REAL-TIME POLYMERASE CHAIN REACTION FOR IDENTIFICATION OF A HIGHLY PATHOGENIC GROUP OF *FUSARIUM OXYSPORUM* F.SP. *CHRYSANTHEMI* ON *ARGYRANTHEMUM FRUTESCENS* L.

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SUMMARY

A real-time Polymerase Chain Reaction (PCR) system based on Taqman® chemistry was developed in order to identify a new group of *Fusarium oxysporum* f.sp. *chrysanthemi* highly pathogenic on Paris daisy (VCG 0052). Sensitivity of PCR and real-time PCR was compared on genomic DNA from mycelium. The ability to identify symptomless infections on daisy was also evaluated using selective substrate, PCR and real-time PCR. Real-time PCR could identify infected plants on the fifth day after artificial inoculation, although plants remained symptomless until the 13th day after inoculation. The real-time PCR could detect as little as 300 fg of genomic DNA. The advantages of real-time PCR as a diagnostic system for this new epidemic disease are discussed.

Keywords: Taqman probe, selective substrate, PCR sensitivity, prevention, VCG 0052.

INTRODUCTION

Argyranthemum frutescens L., commonly known as Paris daisy, has become very popular in the last twenty years. In Italy it is mainly grown for northern European markets where it is appreciated as a spring flower. The Albenga area, located in the Liguria region, is the main producer of potted Paris daisy with more than twelve million pots sold every year (Garibaldi *et al.*, 2002).

Recently, probably due to intensified cultivation, a new disease caused by *Fusarium oxysporum* has been reported (Garibaldi *et al.*, 1998). The causal agent has now been further identified as *F. oxysporum* f.sp. *chrysanthemi*, belonging to a new vegetative compatibility group named VCG 0052 (Pasquali *et al.*, 2004a). This group has the peculiarity to be highly pathogenic on Paris daisy, as shown when its pathogenicity was evaluated on other *Chrysanthemum* species (Garibaldi *et al.*, 2002). It has spread in recent years causing concern among growers. RAPD analysis showed that the isolates sampled have a clonal origin sharing the majority of bands (Pasquali *et al.*, 2003). The symptoms are typical of *Fusarium* wilt, including yellowing and progressive wilting, dark-blue or black basal necrosis, and xylem discoloration. They develop at 25°C, so the disease cannot be identified on mother plants during winter or early spring. Thus epidemic spread of the disease is facilitated. No single approach can be entirely effective in eradicating the disease, but detection and early elimination of infected propagating material is fundamental for an integrated disease management aiming to avoid epidemics like those experienced in the years 1998-2000 (Garibaldi *et al.*, 2002; Minuto *et al.*, 2000).

Molecular methods have been developed to distinguish the pathogen by RAPD-PCR of DNA from fungal colonies grown on agar media (Pasquali *et al.*, 2003), or directly from plant tissues by PCR based on a specific sequence obtained analysing the *Fot 1* transposon insertions (Pasquali *et al.*, 2004). The aim of this study was to develop a faster and more sensitive method of identification of the new group of *Fusarium oxysporum* than traditional PCR analysis. The development of real-time PCR based on Taqman® chemistry (Holland *et al.*, 1991, Lee *et al.*, 1993, Heid *et al.*, 1996) is reported. We present the rapid and sensitive identification of *F. oxysporum* isolates in infected mother plants and the comparative sensitivity of PCR, Real-Time PCR and the use of selective substrate for its detection.

MATERIALS AND METHODS

Plant inoculation and sample collection. *F. oxysporum* f.sp. *chrysanthemi* isolate VIG 4, chosen as the representative isolate for pathogenic studies on *A. frutescens*, was grown in 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth 24 g l^{-1} (Difco Laboratories, Detroit, MI, USA) with yeast extract 5 g l^{-1} (Difco Laboratories, Detroit, MI, USA) at 26°C by shaking at 150 rpm under constant light. After 6 days, the fungal culture was aseptically filtered through 4 layers of cheesecloth and conidia were diluted serially in sterile Ringer solution to

