

Methods

Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction

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Summary

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- A rapid method to quantify the colonization of the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* in *planta* using quantitative real-time polymerase chain reaction (qRT-PCR) technique.
- Specific PCR primers for the fungus (28S rDNA sequence) and host root tissue (chitinase and chalcone synthase gene) were developed and their respective specificity determined.
- The plant specific primers for *Lycopersicon esculentum*, *Medicago truncatula* amplified linearly over a concentration range of: 6.4 pg to 20 ng. The *G. intraradices*-specific primer amplified as low as 1 pg of its target DNA, which allowed us to detect a single spore of the fungus. High degrees of correlation were obtained when threshold cycle (Ct) was plotted against vesicular, hyphal and total colonization using microscopically quantified host roots.
- This is the first report of the application of the qRT-PCR technique for quantification of AMF colonization in *planta*. The success of its application should open up the possibility of its wider application in AM research.

Key words: quantitative real-time PCR (qRT-PCR), arbuscular mycorrhiza, *in vitro* culture, tomato, *Medicago truncatula*, *Glomus intraradices*.

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Introduction

Arbuscular mycorrhizal (AM) fungi belong to the members of the order Glomeromycota (Zygomycetes), which symbiotically colonize the roots of *c.* 80% of terrestrial plant species (Smith & Read, 1997). Assessment of colonization levels within a host tissue is central to every experimental setup that involves the AMF–plant interaction. To date, microscopy (Phillips & Hayman, 1970; McGonigle *et al.*, 1990) and biochemical analysis (Schmitz *et al.*, 1991) have been the methods of choice for most quantitative analysis of AMF in *planta*. Microscopic analysis utilizes specific stains to visualize the AMF

and then to quantify the fungus. Many variants have been published to date, each differing in its sensitivity, specificity and, most importantly, operating complexity. Biochemical analysis, a less popular method, relies on the quantification of sterols or chitin, neither of which are exclusive to AMF.

Of the various methods used for AMF identification, techniques based on the polymerase chain reaction (PCR) have been highly popular in addressing some of the key issues of AMF biology (e.g. detection and subsequent quantification of fungal propagules in soil and in *planta*). Many PCR primers have been described (Simon *et al.*, 1993; Abbas *et al.*, 1996; van Tuinen *et al.*, 1998; Redecker, 2000; Millner *et al.*, 2001).

In addition to AMF detection, these primers have facilitated the clarification of ambiguities in AMF taxonomy (Bago *et al.*, 1998). As data obtained with the aid of these primers is strictly qualitative, quantitative determination of the parameters of the target (fungus) remains a problem. Only one published report has addressed the quantification of the AMF (*Glomus mosseae*) in a host plant by means of PCR in conjunction with microscopy (Edwards *et al.*, 1997).

The development of the quantitative real-time (qRT-PCR) technique is a step forward in quantitative diagnostics, and the technique promises to be useful in areas of research such as medicine (Orlando *et al.*, 1998) and plant pathology (Böhm *et al.*, 1999; Bates *et al.*, 2001; Winston *et al.*, 2002), which require high sensitivity in both detection and quantification (Gibson *et al.*, 1996). This technique uses fluorogenic probes with specialized instrumentation to detect the PCR product during the exponential phase of the cycle (Heid *et al.*, 1996), which removes the limitations that characterize the endpoint detection methods used in conventional PCR. Recent reports of its application in AMF biocontrol studies has underlined its utility as a technique of choice in areas that require rapid, accurate and sensitive enumeration of fungal propagules (Filion *et al.*, 2003a,b).

We report here exploitation of the inherent advantages of the qRT-PCR technique in the detection and quantification of AMF *Glomus intraradices in planta*. A reliable quantification system was achieved by using conventional microscopy to calibrate the qRT-PCR detection signal and thus to generate standard calibration curves.

Materials and Methods

Plant and fungal material

Plant materials used in the study were *Medicago truncatula* cv. R108, *Lycopersicon esculentum* L. cv. Micro-Tom (Schwartz *et al.*, 2001) and the hairy root culture of *Daucus carota* L. clone DC2 (Bécard & Piché, 1992). The AMF isolates used for calibration were *G. intraradices* (BEG141) and *G. intraradices* (DAOM 181602); the former was maintained under pot conditions with leek used as host (Schwartz *et al.*, 2003) and the latter was maintained *in vitro* in association with carrot (St Arnaud *et al.*, 1996). The *G. intraradices* (MUCL 43194) isolate was purchased from MUCL, Belgium as *in vitro* sterile spores and maintained under carrot root organ culture (ROC) thereafter.

