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Conventional PCR and real time quantitative PCR detection of *Phytophthora cryptogea* on *Gerbera jamesonii*

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Abstract A conventional PCR and a SYBR Green real-time PCR assays for the detection and quantification of *Phytophthora cryptogea*, an economically important pathogen, have been developed and tested. A conventional primer set (Cryp1 and Cryp2) was designed from the *Ypt*1 gene of *P. cryptogea.* A 369 bp product was amplified on DNA from 17 isolates of *P. cryptogea.* No product was amplified on DNA from 34 other *Phytophthora* spp., water moulds, true fungi and bacteria. In addition, Cryp1/Cryp2 primers were successfully adapted to real-time PCR. The conventional PCR and real-time PCR assays were compared. The PCR was able to detect the pathogen on naturally infected gerbera plants and on symptomatic artificially infected plants collected 21 days after pathogen inoculation. The detection limit was 5×10^3 *P. cryptogea* zoospores and 16 fg of DNA. Real-time PCR showed a detection limit 100 times lower (50 zoospores, 160 ag of DNA) and the possibility of detecting the pathogen in symptomless artificially infected plants and in the recirculating nutrient solution of closed soilless cultivation systems.

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Introduction

Gerbera is one of the top 10 cut flower crops in Europe and in recent years has been steadily exported to the USA from The Netherlands, France, Italy, Japan, India, Australia and New Zealand (Heinrichs 2005). Gerbera flowers are grown also in South America (Colombia), but the majority of these are exported to Europe and Japan (Heinrichs 2005). It is one of the largest cut flower crops in the United States where 100,598,000 gerbera stems (98% in California) were produced in 2004 (USDA 2004) and the acreage devoted to this crop continues to increase. In 2003, gerbera was grown on 366 ha in Italy and on 243 ha in the Netherlands (Heinrichs 2005). It is a high value cut flower crop with retail prices as high as €3.50/stem. In Italy as well as in many other production areas where gerbera is grown, *P. cryptogea* caused root rot resulting in severe losses every season.

Phytophthora is a genus in the Oomycota, responsible for some of the most serious and economically important plant diseases (Judelson and Blanco 2005). *Phytophthora cryptogea* is a zoospore-producing chromista recognized as an important pathogen of ornamental plants, particularly container-grown

plants. The host range is large, mostly belonging to the Asteraceae or sunflower family (Erwin and Ribeiro 1996). The pathogen is easily recovered from irrigation water and from soil (Garibaldi et al. 2003; Stanghellini and Rasmussen 1994) where it can survive in the absence of a suitable host. Root rot and crown rot commonly occur on gerbera: the causal agent can spread at a devastating speed in greenhouses destroying this crop (Ehret et al. 2001).

The availability of a specific and sensitive diagnostic method for the detection and identification of *P. cryptogea* could be very useful for gerbera growers. Rapid detection of the pathogen, where it is harboured, how it may have entered the production system and how it can spread is crucial to formulating effective, long-term management strategies. Conventional and real-time PCR are important tools for the diagnosis and study of phytopathogenic fungi and have contributed to the alleviation of some of the problems associated with the detection, control and containment of plant pathogens (Bates et al. 2001; Filion et al. 2003; Schena et al. 2004).

The early, rapid and accurate identification and detection of *P. cryptogea* either in the plant or in water is essential in order to limit its spread and to improve prophylaxis (Ehret et al. 2001). PCR and real-time PCR technologies open increasing opportunities for detecting and studying phytopathogenic organisms. Most of the detection methods of phytopathogenic microorganisms are based on the ITS regions (Tomlinson et al. 2005; Martin et al. 2004; Hayden et al. 2004; Martin and Tooley, 2003). Hong et al. (1999) developed PCR primers derived from the ITS region of the nuclear encoded ribosomal RNA genes (rDNA) for the detection of the *P. cryptogea–Phytophthora drechsleri* complex group in plants using conventional PCR assay. Ribosomal DNA provide attractive targets to design specific primers since they are highly stable, can be amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (White et al. 1990). However in some cases the ITS sequences are not sufficiently variable, making the design of primers for identifying and detecting closely related taxa very difficult or impossible.

The highly polymorphic nature of the *Ypt*1 gene sequences obtained from different species of *Phytophthora* enables the differentiation of closely related species that have almost identical ITS regions (Schena and Cooke 1996). Furthermore, the levels of the sequence diversity appear sufficient to design species-specific primers and it seems that the *Ypt*1 gene is not subject to intraspecific variation that could cause problems for diagnostic assays (Schena et al. 2006).

The main goal of this work was to develop a sensitive and effective diagnostic method for identifying and detecting *P. cryptogea* in plant tissue and re-circulating nutrient solution of closed soilless cultivation systems.

