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FULL RESEARCH PAPER

Quantitative detection of *Monosporascus cannonballus* in infected melon roots using real-time PCR

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Abstract A method was developed for the specific detection, identification and quantification of *Monosporascus cannonballus* in infected melon roots based on real-time PCR (SYBR® Green chemistry) targeting the ITS1 region of the rDNA conserved between different strains of the pathogen. The specificity of the reaction was assessed using a number of fungi taxonomically and ecologically related to *M. cannonballus*. The method was highly sensitive and *M. cannonballus* was first detected in the roots of a susceptible Piel de Sapo cultivar 2 days after inoculation, before symptom appearance. Although conventional PCR methods could also provide such a specific and sensitive detection, real-time PCR was also able to produce reliable quantitative data over a range of 4 orders of magnitude (from 5 ng to 0.3 pg). The method allowed the quantitative monitoring of fungal growth from the very first stages of infection, and was successfully employed in the early screening of resistance. The assessment of disease progress and severity obtained with real-time PCR was more accurate than that obtained with the visual scoring of root lesions or root biomass losses. Therefore, there exists a great potential for its implementation in those steps of breeding programmes where high accuracy is required.

Keywords *Ascomycetes* · *Cucumis melo* · Disease assessment · ITS-regions · Root rot/vine decline · SYBR® Green

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Introduction

Monosporascus cannonballus a root-infecting ascomycete, causes the *Monosporascus* vine decline of muskmelon. This fungus is found in warm habitats and causes root rot and necrosis which result in reduced growth, progressive defoliation and partial or complete collapse of the plants towards the end of the season. This destructive disease is significant in the US, Israel, Spain and Japan and is spreading to new countries (Martyn and Miller 1996; Dias et al. 2004).

Strategies for controlling vine decline based on the development of *Monosporascus*-resistant cultivars are being conducted in the most affected countries (Cohen et al. 2000; Crosby et al. 2000; Dias et al. 2002). Breeding populations are assayed in naturally-infected fields or under artificial inoculation conditions. Ascospores serve as the primary inoculum in fields. Nevertheless, since ascospore germination is rare

under standard laboratory conditions, active cultures of the fungus grown in a sand/oat hull medium are used in artificial inoculations. The inoculum pressure is most often controlled by the enumeration of colony-forming units (CFU) in fungal cultures (Bruton et al. 2000). The advance of breeding programmes is limited by the lack of reliable methods for the identification and quantification of the pathogen. Until now the identification of the pathogen in damaged roots has relied on conventional methods such as fungal isolation in selective media and the examination of mycelial characteristics. Current methods for disease severity assessment involve the visual scoring of root lesion intensity coupled with the measurement of root biomass reduction in comparison with healthy controls (Crosby et al. 2000; Dias et al. 2002). These procedures for fungal identification and quantification in roots are time- and labour-consuming, have low accuracy, and require skilled expertise to identify the pathogen and to score the lesions. Another drawback of these methods is that detection of the pathogen is only possible in the late stages of infection.

The early, rapid, and accurate identification and quantification of *M. cannonballus* in plant roots is essential in order to assist breeding programmes and to optimize strategies for disease management. Lovic et al. (1995) developed a conventional PCR-based method for detecting *M. cannonballus* by targeting the ribosomal DNA (rDNA). The ITS-region sequence of *Monosporascus* spp was also used in the recognition of the new species *Monosporascus ibericus* (Collado et al. 2002). The ITS regions are conserved at the species level but vary in higher taxa. Therefore, they are especially valuable for species differentiation. Lovic et al. (1995) reported a high conservation of ITS sequences among a set of representative isolates of *M. cannonballus* and *M. eutypoides* (now considered the same species), whereas Collado et al. (2002) reported a similarity of 83% for the ITS sequences of *M. ibericus* and *M. cannonballus*.

Conventional PCR has contributed to the alleviation of some of the issues associated with the identification of *M. cannonballus*. However, end-point PCR methods do not allow accurate quantification. These difficulties may be overcome to a certain extent by real-time PCR, a technology which combines the sensitivity of conventional PCR with the potential for an accurate quantification of target DNA by measuring the intensity of a fluorescent signal that is proportional to the amount of DNA generated during the amplification. There are increasing reports of quantitative real-time PCR applied to detect soil-borne fungi in soils and roots (Cullen et al. 2001; Filion et al. 2003; Van de Graaf et al. 2003; McCartney et al. 2003; Atkins and Clark 2004; Gao et al. 2004; Ippolito et al. 2004; Atkins et al. 2005).