Time course of colonization of host plants

Tomato and *M. truncatula* were inoculated under greenhouse conditions with two different isolates of *G. intraradices* (BEG141 and DAOM 181602). Inoculation was performed in 50-ml pots to maximize and accelerate the spread of the fungus. The growth substrate was a sterilized mixture of dune sand (amended

with super phosphate to give 15 ppm available phosphorus) and vermiculite (1 : 1). The inoculum was added at a concentration of 300 spores per pot for the DAOM isolate, and 10% of the total pot volume for the BEG141 isolate, which consisted of a mixture of spores, hyphae and colonized leek roots (Schwartz *et al.*, 2001). Three pre-germinated seedlings of the host plant were transplanted into each pot and watered daily and fertilized weekly with half-strength Hoagland's solution (Hoagland & Arnon, 1938) without phosphorus. Fourteen days post inoculation (dpi), two randomly selected pots of each host plant were harvested and the roots were recovered, washed and then chopped into pieces 1–3 cm long. The root pieces were thoroughly mixed, and randomly sampled to provide about 20–30% of the total biomass. The root samples were covered in storage solution (a 3 : 1, v : v, mixture of 99% ethanol and 60% acetic acid) and kept at 4°C until all the samplings had been completed; the remaining root pieces were frozen at –70°C for genomic DNA extraction. This harvesting procedure was conducted every 7 d for 8 wk following the first harvest. Root samples in the storage solution were at the end of the experiment stained with Trypan blue (Phillips & Hayman, 1970). Subsamples of these stained roots were mounted onto slides (four slides each with at least 10 randomly chosen root pieces) and fungal colonization was determined at ×200 magnification by means of the magnified intersection method (McGonigle *et al.*, 1990). At least 10 intersects were examined in each root sample, and the vesicular, and hyphal colonization rates were calculated, to assess the mycorrhizal density of the root sample. Arbuscular quantification was not performed anytime during our assessment procedure.

Design of PCR primers

Plant PCR primers were designed from previously published expressed sequences tag (EST) sequences (Table 1), deposited in GenBank (NCBI). The *G. intraradices*-specific primer was designed on the basis of the 28S ribosomal subunit sequences also deposited previously in GenBank (Table 1). The PCR primers were designed using the Primer Express software (Perkin-Elmer Biosystems, Norwalk, CT, USA), in accordance with the criteria required for qRT-PCR primer design. The primers were synthesized from Sigma-Genosys (Rehovot, Israel) and tested for specificity by two methods: first by finding the homologous DNA sequences by means of GenBank BLAST tool; and second by amplifying the respective target DNA with regular PCR, and visualizing the amplicon on 3% agarose gel with ethidium bromide. The *G. intraradices*-specific primer is designated as GiAM, the tomato-specific one as LeChs2 and the *M. truncatula*-specific one as MtChit1.

Quantitative real time-PCR

The qRT-PCR reactions were set up with the components supplied in the SYBR Green I kit (Eurogentec SA, Seraing,

Table 1 The quantitative real-time polymerase chain reaction primers designed for the present study

| Primer name | Organism | Target sequence | GenBank Accession No. | Primer sequence and position (5' to 3') | Length, T_m |
|-------------|---|---------------------|-----------------------|---|---------------|
| LeChs2 | <i>Lycopersicon esculentum</i> cv. Micro-Tom | Chalcone synthase 2 | X55195 | TTCGGTTAACCGGCTCATGA (F) CTCGAGCACCCCTTGTGTCTC (Rev) (482–582) | 100 bp, 60°C |
| MtChit1 | <i>Medicago truncatula</i> R108 | Chitinase 1 | Y10373 | GGTATGGTTGTCGAGTCAATG (F) CTGTCGTCACGGTGCTTGAG (Rev) (193–293) | 100 bp, 60°C |
| GiAM | <i>Glomus intraradices</i> | 28S rDNA subunit | AF396797 | GCTCTGGTCCCGAAAGCTT (F) TAACCCGTTCTAACCTATTGACCAT (Rev) (52–115) | 64 bp, 60°C |

The plant-specific primers were designed on expressed sequences tag sequences and *G. intraradices* on 28S ribosomal DNA sequence.

Belgium). The 25 μ l of reaction mixture contained the following components (final concentrations): reaction buffer (1 \times), 2.5 mM MgCl₂, 200 μ M dNTP solution, 0.4 μ M forward and reverse primers, 0.2 U of Hot GoldStar (Eurogentec SA, Seraing, Belgium) enzyme, 0.75 μ l of SYBR Green I (1 : 2000 dilution in DMSO), 5 μ l of the template DNA (as described below) and, finally, PCR-grade water to make up the final volume. Routinely, three duplicate reactions were used for each sample, and each set included template controls containing water to check for contamination in the reaction components. The qRT-PCR was carried out in an ABI PRISM 7000 instrument (Perkin-Elmer Biosystems, Norwalk, CT, USA), according to the following program: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The threshold cycle (Ct) was calculated by the ABI PRISM 7000 software, to indicate significant fluorescence signals rising above background during the early cycles of the exponentially amplification phase of the PCR amplification process.