Materials and methods

Isolates, culture, propagules and plant material

Cultures used in this study included 17 *P. cryptogea* isolates (Table 1) from various hosts and continents, chosen to provide geographical and pathological diversity. Isolates numbered ATCC60786 and ATCC10924 belong to *P. cryptogea* molecular group A; CBS 290.35, ATCC36301, IMI 45168, ATCC15402 and CBS113.19 to group B; ATCC 46722, ATCC 34301 and ATCC 46723 to group C; ATCC 54402 and ATCC52401 to group D (Mills et al. 1991). All species of *Phytophthora* (Table 1), *Pythium* and other water moulds (Table 2) were grown on V8 Juice Agar (for 1 l: 200 ml Campbell's V8 juice (Campbell Sauce Company, USA), 15 g agar, 800 ml deionized water) for 1 week at 25°C. Bacteria were grown on Luria Bertani medium (Invitrogen) at 37°C. Gerbera (*Gerbera jamesonii*) plants were grown in a growth chamber at 25°C and a photoperiod of 16 h light and 8 h darkness. When plants were 2 months old they were placed into independent pots for further inoculation and a total of 40 infected plants were placed into two independent closed soilless cultivation systems.

Pathogen inoculation of plants

One month after transplanting gerbera plants in closed soilless systems, one isolate of *P. cryptogea* (ATCC 66770) artificially propagated on wheat and hemp kernels was introduced into the cultivation system. Twenty artificially inoculated plants and 20 naturally infected plants were placed in two different closed soilless systems in order to facilitate the infection of 100 healthy plants throughout the recirculation of non-disinfected nutrient solution. Plants were artificially

Table 1 Species of *Phytophthora* analysed in this study, their designations and origins

Species	Isolate numbers	Isolated from	Geographic origin	ITS6/ITS4 amplification	Cryp1/Cryp2 amplification
Phytophthora boehmariae	Agroinnova collection (csl)	*Boehmeria nivia*	UK	+	−
Phytophthora capsici	Agroinnova collection (C4)	*Capsicum annuum*	Italy	+	−
Phytophthora cinnamomi	Agroinnova collection (A)	*Myrtus communis*	Italy	+	−
Phytophthora cinnamomi	Agroinnova collection (B)	*Myrtus communis*	Italy	+	−
Phytophthora cinnamomi	Agroinnova collection (B)	*Azalea* sp.	Italy	+	−
Phytophthora cinnamomi	Agroinnova collection (10A5)	*Persea americana*	Italy	+	−
Phytophthora cinnamomi	Agroinnova collection (10A9)	*Persea americana*	Italy	+	−
Phytophthora citricola	Agroinnova collection (cit1)	*Citrus* sp.	Italy	+	−
Phytophthora cactorum	Agroinnova collection (cact1)	*Fragaria* sp.	Italy	+	−
Phytophthora cambivora	IMI 77374	Not known	Not known	+	−
Phytophthora citrophthora	Agroinnova collection (magn.)	*Citrus limon*	Italy	+	−
Phytophthora clandestina	CBS 347 86	*Trifolium subterranean*	Australia	+	−
Phytophthora cryptogea	Agroinnova collection (dimorf)	*Osteospermum* sp.	Italy	+	+
Phytophthora cryptogea	Agroinnova collection (gerb1)	*Gerbera jamesonii*	Italy	+	+
Phytophthora cryptogea	Agroinnova collection (gerb2)	*Gerbera jamesonii*	Italy	+	+
Phytophthora cryptogea	Agroinnova collection (gerb3)	*Gerbera jamesonii*	Italy	+	+
Phytophthora cryptogea	Agroinnova collection (gerb4)	Not known	Italy	+	+
Phytophthora cryptogea	Agroinnova collection (magn1)	*Pistacia lentiscus*	Italy	+	+
Phytophthora cryptogea	ATCC 66770	*Lycopersicon esculentum*	Bulgary	+	+
Phytophthora cryptogea	CBS 290.35	*Aster* sp.	USA	+	+
Phytophthora cryptogea	ATCC 52402	*Solanum marginatum*	Ecuador	+	+
Phytophthora cryptogea	ATCC 52401	*Begonia elatior hybrid*	Germany	+	+
Phytophthora cryptogea	CBS 113 19	*Lycopersicon esculentum*	Ireland	+	+
Phytophthora cryptogea	ATCC 46722	*Simmondsia chinensis*	California	+	+
Phytophthora cryptogea	ATCC 46723	*Pinus radiate*	California	+	+
Phytophthora cryptogea	ATCC 34301	*Pseudotsuga menziesii*	Oregon	+	+
Phytophthora cryptogea	ATCC 36301	*Solanum tuberosum*	Ohio	+	+
Phytophthora cryptogea	IMI 45168	*Lycopersicon esculentum*	New Zealand	+	+
Phytophthora cryptogea	ATCC 154002	*Aster* sp.	California	+	+
Phytophthora drechsleri	CBS 359.52	*Solanum tuberosum*	Argentina	+	−
Phytophthora drechsleri	CBS 149 88	Soil	USA	+	−
Phytophthora drechsleri	ATCC 60786	*Capsicum* sp.	Mexico	+	−
Phytophthora erythroseptica	ATCC 10924	Soil	USA	+	−
Phytophthora erythroseptica	Agroinnova collection (ery1)	*Solanum tuberosum*	Italy	+	−
Phytophthora fragariae	Agroinnova collection (frag1)	*Fragaria* sp.	Canada	+	−
Phytophthora fragariae	CBS 309 62	*Fragaria* sp.	UK	+	−
Pytophthora gonapodyides	CBS 114.340	*Prunus persica*	France	+	−
Phytophthora heveae	CBS 296.29	*Theobroma cacao*	Malaysia	+	−
Phytophthora multivesiculata	CBS 545.96	*Cymbidium* sp.	The Netherlands	+	−
Phytophthora hybernalis	Agroinnova collection (csl)	*Citrus sinensus*	UK	+	−