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a final cell density of $1 \cdot 10^7$, $1 \cdot 10^6$, $1 \cdot 10^5$ CFU ml^{-1}. The inoculum was applied by dipping 20-day-old rooted cuttings of the susceptible cv Camilla Ponticelli in VIG 4 conidial suspension for 30 sec at transplant. Moreover, 5 ml of conidial suspension were added to the soil. A control was added by dipping daisy cuttings in sterile distilled water at transplant. Plastic pots (one plant per pot, 1 l capacity) were filled with a steam-disinfested potting mixture (pH 5.5) containing two parts soil (pH 6.9, P 352, K 1700, Ca 1500, Mg 415, Zn 29, Mn 8.7, Fe 130 mg ml^{-1}) and one part peat moss (v/v). Pots were kept in a temperature-controlled room (28°C; relative humidity 50-90%; daily light conditions: 50-60 KLux per m^2). From day 1 to day 13 after transplant, every day one plant for each treatment (VIG4 and water) was uprooted and cut lengthwise. Four more plants for each treatment were maintained in the growth chamber up to the 27^{th} day after inoculation, to measure development of the disease. Stems and roots were isolated, washed and brushed with sterile water, surface-sterilized by dipping once in 3% sodium hypochlorite and twice in sterile distilled water, air-dried, weighted and frozen in liquid nitrogen for 5 minutes. Total DNA was then extracted from plants treated with the conidial concentrations stated above. Every day a small fraction of the internal vessels of the stem and pieces of root were plated on *Fusarium*-selective Komada substrate (Komada, 1975) in order to check internal colonization by the VIG 4 isolate. The experiment was repeated once.

DNA preparation. Genomic DNA from pure fungal cultures of all the isolates reported in Table 1 was extracted as described by Chiocchetti *et al.* (1999). There were 11 isolates pathogenic to Paris daisy and reference controls from the American Type Culture Collection, belonging to the two known ff.spp. *chrysanthemi* and *tracheiphilum* that can be isolated from *Chrysanthemum* species.

DNA from artificially infected Paris daisies was extracted according to Chiocchetti *et al.* (1999) with slight modification. Briefly, 100 mg of surface-sterilized plant stems or roots were ground in a mortar with liquid nitrogen. One milliliter of extraction buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 1% polyvinylpyrrolidone, 2% hexadecyltrimethyl-ammonium bromide, pH 8), containing 100 µg proteinase K, was added, and the lysate was incubated for 15 min at 37°C. After centrifugation at 13000 rpm for 2 min, the supernatant was phenol-extracted once, treated with one volume of isopropanol, rinsed with ethanol and resuspended in TE pH 8.0.

All purified DNAs (from pure fungal cultures and from infected plants) were stored at 4°C and quantified by gel and Bio-Photometer (Eppendorf, Hamburg, Germany) analysis. All DNA samples were brought to a concentration of 100 ng μl^{-1}. To verify PCR sensitivity all samples belonging to VCG 0052 were serially diluted ten-fold from 100 ng μl^{-1} to $1 \cdot 10^{-3}$ ng μl^{-1}. To obtain a calibration curve and to verify the sensitivity of real-time PCR, serial ten-fold dilutions from 150 ng μl^{-1} to 1.5 fg μl^{-1} of pure genomic DNA of the isolate SCO11 were prepared in TE 0.1 (10 mM Tris pH 8, 0.1 mM EDTA).

PCR and real-time PCR reactions. PCR reactions were performed as described by Pasquali *et al.* (2004) using primers Mg5 (5'-GGGGTCGGTTACATGGG TG-3') and Mg6 (5'-CAACAACAAGGCGAAGAGG G-3'). PCR products were visualized by loading 5 µl onto a 1.5% agarose gel containing 0.5 µg of ethidium bromide ml^{-1}, followed by electrophoresis in the Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time PCR primers and the probe were designed using the Input3 program (S. Rozen and H.J. Skaletsky, 1998. Primer3. Code available at the website: htpp://www.genome.wi.mit.edu/genome_software/other/primer 3.html). The primers and the probe were based on the Gene Bank deposited sequence AF282999 (Pasquali *et al.*, 2004). In particular, three primer pairs were designed (Fig. 1): the previously designed Mg5 and Mg6 and the pairs Mg1 (5'-TCTGAAGACGTGTCTGAGGT-3') + Mg2 (5'-GGCCAAAACCGTGTAGGATA-3'), and Mg3 (5'-GTCGGTTACATGGGTGCTTG-3') + Mg4 (5'-GC-CAAAACCGTGTAGGATAATC-3'). Each primer pair was used together with the probe Marge (5' FAM d(TAAGCAGGCGAGTCCTTGATTTGAT) TAMRA 3') on genomic DNA to evaluate the efficiency of the three systems.