In this paper, we describe for the first time a sensitive, quantitative and specific detection of *M. cannonballus* from pure cultures, artificially inoculated soil and artificially infected roots, by real-time PCR with the SYBR® Green I dye. This method allowed the quantitative monitoring of the fungal colonization of roots of two melon genotypes, one susceptible and one partially resistant to the fungus, from the first stages of infection.

Materials and methods

Fungal isolates

Nine isolates of *M. cannonballus* from different origins (USA, Spain and Egypt), and one Spanish isolate of *M. ibericus* (Collado et al. 2002) were assayed, along with other fungal species taxonomically and ecologically related to *M. cannonballus* (Table 1). The genus *Monosporascus* was first believed to be related to Sordariales, but more recent studies based on the 18S rDNA sequences indicated that it might be closer to Xylariales (Lovic et al. 1995; Collado et al. 2002). Representative species of the genera considered taxonomically most closely related to *M. cannonballus,* such as *Xylaria carpophila* (Xylariaceae, Xylariales), *Daldinia concentrica* (Xylariaceae, Xylariales) and *Gelasinospora endodonta* (Sordariaceae, Sordariales), were included in the analysis. Other fungal pathogens of melon, some of them believed to be involved in the melon vine decline syndrome, were also tested. These were *Acremonium cucurbitacearum, Didymella bryoniae, Fusarium oxysporum* f.sp. *niveum, Fusarium oxysporum* f.sp. *melonis*, *Macrophomina phaseolina, Phytophthora capsici, Pythium aphanidermatum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*.

In all cases, bulk mycelia for DNA extractions were harvested from the surface of 2 week old PDA cultures. The mycelia were ground in liquid nitrogen

Table 1 CT value for each fungal isolate amplified in a real-time PCR with primer pair 2

	Isolate code[a]	Origin/source[b]	CT value[c]
Monosporascus spp. and taxonomically related species			
M. cannonballus (Spain)	McG	Granada, UPV-COMAV	17.20
	McV(p)	Valencia, P. Nuevo, CECT, 2963	17.08
	McC	Castellón, Almenara, UPV-COMAV	17.01
	McV(a)	Valencia, Alboraya, UPV-PPG, Mc0504	17.70
M. cannonballus (USA)	McTx	Texas, B. B., Tx-3035-4	17.56
	McA	Arizona, M. St, Mc 13	17.23
	McC1	California, M. St, Mc 30	17.56
	McC2	California, M. St, Mc 14	17.58
M. cannonballus	McE	Egypt, CBS, 586.93	17.63
M. ibericus	Mi	Spain, Tarragona, CBS, 110550	Und.
Xylaria carpophila	Xc	UK, CBS, 331.70	Und.
Daldinia concentrica	Dc	Greece, CBS, 117124	35.9
Gelasinospora endodonta	Ge	Spain, CBS, 371.83	31.7
Fungal species ecologically related to *M. cannonballus*			
Acremonium cucurbitacearum	Ac	Spain, Valencia, UPV-PPG	Und.
Didymella bryoniae	Db	Netherlands, CBS, 386.65	Und.
Fusarium oxysporum fsp. niveum	Fon	Spain, CECT, 2871	Und.
Fusarium oxysporum fsp. melonis	Fom	Spain, Almeria, CECT, 20474	Und.
Macrophomina phaseolina	Mp	Spain, CECT, 2026	35.01
Pythium aphanidermatum	Pa	Japan, CECT, 2966	Und.
Rhizoctonia solani	Rs	Spain, Sevilla, CECT, 2815	Und.
Phytophthora capsici	Pc	Spain, Valencia, UPV-PPG	Und.
Sclerotinia sclerotiorum	Ss	Spain, CECT, 2823	33.30
Pestalotiopsis palmarum	Pp	Papua, Nueva Guinea, CBS, 336.97	Und.

[a] Code used in this paper to refer to each fungal species

[b] Area in which the isolate was collected/institution that provided the isolate: *UPV-COMAV* isolates obtained by the authors; *CECT* Spanish Type Culture Collection at the University of Valencia (UV); *UPV-PPG* isolates provided by the plant pathology group at the UPV; *B.B.* isolates kindly provided by Dr. B. Bruton; *M. St.* isolates kindly provided by Dr. M. Stanghellini; *CBS*, Centraalbureau voor Schimmelcultures (Netherlands). The code of each isolate provided by the donating institution is also included.

[c] Ct values obtained in real-time PCR amplifications. Ct values above 31 are considered negative; *Und.* Undetermined value=Ct>40

and DNA was extracted using the Plant Mini Kit (Qiagen). Equal concentrations of genomic DNA of *M. cannonballus* and *F. oxysporum* f.sp. *niveum* were mixed to determine the efficiency of the amplification of *M. cannonballus* in the presence of unspecific DNA.