Table 1 (continued)

Species	Isolate numbers	Isolated from	Geographic origin	ITS6/ITS4 amplification	Cryp1/Cryp2 amplification
Phytophthora humicola	CBS 200 81	Soil	Taiwan	+	−
Phytophthora infestans	Agroinnova collection (inf1)	*Solanum tuberosum*	UK	+	−
Phytophthora inundata	Agroinnova collection (inu1)	*Prunus persica*	Italy	+	−
Phytophthora katsurae	CBS 587 85	*Cocos nucifera*	Taiwan	+	−
Phytophthora megasperma	Agroinnova collection (meg1)	*Medicago sativa*	Ireland	+	−
Phytophthora megakarya	CBS 238 83	*Theobroma cacao*	Cameroon	+	−
Phytophthora insolita	CBS 691 79	*Citrus* sp. soil	Taiwan	+	−
Phytophthora nicotianae	Agroinnova collection (N3)	*Dianthus chinensis*	Italy	+	−
Phytophthora nicotianae	Agroinnova collection (N4)	*Lavanda officinalis*	Italy	+	−
Phytophthora nicotianae	Agroinnova collection (N5)	*Solanum melongena*	Italy	+	−
Phytophthora nicotianae	Agroinnova collection (N6)	*Lycopersicon esculentum*	Italy	+	−
Phytophthora nicotianae	Agroinnova collection (N7)	*Skimmia japonica*	Italy	+	−
Phytophthora palmivora	Agroinnova collection (magn)	*Olea europea*	Italy	+	−
Phytophthora porri	CBS 688 79	*Brassica chinensis*	Canada	+	−
Phytophthora pseudotsugae	CBS444 84	*Pseudotsugae menziesii*	USA	+	−
Phytophthora psychrophila	CBS 803.95	Soil	Germany	+	−
Phytophthora quercina	CBS 781.95	*Quercus robur*	Hungary	+	−
Phytophthora ramorum	Agroinnova collection (ger)	*Rhododendron* sp.	Germany	+	−
Phytophthora ramorum	Agroinnova collection (domen)	*Rhododendron* sp.	Italy	+	
Phytophthora richardiae	CBS 240.30	*Zantedeschia aethiopica*	USA	+	−
Phytophthora richardiae	ATCC 46734	*Zantedeschia aethiopica*	USA	+	−
Phytophthora tentaculata	CBS 412.96	*Chrysanthemum leucanthemum*	Germany	+	−
Phytophthora tropicalis	Agroinnova collection (trop1)	*Dianthus caryophyllus*	Italy	+	−
Phytophthora colocasiae	CBS 955.87	*Colocasia esculenta*	India	+	−
Phytophthora idaei	CBS 968.95	*Rubus idaeus*	UK	+	−

inoculated by removing them from growing pots and placing infected wheat and hemp kernels directly in contact with the root system; plants were then potted again. Ten gerbera plants were grown as non-inoculated control.

Sampling

Roots from 10 gerbera artificially inoculated plants and 10 plants infected by re-circulating nutrient solution were collected separately 7, 14, 21, 28 days after inoculation. Roots from 10 naturally infected plants obtained from Italian ornamental plant growers and from 10 non-inoculated plants were also analyzed. Three litres of re-circulating nutrient solution were collected separately from each of the two different closed soilless cultivation systems at 7, 14, 21, 28 days after placing the infected plants in the systems. The two solutions (500 ml each) were filtered through two thin nitrocellulose membranes (pore diameter of 0.5 μm) in order to collect pathogen zoospores. Membranes were then used for DNA extraction. DNA was extracted in triplicate for each sample. Infected plant roots were plated in triplicate on oomycete selective medium (Tsao 1970). After incubation at 25°C for 5 days the identity of the pathogen was confirmed using classical taxonomy techniques.