Preparation of DNA templates

Pure genomic DNA was extracted from plant and fungus with the Plant DNeasy Mini Kit (Qiagen, Hilden, Germany) for constructing standard calibration curves. Roots of tissue culture-grown plantlets of tomato and *M. truncatula* were used as the starting materials for extracting plant genomic DNA and extraction procedure was as per the manufacturers instructions. For the fungal genomic DNA, spores of *G. intraradices* (DAOM 181602), produced in split-plate culture (St Arnaud *et al.*, 1996) with Ri T-DNA transformed carrot roots, axenically, were harvested by solubilizing the gelling agent Phytigel (Sigma Chemicals, St. Louis, MO, USA), according to Doner and Bécard (1991). The *G. intraradices* (BEG141) spores were isolated from pot cultures of leek, by the wet sieving and decanting method of Gerdemann & Nicholson (1963). These spores were surface-sterilized with 2% chloramine-T, before genomic DNA extraction. The genomic DNA of each preparation was quantified and its purity was verified spectrophotometrically at 260 nm/280 nm; it was then stored in aliquots at –20°C until use.

DNA extraction from single spores

Owing to the unsuitability of the Plant DNeasy Mini Kit to extract genomic DNA from single spores of *G. intraradices*, the Chelex-100 resin (30%, w : v, 100–200 mesh size, sodium form, Sigma Chemicals) protocol, as described by Gadkar and Adholeya (2000), was used. This extraction methodology was required to evaluate the sensitivity of the fungal qRT-PCR primers GiAM, to amplify from a minute amount of starting material. The DNA preparation was vacuum dried, resuspended in 20 μ l of sterile PCR grade water, stored at –20°C and used within 2–3 wk. Each extraction was performed at least in triplicate and repeated independently twice.

Standard curve preparation using genomic DNA

Standard curves for plant and fungal genomic DNA were prepared by using spectrophotometrically quantified genomic DNA isolated as described above. Concentration gradients of plant (6.4 pg–20 ng) and fungal DNA (3.2 pg–10 ng) were used for generating the calibration curves. The concentration of input DNA was plotted against threshold cycle (Ct), by means of the ABI PRISM 7000 software. The Ct value was plotted vs. the concentration (logarithmic value) to obtain a standard quantification curve. The assay was repeated twice, with each dilution reaction set up in triplicate.

Spike test for *G. intraradices*-specific qRT-PCR primer

The ability of the *G. intraradices* PCR primer GiAM to identify and amplify its target among an overwhelming concentration of nontarget (plant) DNA was analysed in spiking studies. Varying amount of fungal DNA (0.01 ng, 0.1 ng, 1.0 ng and 10 ng) were spiked into fixed amounts of plant DNA (1, 5 and 20 ng) and then used as templates to detect the signal by means of qRT-PCR. The relationship between the threshold cycle, Ct and the concentration of input template was determined.

Ratiometric analysis of colonized roots

Tomato roots exhibiting various degrees of colonization by *G. intraradices* DAOM 181602 (Schwartz *et al.*, 2003) *in vitro*, were isolated from Petri dishes under a dissecting microscope (WILD M8, Heerbrugg, Switzerland). Root segments with high degree of vesicular colonization were visualized in the dark-field mode and carefully isolated with a scalpel. All the highly colonized root segments (referred to below as '100% colonized') were pooled, and then mixed with noncolonized tomato roots on a fresh weight basis (total weight 100 mg). For example, mixing of 20 mg of '100% colonized' roots with 80% noncolonized roots were, referred to as '20% colonization' and '60% colonized' roots with '40%' noncolonized as '60% colonization'. Each dilution series was then separately subjected to genomic DNA extraction (see Preparation of DNA samples) and 5 ng of the genomic DNA was taken from each sample and used as a template for a qRT-PCR reaction (see Quantitative real time-PCR). Two separate reactions, one containing the GiAM and other the tomato-specific LeChs2 PCR primer, were used for detection.

Calibration of qRT-PCR with mycorrhizal colonized roots

The qRT-PCR signal was calibrated on genomic DNA of tomato and *M. truncatula* roots, temporally harvested during their colonization with the AM fungi *G. intraradices*, as described earlier (see Time course of colonization of host plants).

The qRT-PCR reactions were set up in triplicate with 5 ng of genomic DNA as a template for each time point, and the Ct signal calculated by the software was recorded. Each value obtained was correlated with the corresponding value of the *G. intraradices* colonization (i.e. hyphal and vesicular for each time point for both the isolates, quantified as described earlier by the gridline intersect method).