Artificial inoculum

For the artificial inoculation of melon roots the McG isolate was used. Mycelial plugs obtained from active PDA cultures were used to inoculate a sterilized and hydrated sand/oat hull mixture (1 l/91.5 g). After 4 weeks, the inoculum was quantified by counting the number of colony-forming units (CFU) as described in Bruton et al. (2000). Sterile soil substrate (autoclaved for 60 min at 120°C three times) was mixed with the sand/oat hull inoculum to reach a final concentration of 50 CFU $g^{-1}$of McG in the artificially inoculated soil.

The potential of real-time PCR to detect *M. cannonballus* in the sand/oat hull media was assessed. Six 2 g-samples were taken from the artificial inoculum. Each one was mixed thoroughly by vortexing in order to obtain a uniform sample before dividing it into two 1 g-samples; one was used for CFU-counting and the other (taking a subsample of 250 mg) for DNA extraction. Total DNA was extracted by grinding the sand/oat hull media in liquid nitrogen, followed by an extraction using the Soilmaster DNA extraction kit according to the manufacturer's instructions (Epicenter).

Inoculation of melon roots and disease assessment

Four-day-old plantlets of the muskmelon cv. Piel de Sapo (Semillas Battle), which is highly susceptible to *M. cannonballus* (Dias et al. 2004), were transplanted to 0.5 l pots filled with the artificially inoculated soil described above. The plants were grown in a greenhouse. The effect of infection on the melon roots was monitored on days 0, 2, 4, 6, 8, 10, 12, 14 and 16 after transplanting (DAT). Three inoculated plants and three healthy controls (plants grown in sterilized soil without inoculum) were analyzed on each of these days. The roots were washed carefully, placed on glass submerged in very shallow water and spread apart using dissecting needles. Each root was graded for the severity of the lesions caused by *M. cannonballus* on a scale of 0 (healthy) to 4 (severe discolouration, browning, root rot, and necrosis) as reported by Bruton et al. (2000) and Dias et al. (2004). Roots were also scanned and the total projected area of each root (area occupied by the roots in the two-dimensional scanned image, cm^2) was calculated using specific root analysis software (WhinRhizos, Regent Instruments, Canada). This trait can be used to estimate the effect of *M. cannonballus* on the root biomass (Fita et al. 2006).

The presence of *M. cannonballus* in each root was assessed by real-time PCR. After washing the roots to remove the soil, these were surface-sterilized for 60 s in sodium hypochlorite (1.5% available chlorine), and then washed three times in sterile water. The whole fresh root was ground in liquid nitrogen. Total DNA was extracted from 100 mg aliquots of ground tissue, using the DNeasy Plant Mini Kit (Qiagen). Three different aliquots of each root were extracted from six randomly selected plants to assess variations in fungal concentration and in extraction efficiency among samples from the same root. The presence of *M. cannonballus* in the roots was also confirmed by fungal isolation from root segments on PDA medium.

The utility of the method for resistance screening was validated by quantifying the amount of *M. cannonballus* in the roots of the accessions *C. melo* subsp *agrestis* Pat 81. This accession has been previously reported as partially resistant to the fungal attack (Dias et al. 2004). Plants of Pat 81 were cultivated in inoculated soils and roots were inspected for *M. cannonballus* by real-time PCR as described previously, on 2, 6, 8, 10, 12, and 14 DAT. Piel de sapo plants were used as susceptible controls.

Real-time PCR assay

Three sets of primers were designed from the genomic rDNA (ITS1-5.8S-ITS2) regions of the Japanese strain of *M. cannonballus* (98-62; AB097099; Table 2). This sequence has 100% sequence identity with sequences of all *M. cannonballus* isolates held in the NCBI database (3 from Italy, 14 from Japan and 1 from Spain), but <91% sequence identity with one isolate of *M. ibericus* from Spain (AF340013). Some of the primers had been used by Lovic et al. (1995) for the amplification of *M. cannonballus* by conventional PCR and others were designed by our group. The pair of primers finally selected (pair 2) and the sequence of the amplified region was analyzed for specificity *in-silico* by blast analysis using the NCBI Blast feature (http://www.ncbi.nlm.nih.gov/BLAST). Primer 2R gave hits with *M. cannonballus*, but also with *Pestalotiopsis palmarum*. The sequence of the amplified region gave only significant hits with *M. cannonballus*; one isolate of *P. palmarum* was included to test the specificity of the analysis (Table 1).