Table 2 List of microrganisms used in this study, their designations and origins

Species	Isolates numbers	Isolated from	Geographic origin	NMS1/NMS2 amplification	Cryp1/Cyip2 amplification
Alternaria sp.	Agroinnova collection (A21)	*Pyrus communis*	Italy	+	−
Pythium ultimum	Agroinnova collection (P3)	*Argiranthemum frutescens*	Italy	+	−
Pythium sylvaticum	Agroinnova collection (P4)	*Fragaria vesca*	Italy	+	−
Mortierella sp.	Agroinnova collection (M2)	*Ocymum basilicum*	Italy	+	−
Verticillium dahliae	Agroinnova collection (V2)	*Solanum tuberosum*	Italy	+	−
Monilia sp.	Agroinnova collection (M5)	*Prunus* sp.	Italy	+	−
Coryneum sp.	Agroinnova collection (C6)	*Prunus* sp.	Italy	+	−
Fusarium oxysporum f.sp. *radicis lycopersici*	Agroinnova collection (F10)	*Lycopersicon esculentum*	Italy	+	−
Fusarium oxysporum f.sp. *lycopersici*	Agroinnova collection (F3)	*Lycopersicon esculentum*	Italy	+	−
Fusarium oxysporum f.sp. *melonis*	Agroinnova collection (F9)	*Cucumis melo*	Italy	+	−
Fusarium oxysporum f.sp. *dianthi*	Agroinnova collection (F7)	*Dianthus caryophillus*	Italy	+	−
Rhizoctonia solani	Agroinnova collection (R2)	*Solanum melongena*	Italy	+	−
Sclerotinia sclerotiorum	Agroinnova collection (S6)	*Glycine max*	Italy	+	−
Colletotricum acutatum	Agroinnova collection (C10)	*Citrus* sp.	Italy	+	−
Sclerotium rolfsii	Agroinnova collection (S9)	*Cucurbita* sp.	Italy	+	−
Ophiosphaerella korrae	Agroinnova collection (O4)	*Festuca rubra*	Italy	+	−
Gaeumannomyces incrustans	Agroinnova collection (G8)	*Poa annua*	Italy	+	−
Trichoderma sp.	Agroinnova collection (T5)	Soil	Italy	+	−
Botrytis cinerea	Agroinnova collection (B1)	*Fragaria* sp.	Italy	+	−
Erwinia amylovora	Agroinnova collection (E6)	*Pyrus communis*	Italy	+[a]	−
Pseudomonas sp.	Agroinnova collection (P3)	*Lycopersicon esculentum*	Italy	+[a]	−
Xanthomonas sp.	Agroinnova collection (X1)	*Citrus* sp.	Italy	+[a]	−

[a] Amplification tested with 704f/1495r primers

Zoospore production

Phytophthora cryptogea was cultured on V8 medium for 7 days at 24°C in the dark. With the aid of a scalpel, small orthogonal pieces (1×3 cm) were cut from the periphery of the colony, and transferred to soil organic matter for stimulating the production of sporangia and zoospores. The cultures were incubated at 24°C for 1 week with light, chilled to 13°C for 3 days in the dark, returned to 24°C for 3 days with light, and chilled again at 13°C for a further 1–3 days in the dark to allow the development of sporangia. Zoospore release was achieved by chilling cultures containing sufficient sporangia at 4°C for 1 h. Suspensions of zoospores were collected and diluted after a further 1 h at room temperature. Zoospores were collected by passage of the suspension through Whatman No. 541 filter paper to remove chlamydospores and mycelial fragments and counted using a haemocytometer (BioKobe). Suspensions were adjusted to a final concentration of 5×10^4, 5×10^3, 5×10^2, 5×10^1 zoospores ml^{-1} of sterilized water.

DNA extraction

DNA from all microorganisms listed in Tables 1 and 2 and from *P. cryptogea* (ATCC66770) zoospores was extracted using the Nucleospin Plant kit (Macherey Nagel) according to the manufacturer's instructions. Zoospores were centrifuged at 11,000×*g* for DNA extraction. About 100 mg of artificially, naturally infected and non-infected roots were ground in liquid nitrogen with a mortar and pestle. The fine powder was used for the DNA extraction. Bacterial lysates were prepared by placing individual colonies in filter-sterilized distilled water at 95°C for 5 min and on ice for 2 min. Centrifugation at 14,000×*g* for 5 min followed, and the supernatant was used in PCR

without further purification. Membranes used for the filtration of re-circulating nutrient solutions containing zoospores were ground in liquid nitrogen and DNA extracted using the same kit described above.