Accuracy of detection of qRT-PCR and microscopy

Tomato plants colonized to various degrees with *G. intraradices* (BEG141) were used to test the accuracy of the calibration curves. The test plants were coded from 1 to 5, and roots from each plant were processed as described above. Specifically, each sample was divided in half, and one portion was quantified under the microscope, while the other subjected to qRT-PCR assay with the *Gl. intraradices*- and tomato-specific PCR primers. The Ct values obtained by qRT-PCR were used to calculate the colonization from the standard regression curves and the results were compared with the values obtained by microscopy.

Statistical analysis

The data were statistically analysed with the COSTAT (version 6.204) statistical software package (CoHort Software, Monterey, CA, USA; www.cohort.com).

Results

Optimization of specific primers and standard curve preparation

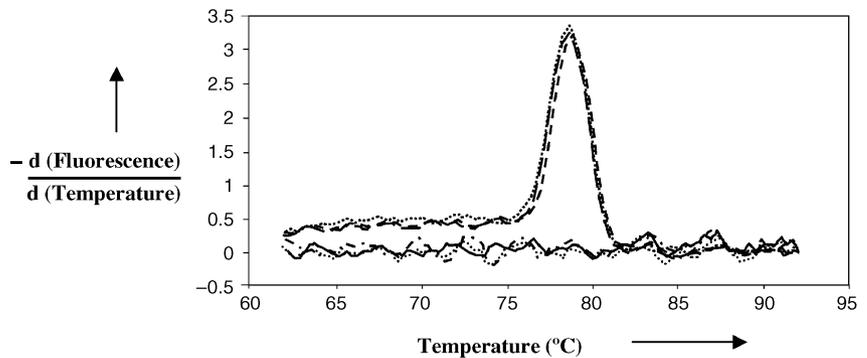
The *G. intraradices*-specific primer GiAM was designed on the 28S rDNA region, and its fidelity confirmed experimentally by regular PCR with target (*G. intraradices*) and non-target (*M. truncatula* and tomato) templates. BLAST alignment of this 64 bp sequence in GenBank revealed homology with members of *Glomus* species at varying degree of similarity (Fig. 1). Because of the small size of the amplicon (64 bp), the specificity of the GiAM *G. intraradices* primer was best visualized by means of the dissociation curve of the qRT-PCR (Fig. 2). The amplification profile shows that the GiAM primer shows an amplification in the presence of the *G. intraradices* template but no such amplification, as seen by the baseline curves are observed, when the template is substitute by plant or water (negative control).

The PCR primers for the plant were designed on EST sequences known to be specific to plants, as listed in Table 1. These primers, designed for qRT-PCR application, had a melting temperature (T_m) of 60°C and amplicon size of 100 bp. The tomato- and *M. truncatula*-specific primers were tested on their respective target plants by amplifying the genomic DNA by PCR with a similar cycling profile to that of

| | GiAM-Fwd | GiAM-Rev | Homology (%) |
|-----------------------------------|----------------------------|---|--------------|
| GiAM | GCTCTGGTGCCGAAAGCTT | GCTTACGGTTATCAAAGTCGATGGTCAATAGGTTAGAACG | |
| <i>G. intraradices</i> (RWI_141) | ----- | ----- | 100 |
| <i>G. intraradices</i> (Duernast) | ----- | -----C----- | 98 |
| <i>G. clarum</i> (LPA64) | -----T----- | -----T-----T-----A----- | 93 |
| <i>G. spp</i> | A----- | -----T-----C-----T-----A----- | 92 |
| <i>G. coronatum</i> (BEG28K) | -----C----- | -----C-----T-----C-----T-----C----- | 90 |
| <i>G. microaggregatum</i> (BEG56) | ---TA----- | ---C---T-T-A---C---T---A-----T-A--- | 87 |
| <i>G. caledonium</i> (SC_658) | --T--AC-TT----- | --T-A-GT-AGGGTATT-C-----A-----G | 76 |
| <i>G. constrictum</i> (BEG130) | A-CT-GAC-TC----- | -T-A-GT-AGGGTATT-C-----A----- | 71 |
| <i>G. geosporum</i> (BEG11) | --T-GAT-TC-----G----- | -T-A-GT-AGGGTATT-C-----A----- | 75 |
| <i>G. mosseae</i> (BEG25) | --T-GAT-TC----- | -T-A-GT-AGGGTATT-C-----A----- | 65 |

Fig. 1 Alignment of the 64 bp GiAM sequence with other members of the *Glomus* species. Various isolates of *Glomus* species with the percentage identity being listed. The sequence of the forward and reverse GiAM primer is underlined in bold letters.

Fig. 2 Dissociation curve of quantitative real-time polymerase chain reaction amplification using the GiAM polymerase chain reaction primer. Specific amplification of *Glomus intraradices* is seen by the amplification of reaction which contained template derived from this fungus. The *G. intraradices* BEG141 (—), *G. intraradices* DAOM 181602 (---), *G. intraradices* MUCL (- - -), *Medicago truncatula* (- - -), *Lycopersicon esculentum* (—) and negative (••••).



qRT-PCR (Fig. 3). The tomato- and *M. truncatula*-specific primers amplified the 100-bp fragments from their respective target templates but not those from the nontarget template (Fig. 3).

