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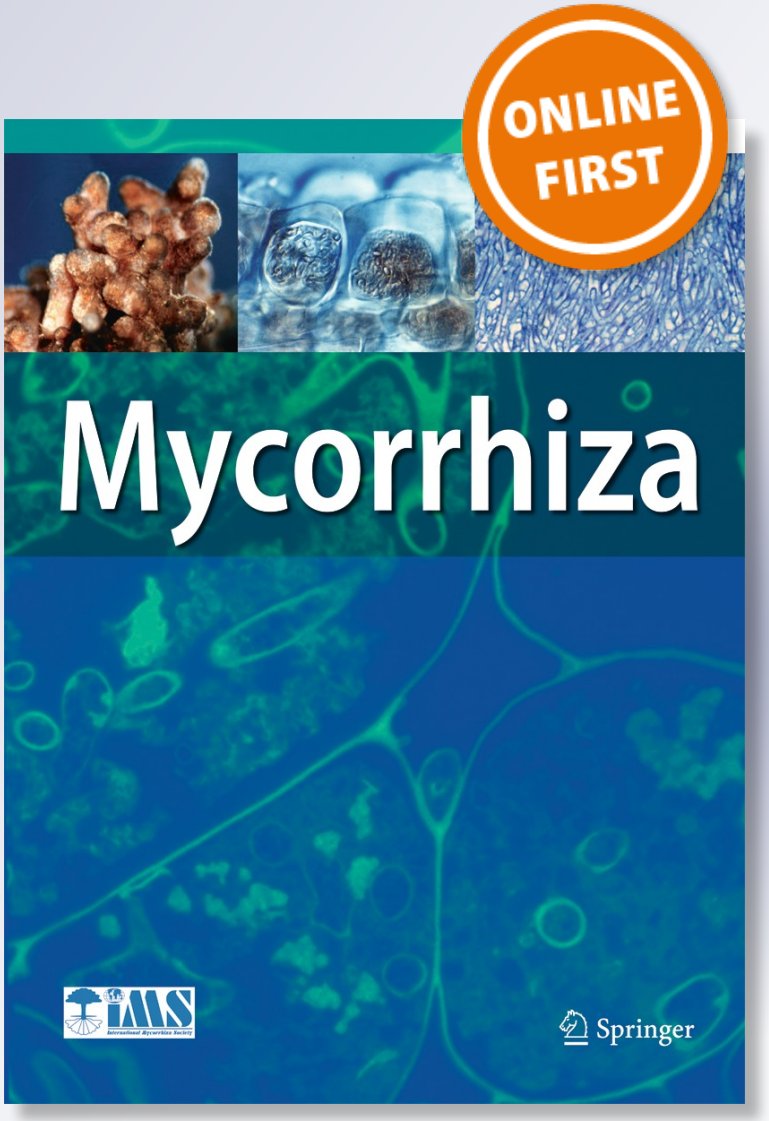

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ORIGINAL ARTICLE

Molecular diagnostic toolkit for *Rhizophagus irregularis* isolate DAOM-197198 using quantitative PCR assay targeting the mitochondrial genome

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Abstract *Rhizophagus irregularis* (previously named *Glomus irregulare*) is one of the most widespread and common arbuscular mycorrhizal fungal (AMF) species. It has been recovered worldwide in agricultural and natural soils, and the isolate DAOM-197198 has been utilized as a commercial inoculant for two decades. Despite the ecological and economical importance of this taxon, specific markers for quantification of propagules by quantitative real-time PCR (qPCR) are extremely limited and none have been rigorously validated for quality control of manufactured products such as biofertilizers. From the sequencing of 14 complete AMF mitochondrial (mt) genomes, a qPCR assay using a hydrolysis probe designed in the single copy *cox3-rnl* intergenic region was tested and validated to specifically and accurately quantify the spores of *R. irregularis* isolate DAOM-197198. Specificity tests were performed using standard PCR and qPCR, and results clearly showed that the primers specifically amplified the isolate DAOM-197198, yielding a PCR product of 106 bp. According to the qPCR analyses on spores produced in vitro, the average copy number of mt genomes per spore was 3172 ± 304 SE ($n = 6$). Quantification assays were successfully undertaken on known and unknown samples in liquid suspensions and commercial dry formulations to show the accuracy, precision, robustness, and reproducibility of the qPCR assay. This study provides a powerful molecular toolkit specifically designed to quantify spores of the model AMF isolate DAOM-197198. The approach of molecular toolkit used in our study could be applied to other AMF taxa and will be useful to research institutions and governmental and industrial laboratories running routine quality control of AMF-based products.