Primer design and PCR amplification

A ClustalW alignment of the *Ypt*1 gene sequences present in databases and belonging to *Phytophthora* species closely related to *P. cryptogea* (DQ162987), *Phytophthora cinnamomi* (DQ162987), *Phytophthora infestans* (AF424657), *Phytophthora citricola* (DQ162968), *Phytophthora citrophthora*: (DQ162968), *Phytophthora drechsleri* (DQ162989), *Phytophthora megasperma* (DQ162986), and *Phytophthora ramorum* (DQ162992) was used to identify conserved and different regions. On the basis of *P. cryptogea* regions of divergence, two primers were designed and called Cryp1: 5′-CCTGGTGGCCGACAAGGTCC-3′ and Cryp2: 5′-GTTGAGTTCGCGCCGTACC-3′. Conventional PCR was used to evaluate primers Cryp1/Cryp2 with DNA extracted from pure cultures, re-circulating nutrient solutions, and plant root samples. Each 20 μl PCR reaction contained 2 μl of DNA templates (30 ng), 250 mM each deoxynucleoside triphosphate, 2 μl of 10× buffer (Taq DNA Polymerase, Invitrogen), 0.7 mM of each primer, and 1.0 U Taq DNA Polymerase (Invitrogen). The reaction was carried out in a TGradient thermal cycler (Biometra) programmed with touchdown PCR protocol consisting of two phases: phase 1 included an initial step at 94°C for 4 min, followed by 10 cycles of denaturation at 94°C for 15 s, annealing at variable temperature for 30 s, and extension at 72°C for 20 s. In the first cycle, the annealing temperature was set to 63°C and, at each of the subsequent cycles, the annealing temperature was decreased by 0.5°C (i.e., it varied from 63°C to 58°C at 0.5°C decrements along the 10 cycles). Phase 2 consisted of 25 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 20 s. To ascertain that DNA extractions were successful, PCR reactions were performed using ITS4/ITS6 primers (Cooke and Duncan 1997) for *Phytophtora* species, NMS1/NMS2 universal primers for other fungi (Li et al. 1994) and universal eubacterial primers 704f/1495r for bacteria (Zhihong et al. 2002). A minimum of one negative water control was included with each PCR run. The results were only considered where no DNA amplification was visible on gel electrophoresis for such negative controls. A 7 μl aliquot of PCR products from each reaction was electrophoresed in 1.5% agarose gel and then stained with SYBR Safe (Invitrogen). The amplified fragments were cloned into the pCR4 TOPO vector (Invitrogen) using the TOPO TA cloning kit and sequenced by Genome Express (Padova-Italy) using an ABI PRISM 3730XL DNA Sequencer.

Primer specificity and sensitivity

The specificity of the primer pair Cryp1/Cryp2 was assessed by PCR with mycelium DNA from 17 isolates of *P. cryptogea* originating from different hosts and geographic locations (Table 1). These primers were also tested with DNA from 34 other species of *Phytophthora* (Table 1), water moulds, bacteria and true fungi (Table 2). The cross-reactivity of the designed detection system with gerbera tissue and its micro-flora was checked by testing different extracts prepared from plants tissues (stems and roots) free of *Phytopthora* blight disease symptoms. The performance of the selected primers was further evaluated: 100 and 2 pg μl^{-1} of *P. cryptogea* genomic DNA was amplified in the presence of 40 ng μl^{-1} genomic DNA extracted from a non-inoculated gerbera plant. In addition, specific amounts of target DNA (either 100, 50, 10 pg μl^{-1}) were added to samples containing 10 pg μl^{-1} DNA isolated from organisms listed in Table 2.

The sensitivity of Cryp1/Cryp2 primers was evaluated using spore PCR. Zoospore PCR was performed twice at levels of DNA extracted from 5×10^4, 5×10^3, 5×10^2, 5×10^1 zoospores ml^{-1} of sterilized water. Additional sensitivity tests were performed with a serial dilution of purified culture DNA, ranging from 160 pg to 160 ag eluted μl^{-1} reaction mix. PCR using primers Cryp1/Cryp2 was performed on DNA extracted from naturally infected roots, artificially infected roots collected 7, 14, 21, 28 days after inoculation, nutrient solution of two different closed soilless cultivation systems, zoospores and non-inoculated roots. All tests were repeated at least three times.

Real-time PCR

A portion of the *P. cryptogea Ypt*1 *gene* was amplified using conventional PCR protocol described above. The resulting 369 base pair fragment was ligated into a pCR 4.0 plasmid and subsequently cloned into One Shot Top10 chemically competent *Escherichia coli* with a TA Cloning kit (Invitrogen) using the manufacturer's

instructions. Transformed *E. coli* were grown overnight in a shaking water bath at 37°C in Luria Bertani broth (Invitrogen) supplemented with ampicillin. The *E. coli* were pelleted and plasmids extracted and purified with the FastPlasmid Mini kit (Eppendorf) using the manufacturer's instructions. Five microliters of purified plasmid solution was diluted (1:100) and the DNA quantified in a spectrophotometer. A theoretical number of plasmid copies and reaction efficiency were calculated. A standard curve was constructed by plotting the logarithm of 10-fold serial plasmid dilutions ranging from 3.5×10^7 to 3.5×10^1 copies per reaction. Duplicates of each serial dilution underwent real-time PCR.

Real-time PCR assays were performed in an iCycler (BioRad, Hercules, CA) apparatus and the results were analyzed using the manufacturer's software (Optical System Software, v. 3.0; BioRad). Each reaction mixture (25 μl) contained 2 μl of undiluted DNA, 2x Quanti Fast PCR kit (Qiagen), and 1 μM of Cryp1/Cryp2 primers. Reactions of each DNA sample were run by performing the following thermal cycling protocol: 1 cycle at 95°C for 2 min, 50 cycles at 95°C for 15 s, 66°C for 25 s, 72°C for 30 s. A melting curve (45–95°C) with a heating rate of 0.5°C for 10 s and a continuous fluorescence measurement (at 530 nm) was recorded at the end of every run to assess product specificity (Ririe et al. 1997). Positive results were confirmed by resolving the product on 1.5% agarose gel.