A linear relationship was obtained between Ct of the qRT-PCR amplification reaction and DNA concentration. The 64-bp diagnostic *G. intraradices*-specific fragment was amplified linearly over log concentration ranges of 3.2×10^{-3} to 10 ng and 1.5×10^{-3} to 15 ng for DAOM ($R^2 = 0.99$) and BEG141 ($R^2 = 0.98$), respectively (Fig. 4a). The Ct for plant qRT-PCR also showed a linear response to logarithmically increasing DNA concentrations ranging from 6.4×10^{-3} to 20 ng for both the host tomato and *M. truncatula* plants (Fig. 4b). The regression coefficient value (R^2) for both was 0.99.

qRT-PCR using *G. intraradices*-specific primer

Analysis of single spores Amplification of the 28S rDNA fragment from *G. intraradices* (DAOM and BEG141) with the GiAM primer consistently amplified the 64 bp fragment from the fungus. This fragment was consistently amplifiable in the presence of as little as single spore, over two orders of dilution (1 : 5 and 1 : 20). A linear response curve for the logarithm of the number of spores vs. threshold cycle Ct was obtained, the regression coefficients for 1 : 5 and 1 : 20 dilution being 0.92 and 0.89, respectively (Fig. 5). Under uniform amplification conditions, significant ($P < 0.0001$) correlation

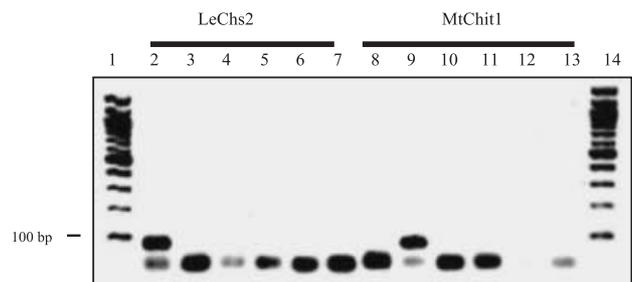


Fig. 3 Amplification of plant target sequences with specific polymerase chain reaction primers designed for quantitative real-time polymerase chain reaction application. Test for tomato-specific LeChs2 primer from lanes 2–7; only genomic DNA of tomato amplified. Test for *Medicago truncatula*-specific MtChit1 primer from lanes 8–13; only genomic DNA of *M. truncatula* amplified. Lanes 1 and 14 have a 100-bp ladder (New England Biolabs, Beverly, MA, USA). Lanes 2 and 8: tomato, Lanes 3 and 9: *M. truncatula*, Lane 4 and 10: *Daucus carota*; lanes 5 and 11, *Glomus intraradices* (DAOM isolate); lanes 6 and 12, *G. intraradices* (BEG141); lanes 7 and 13, negative control.

between the two independent assays was observed, with a regression coefficients (R^2) of 0.8 (1 : 5 dilution) and 0.6 (1 : 20 dilution), between the two assays.

Spike test for *G. intraradices* amplification The GiAM PCR primer amplified the 64 bp fragment from among a defined, but overwhelmingly high concentration of *M. truncatula* genomic DNA. The amounts of plant genomic DNA used

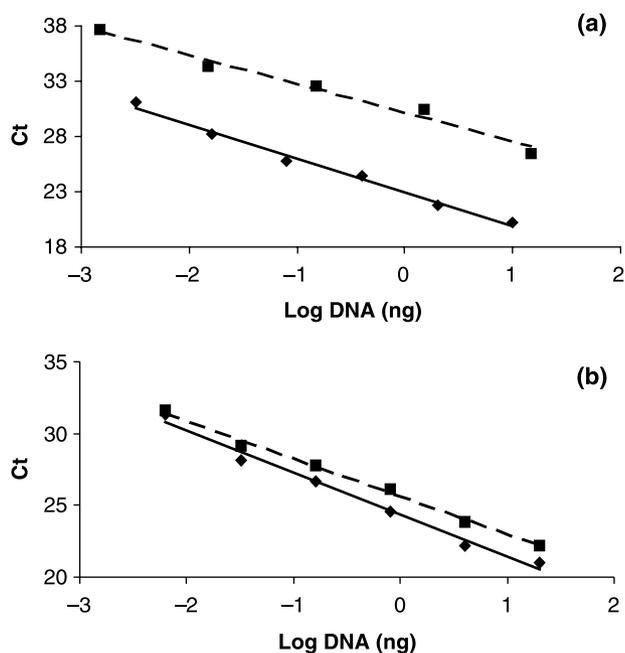


Fig. 4 Standard curves obtained by plotting threshold cycle (Ct) against genomic DNA concentration. (a) *Glomus intraradices* BEG141 (squares; $y = -2.63x + 30.15$, $R^2 = 0.98$) and *G. intraradices* DAOM 181602 (diamonds; $y = -3.05x + 22.95$, $R^2 = 0.99$). (b) *Lycopersicon esculentum* (squares; $y = -2.65x + 25.6$, $R^2 = 0.99$) and *Medicago truncatula* (diamonds; $y = -2.93x + 24.35$, $R^2 = 0.99$).