Five nanograms of total DNA from the pure fungal cultures, from the artificial inoculum and from the inoculated and healthy roots were used as a template in each PCR. This was selected as the optimal concentration of total DNA, using a set of root samples representing different states of *M. cannonballus* infection. The real-time PCR reaction was performed in a total volume of 25 µl that contained SYBR® Green PCR Master Mix (Applied Biosystems). Nine combinations, using 50, 300, and 900 nM each of forward and reverse primers, were tested. The minimal forward and reverse primer concentrations that yielded the maximum fluorescent intensity were chosen as the optimal primer concentrations (Table 2). The PCR was monitored on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The PCR reaction was initiated with a pre-incubation at 50°C for 2 min and denaturing at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s and annealing and extension together at 60°C for 1 min/cycle, followed by a final extension step of 72° C for 10 min. Immediately after the final PCR cycle, a melting curve analysis was performed to determine the specificity of the reaction. All of the reactions were also analysed by acrylamide gel electrophoresis.

Each sample was loaded in duplicate onto a single PCR plate. Common plant samples were run on every

Table 2 Specific primers used for the amplification of *Monosporascus cannonballus* by real-time PCR, designed from the genomic rDNA sequence of the Japanese strain of *M. cannonballus* (98-62; GenBank sequence AB097099)

	Primer Sequence	Primer location[a]	Region amplified	Amplicon length (bp)	Optimal primer concentration
Pair 1	F: 5′-CAC ATT GCG CCC ATT AGT ATT C-3′	298–320	5.8 gene	79	300nM
	R: 5′-ACA CCA AGC AAC TAA GGC TTG AG-3′	354–376	and ITS2		900nM
Pair 2	F: 5′-CTT ACC TAT GTT GCC TCG GCG-3′[b]	37–57	ITS1	112	300nM
	R:5′-AAG AGT TTA GAT GGT CCA CCG G-3′[b]	128–148			300nM
Pair 3	F: 5′-GCT TGG TGT TGG GAG CTT ATC CC-3′	368–390	ITS2	134	900nM
	R: 5′-GGT TTA GTG GCC AGA AGC CAG CG-3′[b]	479–501			900nM

[a] Numbers indicate positions of the sequence AB097099 to which primers correspond

[b] Lovic et al. (1995) recommended the use of primer 3R along with an additional primer (430 bp) for fungal detection by conventional PCR on infected root tissue, and a nested PCR, using 3R and 2F (464 bp) followed by 2R and an additional primer (68 bp), was suggested as more suitable for *M. cannonballus* detection on infected soils.

plate to study the reliability of the method, as well as no-template controls (healthy root DNA and water). A total of eight fourfold dilutions (ranging from 5 ng to 0.3 pg) of the genomic DNA of *M. cannonballus* (isolate McG), were also run in duplicate on every plate. DNA from healthy roots was used as unspecific DNA background to dilute the known amounts of *M. cannonballus*. These dilution series were used to construct a standard regression line by plotting the Ct values (the cycle number in which amplification first entered the logarithmic phase) versus the logarithm of the starting DNA concentration of *M. cannonballus* in each fourfold dilution (logCo). The amount of *M. cannonballus* in each sample (expressed as pg of target fungal DNA per ng of total DNA in the original sample, pg ng^{-1}) was estimated by comparing the Ct value of each sample to the Ct values of the standard regression line. Means and standard errors, regression lines, and correlation coefficients were calculated using Statgraphics plus (version 4.0).

Results

Reaction sensitivity and specificity

The amplification of the standard dilution series of *M. cannonballus* genomic DNA with primer pairs 2 and 3 yielded the expected single amplicons (Fig. 1a). The predicted PCR product length (112 and 134 bp for pair 2 and 3 respectively) was confirmed by both the melting curve analysis and agarose gel electrophoresis. Primer pair 1 amplified the expected product (79 bp) along with a second fragment which was very weakly amplified. Non-specific primer-dimer products were apparent with primer pair 1 in fungal DNA concentrations <5 pg (Fig. 1a), so this primer pair was discarded for the following analysis.

As expected, Ct values decreased as the starting template concentrations increased. Amplifications with primer pair 3 were erratic and the slope of the standard regression line ($Ct=-3.89\log Co+19.1$, $R^2>0.995$) was more pronounced than that of primer pair 2 ($Ct=-3.23\log Co+19.1$, $R^2>0.999$), which was near the optimal slope for a dilution series. The regression line allowed quantification of *M. cannonballus* DNA over more than 4 orders of magnitude, with Ct values between approximately 17 and 31 for 5 ng and 0.3 pg template respectively. As only primer pair 2 gave linear and reliable results over the whole range of DNA concentrations examined, primer pair 3 was also discarded for future analysis.