Analysis of sequence data

Amino acid and nucleotide sequences were retrieved from the GenBank, EMBL and PIR databases. The ClustalW programme (Altschul et al. 1997) was used for pairwise and multiple alignments using default gap penalties. BLAST probing of the DNA and protein databases was performed with the BLASTn, BLASTP and BLASTX programmes.

Results

Specificity of Cryp1/Cryp2 primers and detection limit of conventional PCR

PCR with primers Cryp1/Cryp2 amplified DNA from all *P. cryptogea* isolates (Table 1) producing a specific amplicon of 369 bp. An amplified fragment was obtained from all the *P. cryptogea* belonging to molecular groups B, C, D (Mills et al. 1991). No signal was obtained from *P. drechsleri* and *P. erythroseptica* of molecular group A (Mills et al. 1991).

No amplicon was obtained from any of the 34 other *Phytophthora* species tested (data not shown), including three isolates of *P. drechsleri* and from non-*Phytophthora* species and soil bacteria DNA (Table 2). Direct sequencing of every amplified fragment showed a nucleotide sequence exhibiting 100% identity with *Ypt*1 gene of *P. cryptogea*. To ensure good quality DNA, extracts were amplified with primers ITS4 /ITS6 (Cooke and Duncan 1997), NMS1/NMS2 (Li et al. 1994) or 704f/1495r (Zhihong et al. 2002). These primers yield a fragment of about 900, 600 and 700 bp, respectively (data not shown).

PCR performed on DNA extracted from 5×10^4, 5×10^3, 5×10^2, 5×10^1 zoospores of *P. cryptogea* showed that an amplified fragment of the expected size was detected in the case of 5×10^4 and 5×10^3 zoospores. No signal was detected on DNA extracted from 5×10^2 and 5×10^1 zoospores. The concentration of recombinant plasmid DNA was calculated to be 80 ng ml^{-1} equivalent to 3.5×10^7 *Ypt* 1 copies. Ten-fold serial recombinant plasmid dilution ranging from 3.5×10^7 to 3.5×10^1 copies was prepared and used as template in conventional PCR. The detection limit was 3.5×10^3 copies equivalent to 16 fg.

Detection of *P. cryptogea* in planta and in re-circulating nutrient solution

After 21 days from *P. cryptogea* inoculation the first symptoms were observed on gerbera plants. Using the Cryp1/Cryp2 primers no amplification was observed on DNA extracted from non-inoculated plants, whereas a fragment of the expected size was found on DNA extracted from gerbera roots artificially infected and collected 21 days after inoculation and from naturally infected roots. No amplicon was revealed on the two re-circulating nutrient solutions collected at 7, 14, 21 days after inoculation and in symptomless roots collected on the 7th and 14th day. Sequence analysis of 350 bp amplified fragment showed 100% homology with the *P. cryptogea Ypt*1 gene.

Real-time PCR: sensitivity of the assay

Real-time PCR assays using Cryp1/Cryp2 primers were performed on DNA extracted from organisms

listed in Tables 1 and 2 for any cross-reaction. A standard curve was constructed by plotting recombinant plasmid 10-fold dilution ranging from 3.5×10^7 to 3.5×10^1 copies against the Ct values obtained from real-time PCR. Plotting of fluorescence intensity against the cycle number resulted in a characteristic sigmoidal kinetic function for the various concentrations of target DNA (Fig. 1a). The calibration curve resulting from linear regression is shown in Fig. 1b. An average squared regression (R^2) of 0.999 indicated good correlation between the amount of template and Ct values. The minimum plasmid copy number was 3.5×10^1 equivalent to 160 ag of DNA.

A melting curve programme was run following the PCR to characterise the amplification products present in each sample and in the external standard. The melting curves resulting from the DNA extracted from all the *P. cryptogea* isolates showed a Tm maximum at 88.0°C (Fig. 1c). No other maximum was visible. To confirm the presence of a specific PCR product of 369 bp, a series of recombinant plasmid DNA was analysed after real-time PCR in