Real-time PCR was performed in a LightCycler device (Roche Diagnostics, Mannheim, Germany) using the following conditions: initial heat denaturation at 95°C for 2 min, followed by 30 to 40 cycles each consisting in a denaturation step at 95°C for 5 sec, annealing at 56°C for 15 sec and extension at 72°C for 20 sec.

Two µl of genomic DNA were amplified in 75 mM Tris-HCl (pH 8.8), 4 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.1% (w/v) Tween® 20, 100 µg ml^{-1} BSA (Sigma, Saint Louis, Missouri, USA), 200 µM of each dNTP (Invitrogen, Carlsbad, CA, USA), 200 nM of probe Marge, 500 nM of each primer, 1 unit of Platinum® Taq DNA Polymerase (Invitrogen) in a total volume of 20 µl.

Real-time PCRs on plant tissues and for calculation of the standard curve were performed with the primer pair Mg5-Mg6 and the probe Marge.

A standard curve for quantification was generated by plotting the log of the DNA concentration of the known standard (SCO 11), previously found to be identical to all the other *F. oxysporum* isolates belonging to VCG 0052, against the cycle number at a defined point, chosen with the threshold method, in the log-linear increase phase in fluorescence of the PCR.

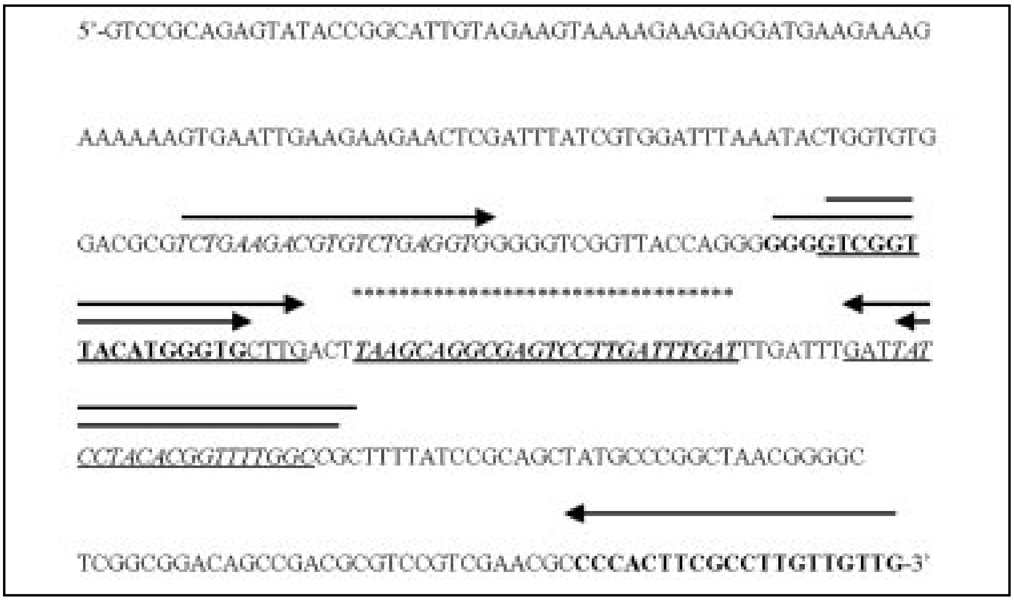

Fig. 1. Primers and probe position on the sequence deposited at GenBank (accession number AF282999) used for real-time PCR amplification. From the left: Mg1(italics), Mg5 (bold), Mg3 (underlined), Marge (stars, underlined, italics, bold), Mg4 (underlined), Mg2 (italics), Mg6 (bold).

Comparison of diagnostic methods on plant samples. Results from Komada isolation, and PCR and real-time PCR on all plant samples were collected under the following criteria. Komada plates containing pieces of root and stem of plants inoculated with VIG 4 conidia were evaluated after 3 days incubation: if typical *Fusarium* mycelium appeared the test was considered positive.