Primer pair 2 was specific for detecting *M. cannonballus*. The nine *M. cannonballus* isolates produced the same PCR product, whereas amplifications on other fungi yielded only non-specific products, with melting temperatures different from that of the specific product. These products were very weakly amplified (Ct>31, Table 1) and no clear bands were visible after gel electrophoresis (Fig. 1b). The specificity and efficiency of the amplification were high regardless of the unspecific background, as no change in the melting curves and in the Ct values could be observed in the amplification of equal amounts of *M. cannonballus* DNA alone or mixed with *F. oxysporum* DNA (Fig. 1b). Results also indicated that the real-time PCR assay

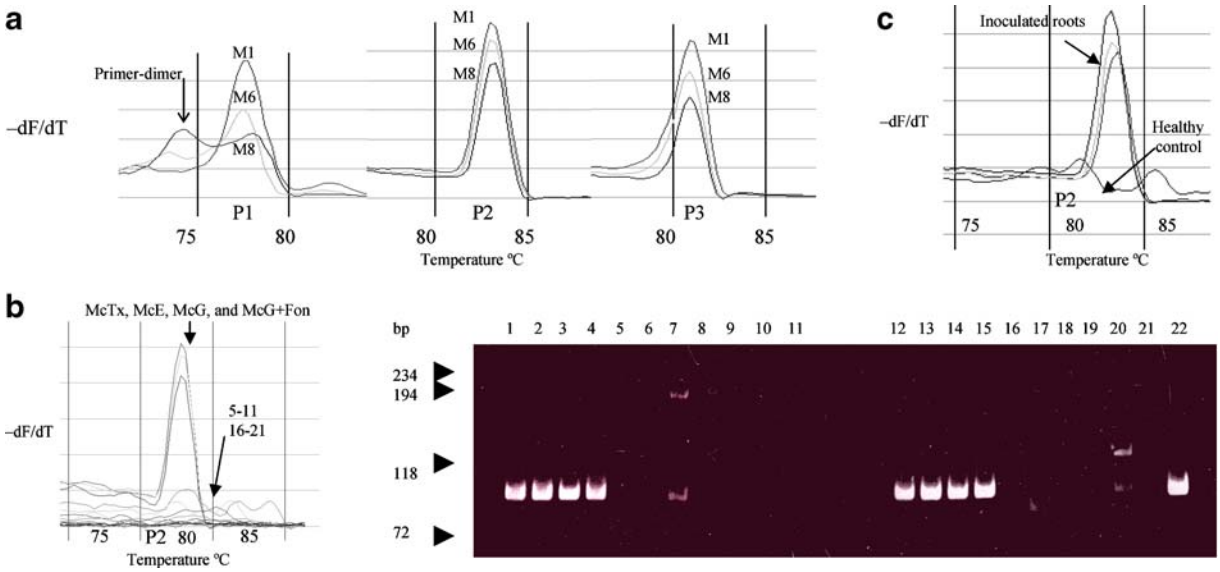

Fig. 1 (**a**) Melting curve profile for real-time amplification with primer pairs 1, 2 and 3 (*P1*, *P2* and *P3*) of a fourfold dilution series of total DNA extracted from pure cultures of *M. cannonballus* (isolate McG). *M1*, *M6* and *M8* correspond with 5 ng, 4.9 pg, and 0.3 pg of genomic DNA respectively. The negative derivative of fluorescence with respect to temperature is plotted as $-dF/dT$ versus temperature to obtain a graphical representation of the melting peaks. (**b**) Melting curve profile for real-time amplification using primer pair 2 of DNA extracted from pure cultures of *M. cannonballus* (one isolate from each origin, USA, Egypt and Spain, is shown: *McTx*, *McE* and *McG*), and other fungal species taxonomically and ecologically related to *M. cannonballus* (from 5 to 11, and from 16 to 21 of the corresponding agarose gel). A mixture of equal amounts of DNA of *M. cannonballus* and *F. oxysporum* DNA was also amplified (McG+Fon). The corresponding agarose gel is included. The correspondence of numbers with isolates included in Table 1 is the following: *1* (McTx), *2* (McA), *3* (McC1), *4* (McC2), *5* (Fom), *6* (Fon), *7* (Ge), *8* (Db), *9* (Pp), *10* (Xc), *11* (Pc), *12* (McV(a)), *13* (McC), *14* (McE), *15* (McV(p)), *16* (Rs), *17* (Dc), *18* (Pa), *19* (Ac), *20* (Mp), *21* (Mi), *22* (McG). (**c**) Detection of *M. cannonballus* in melon roots of Piel de Sapo plants cultivated in soil artificially inoculated with 50 CFU of *M. cannonballus* g^{-1} of soil, analyzed at different dates after inoculation by real-time PCR with primer pair 2. Healthy roots cultivated in uninoculated soil have been also included

