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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.

Keywords
Biological invasions, early detection, international trade, invasive micro-organisms, molecular diagnostics, nursery pathway, *Phytophthora*, qPCR, soil pathway, soilborne diseases.

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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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 StepOnePlus™ 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 ($\text{Eff} = 10^{-1/\text{slope}} - 1$) (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 GenBank. 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.

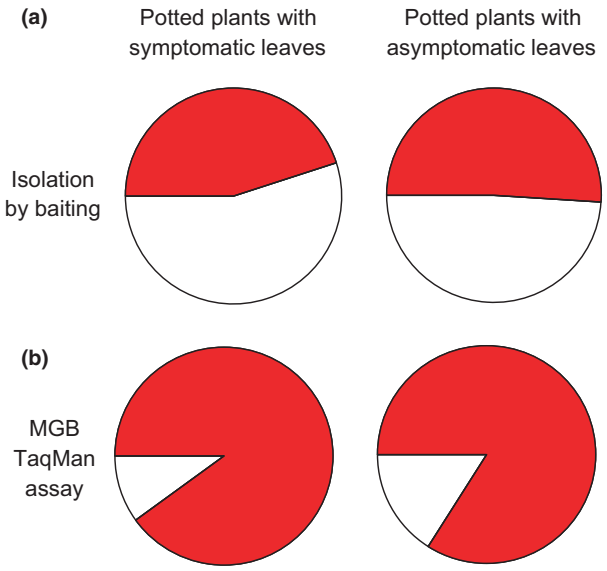

Figure 2 Detection of *Phytophthora* species [coloured = positive, white = negative (%)] in potted plants with or without visible disease symptoms to the crown, by classical isolation on selective medium (a) or by qPCR with a specific MGB TaqMan probe (b).

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

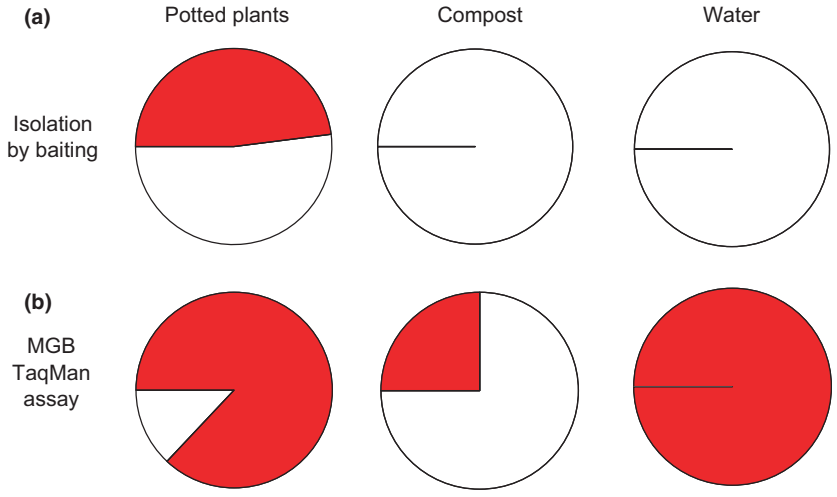

Figure 1 Detection of *Phytophthora* species [coloured = positive, white = negative (%)] in potted plants, compost produced in the nurseries and water from the drainage systems, by classical isolation on selective medium (a) or by qPCR with a specific MGB TaqMan probe (b).

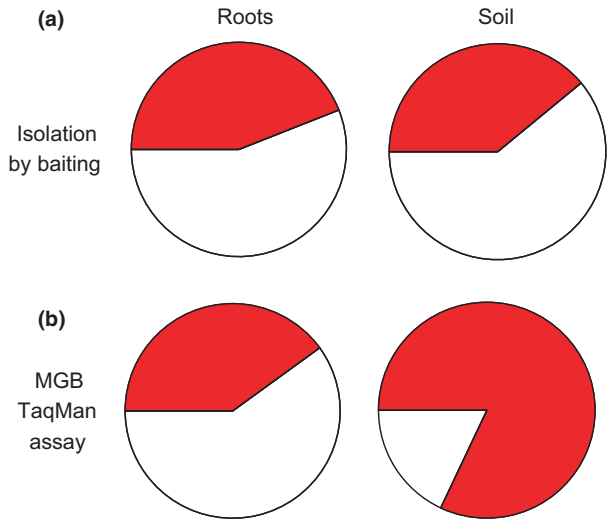

Figure 3 Detection of *Phytophthora* species [coloured = positive, white = negative (%)] in roots or soil samples from all potted plants (with or without visible disease symptoms to the crown), by classical isolation on selective medium (a) or by qPCR with a specific MGB TaqMan probe (b).