PCR performed on DNA extracted from plants inoculated with VIG 4 conidia was evaluated by ethidium bromide staining of a gel under the conditions described above. When a band with the expected molecular size appeared, the test was considered positive.

Real-time PCR tests were taken as positive when the sample reached the threshold level before 45 cycles. When the pathogen was identified with certainty in roots by real-time reaction, no further extractions and amplifications were performed.

The "first day of detection" was determined when both repetitions of the analyses (on three plants inoculated with three different concentration of VIG 4 conidia) gave the same result.

RESULTS

Appearance of plant symptoms. Plants were inoculated with three different concentrations in order to mimic different possible infections that can arise in the field. Plants inoculated with a conidial concentration of $1{\cdot}10^7$ developed the first symptoms 13 after days and died within 7 days. These results confirm previous observations by Pasquali *et al.* (2004). A delay in symptom appearance of about 1 day (14th day) was observed with a conidial concentration at $1{\cdot}10^6$ ml^{-1} and plants died two days later (22nd day). A concentration of $1{\cdot}10^5$ conidia ml^{-1} resulted in first symptoms at the 15th day after inoculation and plants were completely wilted at the 26th day (Fig. 2).

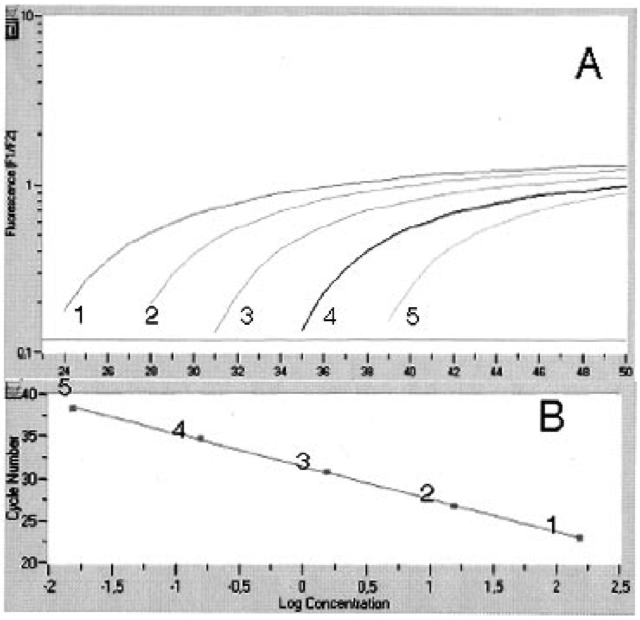

Fig. 2. A) Real-time PCR of DNA of the SCO11 isolate (y axis: fluoroscence ratio; x axis: cycle number; 1: 30 ng; 2: 3 ng; 3: 0.3 ng; 4: 0.03 ng; 5: 0.003 ng). **B)** Standard curve obtained by plotting the log of DNA concentration to the cycle threshold.

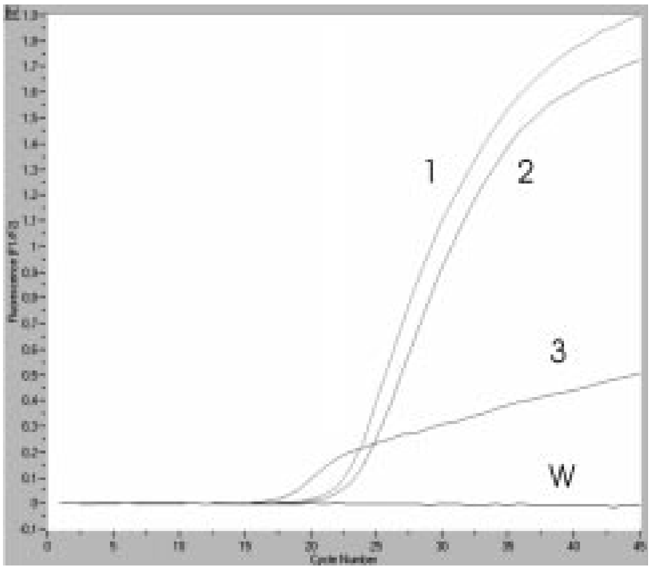

Fig. 3. Real-time PCR of 100 ng of SCO 11 DNA amplified with the primer pairs: **1:** Mg5-Mg6; **2:** Mg3-Mg4; **3:** Mg1-Mg2; **W**: negative control with Mg5-Mg6.