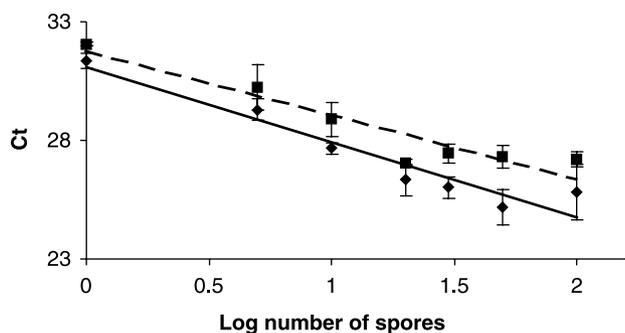


Fig. 5 Standard curves obtained by plotting threshold cycle (Ct) against log number of spores. Numbers of spores used for experimental purposes were, 1, 5, 10, 20, 30, 50 and 100. Two dilution series were used 1 : 5 (diamonds; $y = -3.18x + 31.11$, $R^2 = 0.92$) and 1 : 20 (squares; $y = -2.69x + 31.75$, $R^2 = 0.89$).

were 1, 5 and 20 ng, (i.e. 100, 500 and 2000 times), respectively, as great as the smallest amount (0.01 ng) of the spiked fungal DNA. The regression coefficients were 0.99 at both the 5 ng and 20 ng plant DNA concentrations and 0.98 for 1 ng concentration for various spiking concentrations (Fig. 6). This strongly indicates the high degree of sensitivity of the GiAM fungal primer set in detecting a specific template among an overwhelming heterogeneous sample.

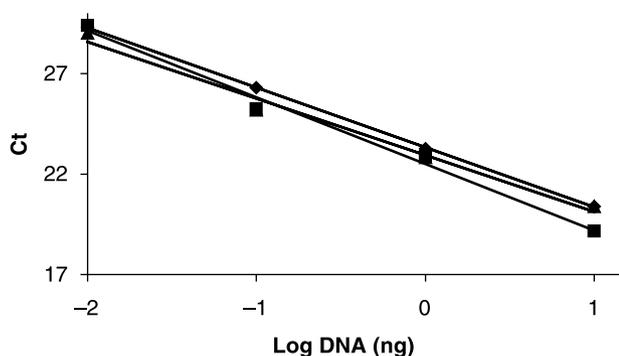


Fig. 6 Spike test analysis curves of varying amounts of *Glomus intraradices* DNA (0.01 ng, 0.1 ng, 1.0 ng and 10 ng) mixed with 1 ng (triangles; $y = -2.82x + 22.94$, $R^2 = 0.98$), 5 ng (squares; $y = -3.31x + 22.51$, $R^2 = 0.99$) and 20 ng (diamonds; $y = -2.82x + 23.32$, $R^2 = 0.99$) of *Medicago truncatula* DNA.

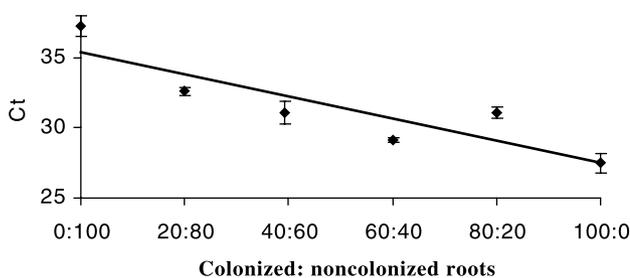


Fig. 7 Ratiometric analysis mixing of colonized: noncolonized roots of tomato plotted against Ct ($y = -0.09x + 35.94$, $R^2 = 0.85$). The 'colonized' roots were prepared by ratiometrically mixing, on a fresh weight basis, highly colonized and non-colonized tomato roots.

Ratiometric analysis of colonized roots The *in vitro*-generated, variously colonized roots were subjected to qRT-PCR to detect the modulation in the Ct signal. The colonization level, as determined by ratiometry, was assumed to model an actual random sample of colonized roots. The modulation of the qRT-PCR signal (Ct) using GiAM primer in response to change in the ratio of colonized to noncolonized roots indicated it possessed the necessary sensitivity to detect actual changes when a colonized host roots were used as a template. Furthermore, there was positive correlation ($R^2 = 0.85$) when the Ct value was plotted against the colonization ratio (Fig. 7).