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–Wallis$_{(1,89)} = 0.11$, $P = 0.74$; 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.

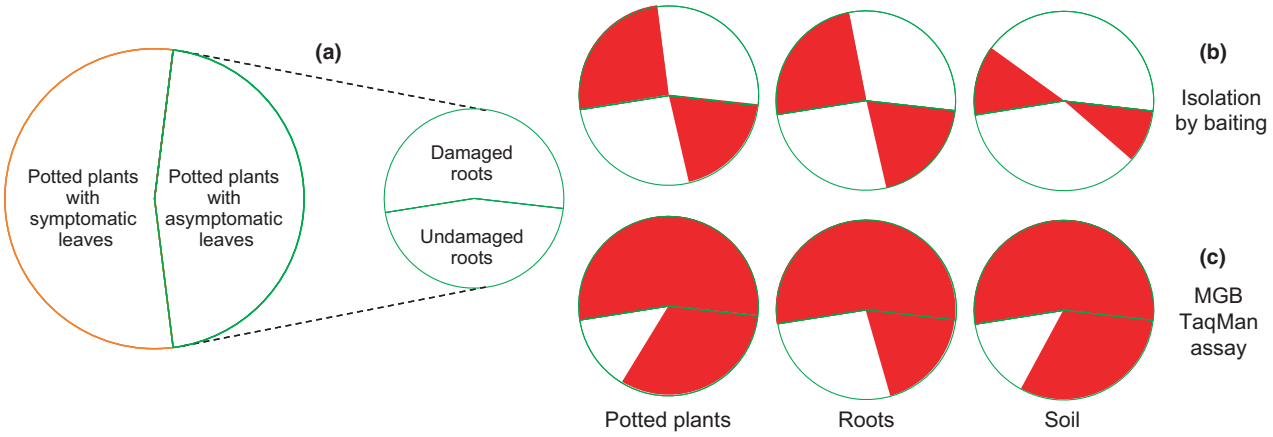

Figure 4 Percentage of asymptomatic plants (i.e. plants without visible disease symptoms to the leaves) that had (damaged roots) or had not (undamaged roots) symptoms to the roots (a). Percentage of samples, from pots containing asymptomatic plants, with either damaged or undamaged roots, which tested positive to *Phytophthora* species by isolation of the fungus on selective medium (b) or by qPCR with a specific MGB TaqMan probe (c).

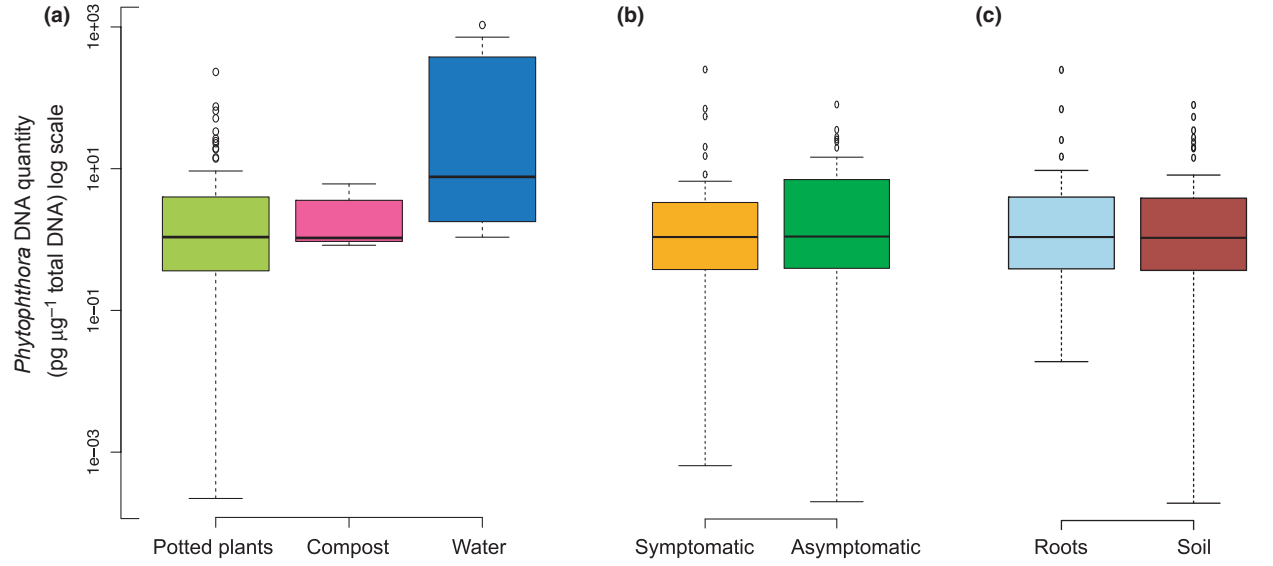

Figure 5 *Phytophthora* DNA quantities (horizontal line = median, box = 1st–3rd quartiles, whisker = min.-max., dots = outliers and extremes; logarithmic scale), estimated by qPCR with a specific MGB TaqMan probe, in tested substrates. Differences between symptomatic and asymptomatic plants (b), and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