PCR and real-time PCR on genomic DNA. To evaluate the best combination in real-time PCR, 100 ng of DNA of isolate SCO11 were amplified with the three different mixes of primer pairs Mg1-Mg2, Mg3-Mg4, Mg5-Mg6 with the probe Marge. The Mg5-Mg6 pair performed better than the other two primer pairs in real-time amplification (Fig. 3) and was therefore chosen in the following studies.

To prove the efficacy of PCR and real-time PCR with the Mg5-Mg6 primers, 100 ng of genomic DNA of all the isolates listed in Table 1 were amplified. As previously described, the specificity of the primers used was confirmed by obtaining the expected amplification product of 166 bp on all the isolates belonging to the highly pathogenic group of *Fusarium oxysporum* f.sp. *chrysanthemi* isolated in Liguria region. Real-time PCR confirmed its specificity, amplifying only the isolates belonging to VCG 0052, highly pathogenic on Paris daisy (Table 1).

Sensitivity comparison of traditional and real-time PCR. DNA from all the isolates from Paris daisy collected in Liguria region (QUA1, QUA2, QUA 3, VIG 4, VIG5, CRI 6, PER 7, REP 8, CER 9, SCO11) were serially diluted and added in different amounts (1 ng, 10^{-1} ng, 10^{-2} ng, 10^{-3} ng) to the reaction mixture. PCR was performed using the primer pair Mg5-Mg6 and sensitivity of the reaction was evaluated on ethidium bromide-stained gels by running 5 µl of the amplification product. Traditional PCR showed a detection limit of $1{\cdot}10^{-2}$ ng (Fig. 4).

The DNA of isolate SCO11 was serially diluted and amounts of $1.5{\cdot}10^{2}$ to $1.5{\cdot}10^{-6}$ ng were used to find the detection limit of the real-time PCR. It was confirmed to be highly sensitive allowing the amplification of 300 fg of genomic DNA of isolate SCO 11 (Fig. 5A). Serial dilution of SCO 11 DNA allowed construction of a standard curve confirming the linearity of the Ct value when plotted against the amount of fungal DNA over a 10^{5} fold range (Fig. 5B) with a correlation coefficient (r) of -1 and a mean squared error of 0.0271.