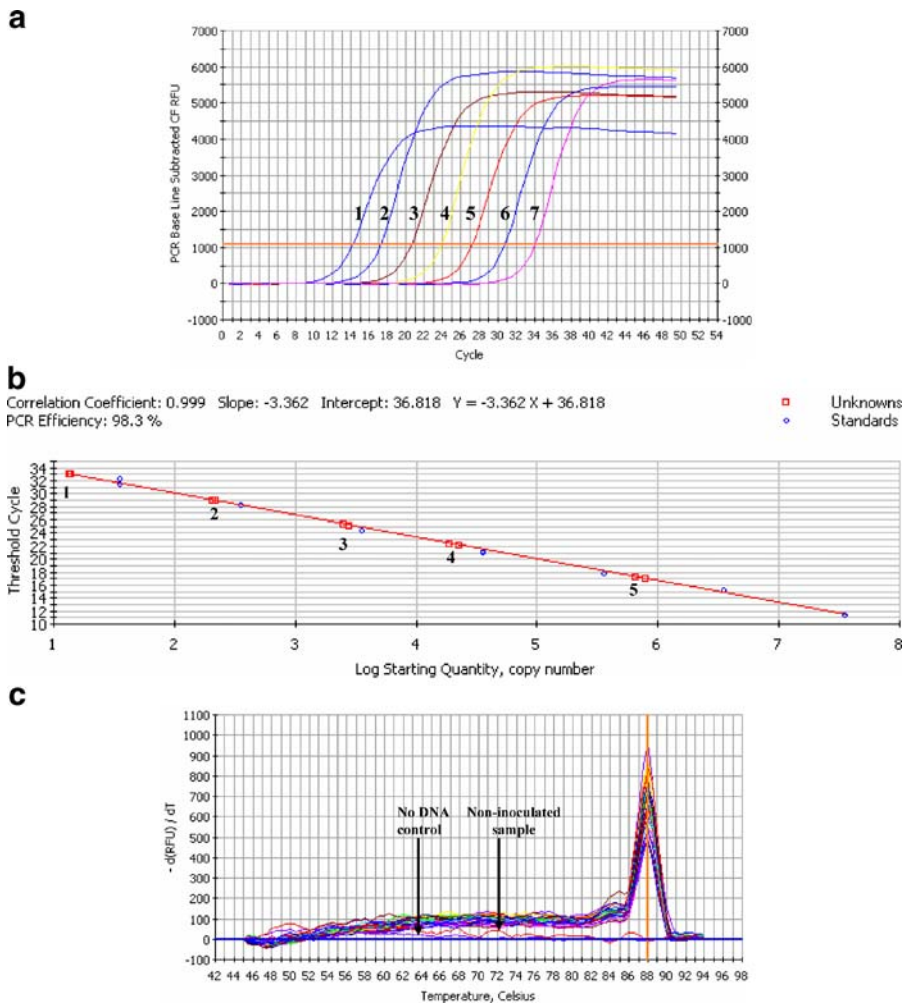

Fig. 1 Quantification of diluted DNA. **a** Kinetic of fluorescence signal at different concentrations of target plasmid DNA (160 pg–160 ag): (*1*) 3.5×10^7 *Ypt*1 gene copies, (*2*) 3.5×10^6 *Ypt*1 gene copies, 3: (*3*) 3.5×10^5 *Ypt*1 gene copies, (*4*) 3.5×10^4 *Ypt*1 gene copies, (*5*) 3.5×10^3 *Ypt*1 gene copies, (*6*) 3.5×10^2 *Ypt*1 gene copies, (*7*) 3.5×10^1 *Ypt*1 gene copies. **b** Standard curve obtained by plotting the log of plasmid DNA concentrations (160 fg–160 ag) values and unknown DNA concentration of symptomless roots collected 14 days after inoculation (*1*), DNA extracted from zoospoores present in the nutrient solution collected 7 days after inoculation (*2*), DNA from roots artificially infected by contaminated nutrient solution collected on 21st day (*3*), DNA extracted from symptomatic roots collected 21 days after inoculation (*4*), DNA from naturally infected roots (*5*). **c** Melting curve profile for real-time PCR amplification on *P. cryptogea* pure genomic DNA and unknown samples

1.5% agarose gel (Fig. 2). All the samples showed the presence of the amplified fragment of the expected size. Blastn analysis of 350 bp obtained from the sequencing of the amplicon showed 100% sequence identity with the *Ypt*1 gene of *P. cryptogea* present in databases. Using the real-time PCR protocol a melting curve with a Tm maximum at 88.0°C was visualized also on DNA extracted from 5×10^1 zoospores.

Quantification of *P. cryptogea* in infected symptomless plants and in re-circulating nutrient solution

Pathogen DNA was quantified in the roots of naturally and artificially infected gerbera plants and in the re-circulating nutrient solution. In the case of symptomless plants a signal was detected on the 14th day after the inoculation (Fig. 1b). The same signal was also found in the re-circulating nutrient solutions collected 7 days after inoculation and in naturally infected plants (Fig. 1b). No signal was detected in the non-inoculated gerbera samples used as negative controls (Fig. 1c). Positive results were confirmed by resolving the product of real-time PCR amplification on 1.5% agarose gel (Fig. 2). Under the PCR conditions tested, the minimum quantity of pathogen DNA that could be accurately quantified in gerbera symptomless artificially infected roots was 1.36×10^1 copies of *Ypt*1 gene equivalent to 62 ag of DNA (Fig. 1b). Inhibitory effects on amplification efficiency occurred with target DNA concentrations above 100 pg μl^{-1} reaction mixture. *Phytophthora cryptogea* DNA amplification was inhibited by the addition of 600 pg or more of plant DNA.