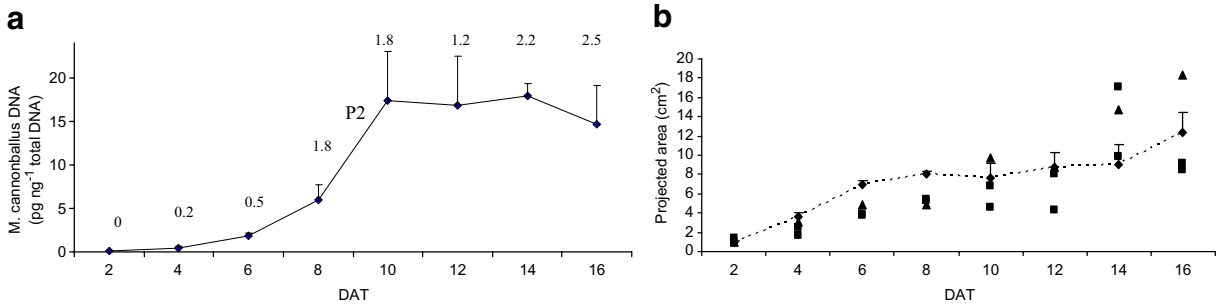

Fig. 2 (**a**) Quantification of *M. cannonballus* in roots of Piel de Sapo plants cultivated in soil artificially inoculated with 50 CFU g^{-1}. Plants were analysed at different dates after transplanting (DAT). The amount of a target fungal DNA (a region of the ITS1 from the rDNA) in each sample was quantified by real-time PCR and used as an estimate of root colonization by the fungus. Each point is the mean of three plants. The *bars* indicate standard errors. The *numbers* indicate the mean severity of lesions observed in roots on each date on a scale of 0 (no symptoms) to 4 (severe rot, browning or necrosis). (**b**) Effect of *M. cannonballus* on the development of Piel de sapo roots. The total area of the roots was obtained from the scanned images with the specific software WhinRhizo. Each *dot* on the line represents the mean area of the three roots analyzed in healthy soil on each date and the *bars* indicate standard errors. The area of each of the three roots analyzed per date in inoculated soil has been represented to show the high variability found in this trait after infection, mainly at the end of the assay

amplified the isolates of *M. cannonballus* with equal efficiency as each isolate had a similar Ct value (ca. 17.5) when amplified at a fixed amount of 5 ng DNA (Table 1).

Quantitative detection in melon roots

Root samples in all stages of infection were amplified with primer pair 2. Single amplicons of the size expected were obtained from inoculated melon roots. No PCR product was obtained from the healthy controls (Fig. 1c). The detection of *M. cannonballus* in samples from the same root was highly reproducible, with Ct differences among the three aliquots analysed from each root ranging from 0.20 to 0.36 cycles (with a mean value of 0.24). These differences are only slightly higher than those found between duplicate amplifications of the same DNA sample (ranging from 0.05 to 0.19, with a mean value of 0.10). These results validate the use of a single sample per root.

Monosporascus cannonballus was first detected in Piel de Sapo at 2 DAT, while first symptoms of the infection appeared at 4 DAT (Fig. 2a). At this early stage of infection, when the amount of fungus in the roots was very low, primer pair 2 allowed the detection of *M. cannonballus* in two of the three analyzed roots (one was negative Ct=32.5, and the other two were positive, Ct=30.6 and 28.6, corresponding with 0.05 and 0.23 pg ng^{-1} respectively).

There was a gradual increase in the fungal DNA concentration detected in the roots, corresponding to the fungal growth during root colonization (Fig. 2a). All plants were positive at 4 DAT (an average of 0.5 pg ng^{-1}), and the *M. cannonballus* concentration in the roots increased significantly from 6 DAT to 10 DAT, when it reached a maximum. Estimates of *Monosporascus* DNA concentration were not significantly different from 10 DAT to 16 DAT. The fungal colonization of the roots estimated by real-time PCR correlated well with the level of infection measured using symptom severity. In fact, the highest increase in symptom severity occurred simultaneously with a significant increase in fungal concentration (from 6 to 10 DAT). At 6 DAT Piel de Sapo roots displayed only mild symptoms (Fig. 3a, b), and at 8 DAT plants already displayed moderate symptoms. Symptoms progressed slowly from 10 DAT to 16 DAT, remaining moderate at the end of the assay (Fig. 3c, d). Healthy plants showed no visible lesions.