Calibration using colonized roots

There was a positive correlation with the level of colonization (total) and normalized threshold cycle values (AM Ct : plant Ct) for both the host plants and the *G. intraradices* isolate. The coefficient of regression, for each *G. intraradices* isolate is listed in Table 2. There was a higher positive correlation in tomato than in *M. truncatula*, which we attribute to the

Table 2 Regression equation of standard curves of *Glomus intraradices* isolates for colonization parameters (total, hyphal and vesicular colonization)

| Fungal colonization | <i>Lycopersicon esculentum</i> <i>G. intraradices</i> isolate | | <i>Medicago truncatula</i> <i>G. intraradices</i> isolate | |
|------------------------|--|-------------------------------------|--|------------------------------------|
| | DAOM | BEG141 | DAOM | BEG141 |
| Hyphal colonization | $y = -0.3x + 1.1$ $R^2 = 0.62$ | $y = -0.12x + 1.1$ $R^2 = 0.62$ | $y = -0.17x + 1.2$ $R^2 = 0.69$ | $y = -0.08x + 1.1$ $R^2 = 0.51$ |
| Vesicular colonization | $y = -0.32x + 1.0$ $R^2 = 0.76$ | $y = -0.11x + 1.05$ $R^2 = 0.62$ | $y = -0.35x + 1.1$ $R^2 = 0.63$ | $y = -0.14x + 1.1$ $R^2 = 0.50$ |
| Total colonization | $y = -0.17x + 1.1$ $R^2 = 0.77$ | $y = -0.06x + 1.07$ $R^2 = 0.60$ | $y = -0.32x + 1.2$ $R^2 = 0.71$ | $y = -0.08x + 1.1$ $R^2 = 0.51$ |

Two separate isolates of the *G. intraradices* were used in the study; the host test plants were tomato and *M. truncatula*.

difficulty in visualizing intraradical structures of the fungus in the latter host. There was a consistent correlation between the normalized Ct signal and the degree of vesicular colonization, therefore, there was greater confidence in enumerating these structures than the other AMF structures (i.e. hyphae and arbuscules). The two isolates differed in their colonization patterns, perhaps because the inoculum originated from two different sources, the DAOM being produced *in vitro* and the BEG141 in pots.

Quantification of unknown samples

To test the accuracy of the standard calibration curves, five tomato plants with differing levels of colonization were taken. The colonization levels determined by qRT-PCR, with the regression equation for total and hyphal colonization, and values obtained by using standard microscopy for the same samples are listed in Table 3. There was a highly significant degree of correlation between values obtained by qRT-PCR and total colonization ($P = 0.0001$) hyphal parameters ($P = 0.0004$) and vesicular colonization ($P = 0.0000$).

Discussion

The main advantages of qRT-PCR are high sensitivity, specificity, excellent efficiency, reduced amplicon size and freedom from post-PCR handling steps (Heid *et al.*, 1996), which make it an ideal tool to assess AMF colonization in large numbers of samples. Accurate detection and quantification of an invasive organism in host plant tissue is important in the study of plant-symbiotic biology. We used the highly sensitive qRT-PCR technique to detect and quantify the AMF *G. intraradices* in roots of two test plants, tomato and *Medicago*.

The colonization data, which express fungal invasion into the root tissue of a host quantitatively, are key to understanding the effects of a test treatment upon the symbiont. These data, which are routinely calculated by means of microscopy, are labor intensive and time-consuming to collect. Of the many variants, the method proposed by McGonigle *et al.*

(1990), called the grid-line intersect method, is unique in that it enumerates, *in planta*, each individual structure of fungal morphology, namely, the arbuscules, hyphae and vesicles, and it has a low operator bias. This enabled us to analyse the correlation between the signal of qRT-PCR (Ct) and each of the fungal structures examined in our sample. Other variants of microscopy procedures for assessing colonization, as described previously (Phillips & Hayman, 1970; Giovannetti & Mosse, 1980; Trouvelot *et al.*, 1986) give a 'composite colonization value'. Moreover, these variants have a high operator bias during the assessment procedure (unlike the grid-line intersect method) and their output does not distinguish among the contributions made by the various fungal structures, namely, arbuscule, hyphae and vesicle, giving aberrant values in the qRT-PCR calibration (data not shown).

