infection compared to controls. In C (visual evaluation of health condition) and D (measure of root dry weight), *Phytophthora* species pathogenicity per plant family is illustrated by the difference between the values observed in infected plants and the measure in the respective control plants.

Figure 8
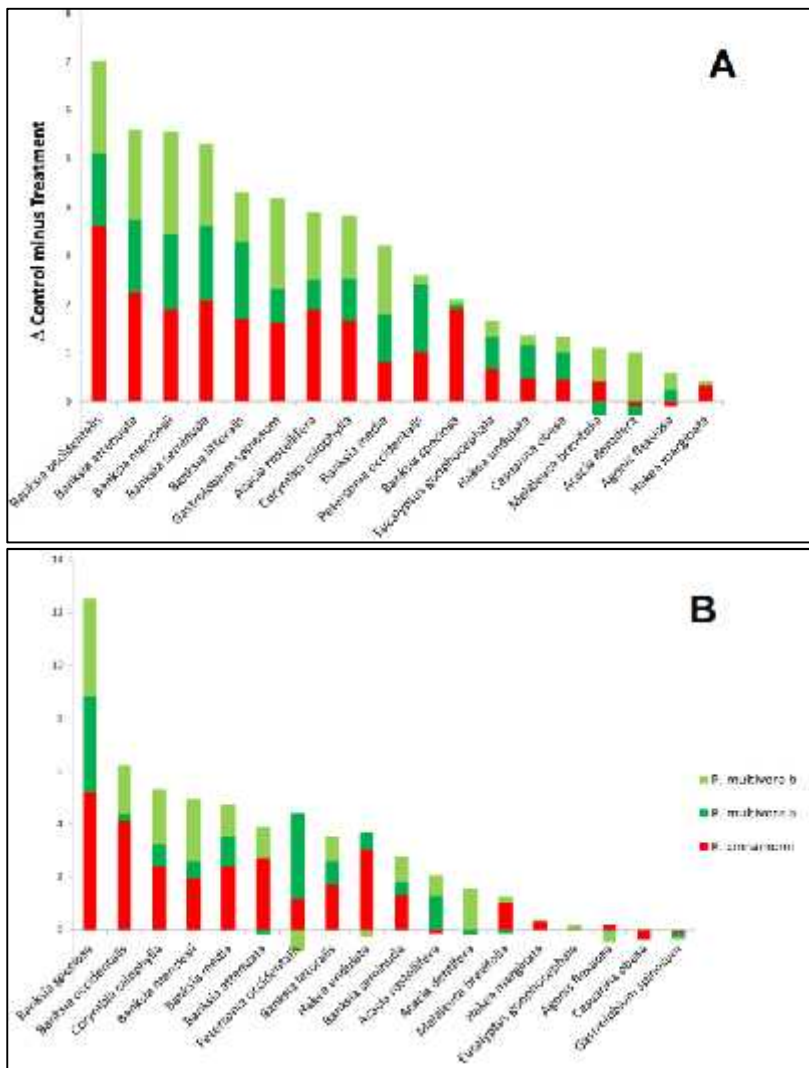
Cumulate differences between treated and control values are shown for each plant species with different colors for the three isolates of *Phytophthora*. Rating in A and DW in B. Columns below the zero value are samples for which the health conditions of roots were better and/or dry weight higher than in control plants.