Monosporascus cannonballus caused a delay in root growth. From 2 DAT to 8 DAT the inoculated roots had a reduced projected area in comparison with healthy roots. However, towards the end of the assay this effect was not so clear, and some inoculated roots achieved a size similar to or even larger than healthy ones (Fig. 2b).

Monosporascus cannonballus was also detected in roots of *C. melo* subsp *agrestis* Pat 81 from 2 DAT on. Real-time PCR allowed quantification of the pathogen in roots of this partially resistant accession (Fig. 4). *Monosporascus cannonballus* concentration was significantly lower in Pat 81 in comparison with the

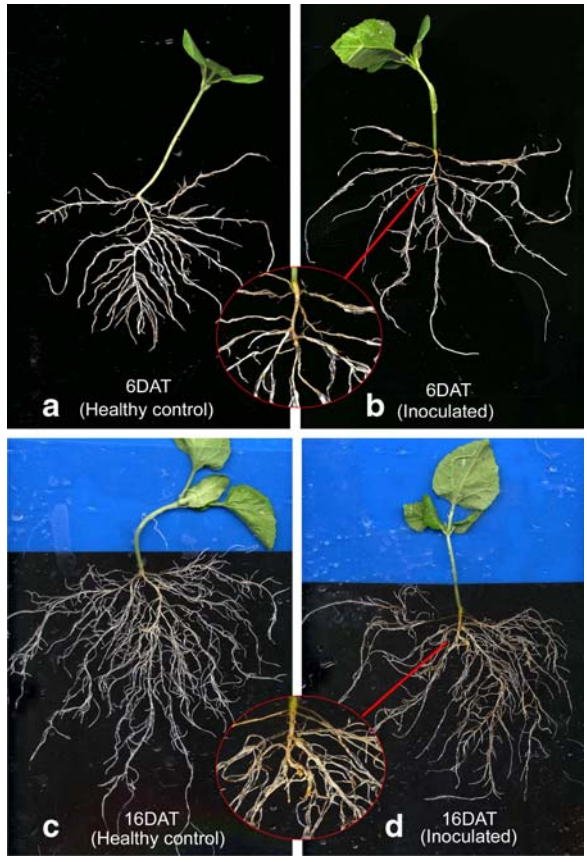

Fig. 3 Roots of Piel de Sapo plants grown in soil artificially inoculated with 50 CFU of *M. cannonballus* g^{-1} (inoculated) in comparison with roots grown in sterilized soil (healthy control): **a** and **b**, roots analysed 6 days after transplanting (DAT) when mild symptoms are almost imperceptible and difficult to score (with scores from 0 to 1); **c** and **d**, roots analyzed at 16 DAT, at this early stage of infection symptoms are still moderate (from 1 to 3), including discrete brownish lesions and discolouration of the tap root and major secondary roots

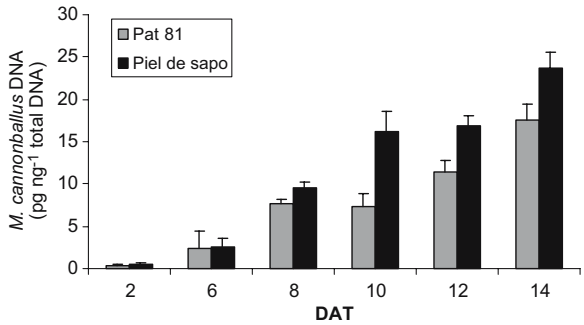

Fig. 4 Quantification of *M. cannonballus* in roots of two genotypes, C. melo subsp. melo cv Piel de Sapo (highly susceptible to *M. cannonballus*), and *C.melo* subsp *agrestis* Pat 81, partially resistant to the fungus, cultivated in soil artificially inoculated with 50 CFU g^{-1}. Plants were analysed at different dates after transplanting (DAT). The amount of a target fungal DNA (a region of the ITS1 from the rDNA) in each sample was quantified by real-time PCR and used as an estimate of root colonization by the fungus. Each *value* is the mean of three plants. The *bars* indicate standard errors

susceptible Piel de Sapo (from 20 to 55% lower) from 8 DAT until the end of the assay.

Quantitative detection in artificial inoculum

Monosporascus cannonballus was also specifically detected in all samples of total DNA extracted from the sand/oat hull mixture with primer pair 2. The estimates of *M. cannonballus* concentration from the six replicates analyzed were quite variable (5 to 12 pg of fungal genomic DNA g^{-1} of sand/oat hull media) in comparison with the uniformity found within replicate samples from the same root. This variation was similar to that found with the CFU counting (2,750 to 5,375 CFU) and is probably a result of the sampling procedure itself.