The target primers for the fungus were designed on the multicopy 28S rDNA and the EST sequences of chalcone synthase (tomato) and chitinase (*M. truncatula*) for the hosts. The specific plant ESTs were chosen primarily because they are members of the plant kingdom, which should reduce any spurious amplification from the fungal DNA. The GiAM primer was sufficiently sensitive to amplify from a single spore of *G. intraradices*, and this amplification was consistent across many replicate reactions from spores originating from differing culture conditions. A single spore of *G. intraradices* has a DNA content in the range of 5–19 pg (Hosny *et al.*, 1998). The amount of DNA added in a single PCR amplification reaction in the 1 : 5 dilution series amounts to 0.21–0.8 pg. The consistent amplification of the diagnostic 64 bp fragment from a single spore of *G. intraradices* with the GiAM primer strongly indicates the sensitivity of the method. The use of purified fungal DNA gave a reliable amplification over a linear range of 3.2 pg to 10 ng ($R^2 = 0.98$).

Devising a PCR-based method for quantitative detection of AMF *in planta* poses a unique challenge by virtue of its infection biology. The proportion contributed by fungal DNA in a typically highly colonized root is not known, but its estimation using fungal specific probe, shows that it varies anything between 5% and 12% (Maldonado-Mendoza *et al.*,

Table 3 Calculation of hyphal and vesicular colonization parameters, for the arbuscular mycorrhizal fungus *Glomus intraradices* BEG141 in tomato roots, by means of quantitative real-time polymerase chain reaction and microscopy

| Sample no. | Normalized Ct | Total colonization Regression equation | Microscopy | Vesicular colonization Regression equation | Microscopy | Hyphal colonization Regression equation | Microscopy |
|----------------------|---------------|---|------------|---|------------|--|------------|
| 1. | 1.34 | 0.0 | 0.07 | 0.0 | 0.0 | 0.0 | 0.07 |
| 2. | 1.03 | 0.66 | 0.78 | 0.18 | 0.2 | 0.58 | 0.58 |
| 3. | 1.21 | 0.0 | 0.01 | 0.0 | 0.0 | 0.0 | 0.01 |
| 4. | 1.02 | 0.83 | 1.0 | 0.27 | 0.31 | 0.67 | 0.73 |
| 5. | 1.04 | 0.5 | 0.63 | 0.09 | 0.11 | 0.5 | 0.52 |
| Correlation analysis | | $R^2 = 0.99$ $P = 0.0001^{***}$ | | $R^2 = 0.99$ $P = 0.0000^{***}$ | | $R^2 = 0.99$ $P = 0.0004^{***}$ | |

Ct, threshold cycle. The regression equations used for colonization are: total colonization, $y = -0.06x + 1.07$; vesicular colonization, $y = -0.11x + 1.05$; hyphal colonization, $y = -0.12x + 1.1$; y is the normalized Ct value. Negative values obtained using this equation are assigned 'zero' colonization value.

2002). We have strong reasons to believe that, because of this low abundance of the fungal target, it becomes highly imperative to use a sensitive detection method, for example qRT-PCR. The qRT-PCR technique has a wide dynamic response range (up to 10^7 -fold) as compared with that of conventional PCR (up to 10^3 -fold) (Heid *et al.*, 1996), making the detection of low-abundance targets very feasible. This greatly enhances the reliability of the quantitative data on AMF colonization *in planta* compared with those obtained by the regular PCR methods.

Evidence of the modulation in the quantitative real-time signal (Ct) was assessed before actual calibration in two separate sets of experiments. The first used a high concentration of the host DNA sample, spiked with varying amounts of fungal DNA; the second used ratiometric mixing (on a fresh weight basis) of highly colonized host roots with varying amounts of non-colonized roots to obtain a set of roots having a series of increasing degrees of colonization. The ability of the real-time signal (Ct) specific to the fungus to detect these changes proves the suitability of this technique for quantification of AMF during its physiological colonization process. The ratiometric technique has a major limitation as it could be done only in AM fungi, which form intraradical vesicles.

Our method of quantitative assessment of AMF colonization is fast and sensitive, and with the necessary calibration it would serve well where large numbers of samples need to be processed. The classical staining methods require a maximum of 24 h (taking the highly variable time required for the destaining of roots) up to the point the roots are ready for microscopic assessment. This also includes the use of the highly toxic stains such as Trypan blue, although safer alternatives have been proposed (Vierheilig *et al.*, 1998). We were able to process at least 20–30 samples for colonization assessment within 6 h, once the calibration curves had been made available. We plan to use this rapid procedure in our screening for the *Myc*⁻ tomato mutant (Schwartz *et al.*, 2003), in which typically 2000–3000 individual plants are subject to individual assessment.

Our method of analysis by qRT-PCR is not a replacement for the existing microscopic methods of fungal estimation. We aimed to calibrate the qRT-PCR against classical microscopy and then to use the standardization equation derived from this correlation for high-throughput colonization assay. We are confident that the qRT-PCR method would be a good aid in operations in which data need to be generated rapidly, for example to screen a large number of host plants and after identification of the target AM fungi. Of the many variants possible using qRT-PCR, the ability to rapidly detecting and quantifying 'living' vs 'dead' AMF would be very useful.

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