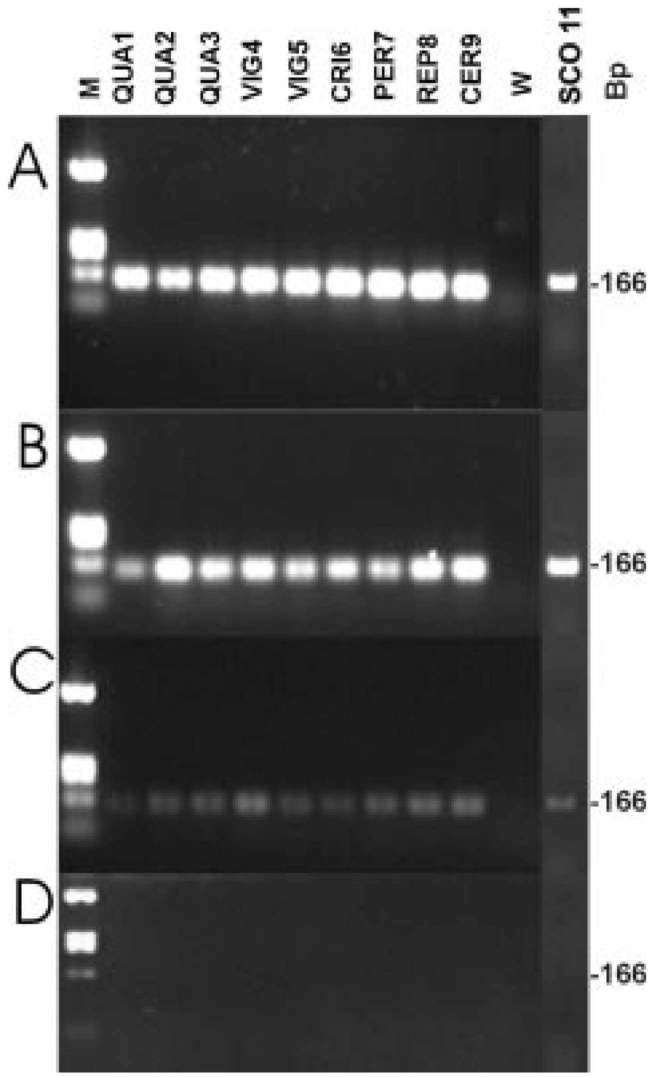

Fig. 4. PCR amplification with the primer pair Mg5-Mg6 of different amounts (**A**, 1 ng; **B**, 10^{-1} ng; **C**, 10^{-2} ng; **D** = 10^{-3} ng) of genomic DNA of isolates QUA1, QUA2, QUA 3, VIG 4, VIG5, CRI 6, PER 7, REP 8 , CER 9, SCO 11 visualized on a 1.5% agarose gel stained with ethidium bromide. (**W**: water; molecular size (bp) indicated on the right).

PCR and real-time PCR on plants extracts. Taqman® PCR and traditional PCR were tested on DNA extracted from infected plants for their ability to identify early infections, as compared with the use of selective substrate.

Isolation on Komada selective substrate proved highly

Table 1. *Fusarium oxysporum* isolates used in this study, and result of real-time PCR with the primer pair Mg5-Mg6 and 100 ng of template DNA**.**

Code	*Forma specialis*	Host plant	Geographic origin and year of isolation	Real-time amplification
QUA 1 (CBS 112084)*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1997)	+
QUA 2*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1997)	+
QUA 3*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1997)	+
VIG 4*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1998)	+
VIG 5*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1998)	+
CRI 6*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1998)	+
PER 7*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1999)	+
REP 8*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1999)	+
CER 9*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (1998)	+
BAC 10 CBS (112085)	*F. oxysporum* f.sp. *unknown*	*Argyranthemum frutescens*	Moncalieri, Torino, Italy (1997)	-
SCO 11*	*F. oxysporum* f.sp. *chrysanthemi*	*Argyranthemum frutescens*	Albenga, Savona, Italy (2001)	+
ATCC66279	*F. oxysporum* f.sp. *chrysanthemi*	*Chrysanthemum morifolium*	California, USA	-
ATCC52422	*F. oxysporum* f.sp. *chrysanthemi*	*Chrysanthemum* sp.	USA	-
ATCC16608	*F. oxysporum* f.sp. *tracheiphilum*	*Vigna unguiculata*	Unknown	-
ATCC16609	*F. oxysporum* f.sp. *tracheiphilum*	*Vigna unguiculata*	Unknown	-
ATCC32724	*F. oxysporum* f.sp. *tracheiphilum*	*Vigna unguiculata*	Nigeria	-
ATCC62913	*F. oxysporum* f.sp. *tracheiphilum*	*Glicine max*	Georgia, USA	-

* isolate belonging to VCG 0052

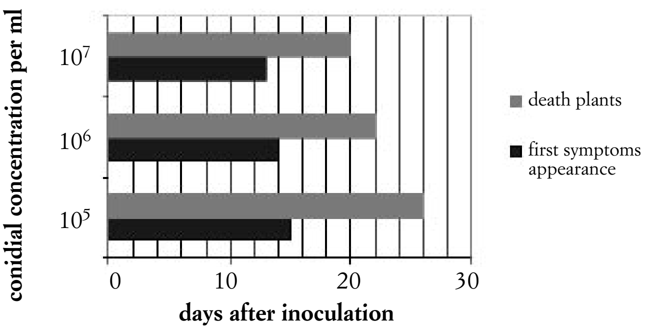

Fig. 5. Appearance of first symptom and complete wilt (death) of daisy plants inoculated with three conidial concentrations of isolate VIG4. The experiment was repeated once.

Table 2. Day of first detection after inoculation, in stem and root extracts (obtained from daisy plants inoculated with $1{\cdot}10^5$, $1{\cdot}10^6$ or $1{\cdot}10^7$ conidia ml^{-1} of VIG 4 isolate) by real-time PCR, PCR and plating on Komada medium. The experiment was repeated once. Tests were performed up to the 13^{th} day after infection.