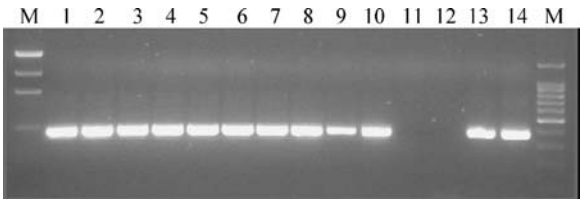

Fig. 2 Agarose gel electrophoresis of real-time PCR products. *Lanes 1 to 7* Tenfold dilution of plasmid DNA in the range of 160 pg–160 ag; lane 8: DNA from symptomless roots collected 14 days after inoculation; *lane 9* DNA extracted from zoospores present in the nutrient solution collected 7 days after inoculation; *lane 10* DNA from roots artificially infected by contaminated nutrient solution collected on 21st day; *lane 11* uninoculated roots; *lane 12* no DNA; *lane 13* DNA extracted from symptomatic roots collected 21 days after inoculation; *lane 14* DNA from naturally infected roots. *M* DNA marker size. DNA Low Mass Ladder (Invitrogen)

Discussion

The primers proposed in the present study were tested for specificity and sensitivity. Specificity was verified by the absence of cross-reaction with DNA from different *Phytophthora* species, several fungal species and the gerbera plant. The results showed that Cryp1/Cryp2 primers amplified DNA extracted from all *P. cryptogea* isolates representing worldwide diversity. No amplification was obtained with 34 other *Phytophthora* species including *Phytophthora drechsleri. Phytophthora cryptogea* and *P. drechlseri* have a long history of taxonomic controversy involving their classification (Mills et al. 1991; Forster et al. 2000). They are characterized by non-papillate sporangia and anphigynous antheridia and they are placed into group VI (Stamps et al. 1990). In addition to subtle differences, *P. drechsleri* is thought to be distinct from *P. cryptogea* by its capability to grow at 35°C. An analysis based on isozymes and mtDNA RFLP (Mills et al. 1991) showed that these two species could not be differentiated. Nine distinct molecular groups with little genetic similarity could be identified (Mills et al. 1991). On the contrary, Forster et al. (2000) demonstrated that *P. cryptogea* and *P. drechsleri* should not be merged into a single species. Primers developed in this work were able to recognise all *P. cryptogea* isolates analyzed belonging to molecular group B, C and D (Mills et al. 1991). DNA extracted from *P. drechsleri* and *Phytophthora erythroseptica* making part of group A (Mills et al. 1991) were not amplified. Primers Cryp1/Cryp2 were not checked on isolates belonging to other molecular groups.

The *P. cryptogea* inoculum was a group B member (Mills et al. 1991) and its DNA was easily detected by primers Cryp1/Cryp2 in artificially infected gerbera roots and re-circulating nutrient solution of closed soilless cultivation systems. Furthermore, no reports are present in the literature about the occurrence of *P. drechsleri* in *Gerbera jamesonii.*

Regarding sensitivity, using a 10-fold dilution series of recombinant plasmid DNA, a visible amplification product was detected down to 16 fg of *P. cryptogea* DNA (equivalent to 3.5×10^3 *Ypt*1 copies) with conventional PCR and to 160 ag with

real-time PCR (equivalent to 3.5×10^1 *Ypt*1 copies). The limit of zoospores detection was 5×10^3 and 5×10^1 using conventional and real-time PCR protocol, respectively. Real-time PCR is 100 times more sensitive; this will be especially important when the target concentration is low or PCR inhibitory substances are present. These results of sensitivity are quite similar to those obtained by other authors with different *Phytophthora* species (Ippolito et al. 2002; Grote et al. 2002; Kong et al. 2003). Our method is about ten times less sensitive than these previously published methods, probably due to the fact that *Ypt*1 is a single copy gene (Schena et al. 2006).

In the second part of this work, the ability of PCR assays to detect fungal DNA in plant tissue and recirculating nutrient solution was tested. The PCR protocols were used effectively to detect *P. cryptogea* in artificially infected plants using conventional and real-time PCR. In this case, conventional PCR was able to detect the pathogen 21 days post-inoculation in roots of symptomatic plants. However, real-time PCR was able to detect the fungal DNA in roots of symptomless plants 14 days after inoculation.

The primers developed were also assayed for their ability to detect zoospores. Zoospores are an important target for detection because they spread the disease in water and infect the host after encystment. The limit of zoospores detection in vitro was 5×10^3 and 5×10^1 by using conventional and real-time PCR protocol, respectively. Low detection levels of zoospores were useful to detect the pathogen in recirculation nutrient solution of the closed soilless cultivation system using the real-time PCR protocol.

The detection technique proposed in this work costs approximately €15–20 for the analysis of 96 samples and requires about 3 h of work. Such costs can be easily sustained by the gerbera industry. This new effective diagnostic tool can be considered a crucial component of plant disease management.

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