Discussion

Soil-borne plant pathogens are notoriously difficult to detect, identify and control. Certain characteristics of *M. cannonballus* make this problem even more pronounced (long-term survival of ascospores, low germination rate, late appearance of perithecia in the roots, influence of environmental conditions, cultural practices and other pathogens on the severity of root damage and the occurrence and severity of vine decline syndrome; Martyn and Miller 1996).The SYBR® Green real-time PCR procedure developed in this study offers a specific and rapid method to detect and quantify *M. cannonballus*, overcoming the constraints of classical methods.

The method has been tested against nine isolates from different regions in USA, Spain, and Egypt. However, it is expected to be valid for amplifying a wider range of *M. cannonballus* isolates, as previous studies (Lovic et al. 1995; Collado et al. 2002), as well as a Blast analysis indicated no sequence divergence in primer sites or in the amplified ITS region among numerous isolates from around the world.

Real-time PCR proved to be highly sensitive. The detection threshold was <0.3 pg, similar to that reported for other fungi using real-time PCR (Filion et al. 2003; Ippolito et al. 2004). This sensitivity allowed the detection and identification of *M. cannonballus* in plant roots as soon as 2 days after inoculation, before the appearance of symptoms. A pre-symptomatic detection is desirable for accelerating breeding programmes and for reducing disease incidence by introducing control measures during the early stages of infection.

Even when symptoms were apparent in the roots, real-time PCR had additional advantages over both symptom rating and the study of root biomass loss in assessing root infection during these early phases. Early symptoms of *M. cannonballus* on melon roots include discrete brownish lesions and discolouration of the tap root and major secondary roots. Necrotic areas appear only on severely infected roots and *M. cannonballus* only forms characteristic perithecia in the late stages of the disease. Scoring these early symptoms is open to errors as root browning itself is a normal developmental process that can be triggered and exacerbated by many external factors. Similarly, the root mass reduction was not a valid criterion for assessing *Monosporascus* infection at the early phases of infection. In fact, previous studies in which root mass losses are used as disease severity criteria, evaluate root mass reduction at least 1 month after inoculation, when necrotic lesions and severe root rot cause the rupture of primary and secondary roots and the loss of fine roots (Bruton et al. 2000; Cohen et al. 2000; Crosby et al. 2000; Iglesias et al. 2000).

Our procedure was quantitative over at least 4 orders of magnitude, which is in accordance with previous studies with other fungi using the SYBR

Green system (Cullen et al. 2001; Filion et al. 2003). The wide range of detection makes it a valuable tool for the study of the dynamics of *M. cannonballus* infection of melon roots. Results indicate that fungal colonization of roots starts very early and that an exponential increase of fungal growth occurs simultaneously with the progression of symptoms. The pattern of fungal colonization found in the susceptible cultivar is similar to that found in the partially resistant accession Pat 81, but with lower levels of *M. cannonballus* in the latter. Real-time PCR allowed discrimination between the susceptible and the resistant cultivar as early as 8 DAT. The delayed colonization of Pat 81 roots is consistent with previous studies that indicated the occurrence of a partial resistance to fungal attack in this accession. This reduced concentration of *M. cannonballus* may account for the mild root lesions and the low percentage of vine decline previously reported in this accession (Dias et al. 2002, 2004). The results validate the utility of the PCR method to resistance testing.

To our knowledge, this is the first reported study on the characterization of the early steps of *M. cannoballus* colonization of melon roots by directly quantifying the DNA of the pathogen. There exists very little quantitative information regarding the development of this disease on melon roots and is nearly all based on the visual symptoms and the assessment of root mass reduction (Bruton et al. 2000; Dias et al. 2004; Stanghellini et al. 2004).

Real-time PCR successfully detected and quantified *M. cannonballus* in media used to produce artificial inoculum. However, like CFU-based quantification (Mertely et al. 1993; Bruton et al. 2000), real-time PCR can be affected by unequal inoculum distribution and sampling errors. An accurate quantification could be possible by analysing a high number of replicates. Further studies on sampling procedures are necessary to determine the real utility of real-time PCR to accurately control inoculum load.

In conclusion, the real-time PCR approach developed in this project is easy, rapid, and sensitive in specifically detecting and quantifying *M. cannonballus* in plant roots and artificial inoculum. The reported procedure, combined with symptom scoring and root mass analysis, may facilitate the standardization of the evaluation of root damage caused by this soil-borne pathogen. This is a valuable tool necessary in the development of strategies for disease management and to assist those steps of breeding programmes where accuracy is required, for example during the indexing of valuable germplasm, for monitoring resistance introgression, and for the characterization of resistance mechanisms.

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