Diagnostic system	Days after infection	
	On root extract	On stem extract
Real time PCR	2	5
PCR	6	9
Komada plating	2	3

sensitive, detecting the pathogen on roots from the second day and inside the stem from the third day after inoculation, but the presence of *Fusarium* was visually detectable on agar medium only after three days from the isolation. The PCR was relatively sensitive allowing secure detection of the pathogen from roots from the sixth day on. On stem extracts PCR detected the pathogen from the 9^{th} day after inoculation. Real-time PCR detected the pathogen on root extracts from the second day, like the selective substrate, showing high sensitivity, and from stem tissue from the fifth day after inoculation. The results are summarized in Table 2.

DISCUSSION

A real-time PCR detection system based on Taqman® chemistry has been developed for early detection of a group of *F. oxysporum* highly pathogenic on *A. frutescens*. This pathogen has spread, through infected mother plants, in recent years in the Liguria region causing concern among growers. The system should allow timely identification of infected mother plants and thus eliminate epidemics initiated by propagative material.

Use of selective substrate, PCR and real-time PCR, for detection of the causal agent in symptomless cuttings were compared. Selective substrate was highly efficient in detecting the pathogen, as already reported (Wang *et al.*, 1999), but it has obvious limits in specificity because it cannot discriminate among slightly pathogenic, highly pathogenic and non-pathogenic isolates. Moreover it requires three days for visual detection of the growth of mycelium.

Traditional PCR was highly specific (Pasquali *et al.*, 2004) but less sensitive than selective medium. The fact that the pathogen was certainly detected in infected roots only from the sixth day may be explained by the efficacy of decontamination of the root surface, avoiding contamination from external conidia, or by a possible inhibition of the PCR caused by root substances.

PCR presents some limits: (i) during the past "it has been adopted with caution by diagnostic laboratories because it is time consuming to confirm the identification of the PCR amplification product" (Schaad and Frederick, 2002); (ii) PCR analysis requires gel visualization increasing the risk of imprecision in the analysis. These are the reasons why we focused on developing a real-time PCR system. In fact, the Taqman® system has the advantage to unify within a single reaction the PCR and the annealing of a specific probe, allowing higher sensitivity when compared to PCR and confirming the specificity of the amplification product in a single step. The same procedure using a PCR test would require to blot the PCR product with a specific probe. Moreover, real-time visualization of the amplification reaction avoids any risk of contamination during gel loading, diminishing at the same time the labour required. In our study we have chosen the FAM-TAMRA system of fluorophores because it is the least expensive, very sensitive and can be read by all thermal-cyclers on the market.

Real-time PCR with a Taqman® probe allowed highly sensitive detection of infected plants using root and stem extracts. No inhibitors appeared to influence the reaction.

The quality of DNA obtained from plant extracts was low when compared with the pure genomic DNA of each isolate. This is mainly due to the protocol adopted for DNA extraction that focused on the rapidity of the procedure. Despite this low quality, the Taqman system performed very well in detecting the pathogen and saving time in the DNA preparation. Therefore within 1.5 hours it is possible to prepare DNA suitable for real-time PCR amplification.

Real-time PCR is sensitive and would permit total automation with the exception of sampling, if coupled with automatic DNA extraction equipment. This opportunity will be evaluated in the future. Our protocol, that still relies on human handling, is very rapid: complete analysis requires 2.5 h. The protocol can be used efficiently in control programs, and large-scale analysis may decrease the unit costs.

This work shows that real-time PCR could be useful for *F. oxysporum* population diagnosis as shown for other plant pathogens (Weller *et al.*, 2000; Bates and Taylor, 2001; Lees *et al.*, 2002; Schena and Ippolito, 2003). A highly sensitive system is required in order to identify possibly infected mother plants in early spring. At this time plants show no symptoms, as these only appear later at temperatures above 25°C. Real-time PCR analysis can identify symptomless infections in late winter and spring, with higher sensitivity than PCR and higher specificity than plating on selective media.

A prospect for real-time PCR development is its use to accurately study fungal growth *in planta*. Future studies will focus on the creation of a quantitative system of detection in order to correlate fungal biomass with the amplification signal. It will then be possible to quantify pathogen growth in infected plants, as showed by Qi and Yang (2002). Moreover, it would be a useful tool to study the spread and physiology of the disease by monitoring the ecology of the pathogen in the soil.

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