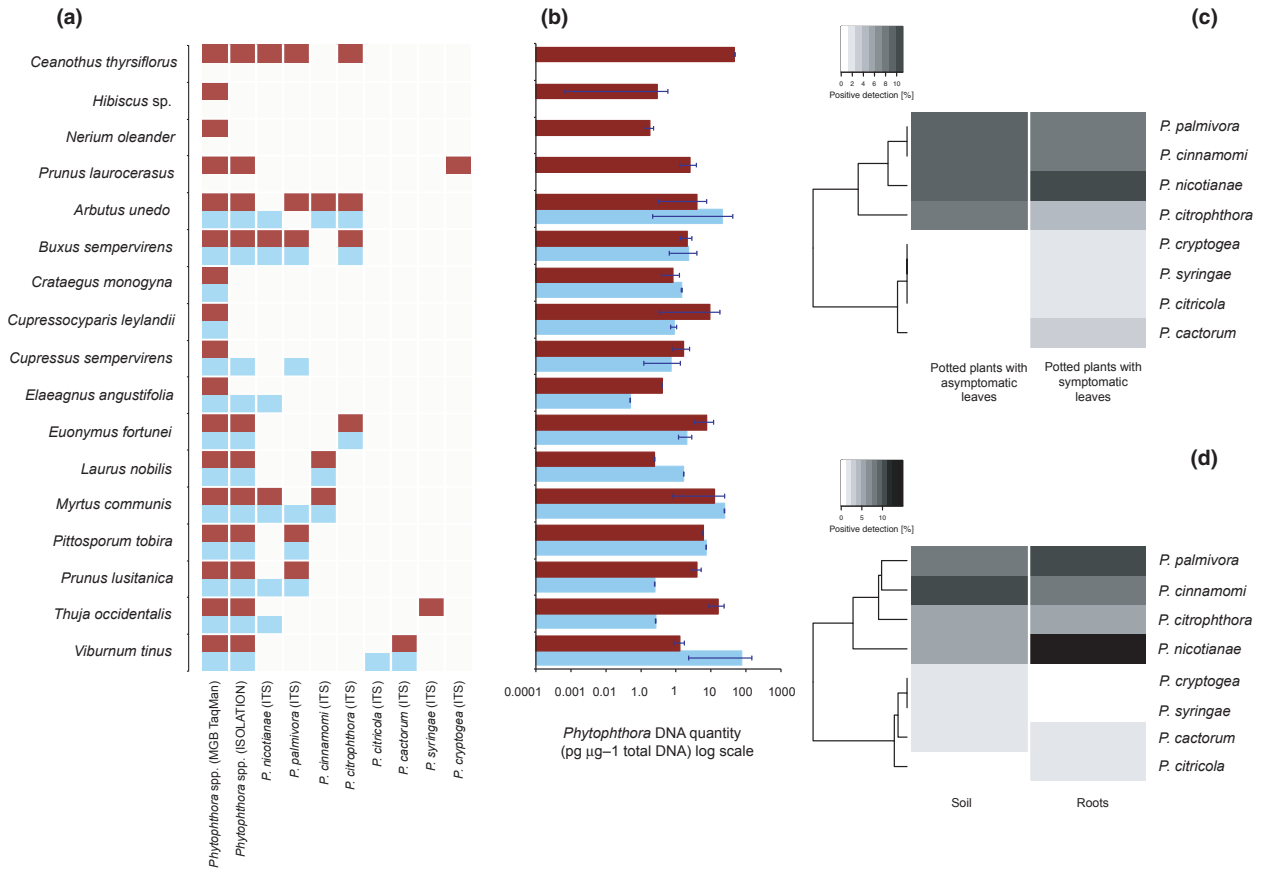

Figure 6 Summary of detection of *Phytophthora* species by isolation on selective medium and ITS analysis, and by qPCR with a specific MGB TaqMan probe. (a) Presence–absence of *Phytophthora* species, and (b) average quantity (±SE, logarithmic scale) of *Phytophthora* DNA estimated by qPCR, in roots (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.

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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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