Keywords Quantitative real-time PCR · Validation · *Rhizophagus irregularis* · Mitochondrial marker · Spore quantification · Biofertilizers · Arbuscular mycorrhizal fungi

Amine Badri and Franck O. P. Stefani contributed equally to this work.

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Introduction

Arbuscular mycorrhizal fungi (AMF) are a ubiquitous group of obligate biotrophic soil fungi which are associated with more than 80 % of terrestrial plants species where they form mycorrhizal symbiosis (Smith and Read 2010). Beneficial effects of this symbiosis on plant fitness include an enhanced nutrient uptake, of phosphorus in particular, and an improved tolerance to biotic and abiotic stress (Koide and Mosse 2004; Smith and Read 2010; Gianinazzi et al. 2010). The use of AMF in agricultural interventions has the potential to benefit not only the health of crops but also the health of the soil itself, especially in the context of sustainable agriculture (Podeszfinski et al. 2002). Using 231 field trials on potato production, Hijri (2016) clearly demonstrated that AMF

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inoculation significantly increased crop yield. In Canada, the surface area of farm fields inoculated in 2015 with AMF-based biofertilizers is expected to double compared to 2014, from 100,000 to 200,000 ha (Fortin et al. 2015).

Arbuscular mycorrhizal fungal species richness and succession are still not well known, and how different assemblages of AMF species benefit the host plant remains poorly understood. AMF identification has historically relied on the analysis of spores and the morphology of spore clusters (size, shape, surface ornamentation, color, contents, spore walls, hyphal attachment, and reaction to staining solutions, e.g., Metzler's). However, the morphological analysis of AMF spores is time consuming and requires specialized expertise because of limited phenotypic differentiation among taxa. Therefore, AMF identification based on morphological and biochemical characters could either fail to identify species or lead to incorrect identification in some situations. This limitation negatively impacts the development of commercial AMF-based biofertilizer inoculants. Indeed, the industrial production of AMF-based biofertilizers requires standardized diagnosis toolkits for monitoring product quality control, assessing shelf-life of formulated products and tracing the inoculants in soil and crop roots.

Few studies have attempted to use molecular-based methods for detecting and quantifying the abundance of AMF. Among these methods, the quantitative real-time polymerase chain reaction (qPCR) has been used in a wide range of routine applications, including medical diagnosis, because of its accuracy and precision. Recent reports of its application in AMF studies have demonstrated its usefulness as a rapid and sensitive technique for the enumeration of fungal propagules or gene copy numbers (Filion et al. 2003; Alkan et al. 2004; Isayenkov et al. 2004; Kiers et al. 2011, Thonar et al. 2012, Couillerot et al. 2013). Several validation steps and improvements have been made to enhance the qPCR sensitivity (Alkan et al. 2006; Gamper et al. 2008). Until now, most of these studies used the ribosomal DNA as a molecular marker in order to quantify AMF by qPCR. However, the genetic variation of this locus within a single spore, the occurrence of multiple copies per genome, and the variation of copy number among isolates (Sanders et al. 1995; Giovannetti et al. 1999; Redecker et al. 1999; Hijri and Sanders 2005; Corradi et al. 2007; Boon et al. 2010) limit the precision of this quantification method and can lead to misestimating the number of spores in samples. In addition, ribosomal DNA (rDNA) cannot discriminate between isolates for a given AMF species. In contrast, the mitochondrial (mt) genome appears to be homoplasmic (Lang and Hijri 2009; Lee and Young 2009; Formey et al. 2012). For example, Lee and Young (2009) showed that the mt DNA of *Glomus intraradices* isolate 494 was homoplasmic, although this has been challenged recently (Beaudet et al. 2015). It was also found that the mt large subunit rDNA gene (mtLSU rDNA, also known as *rnl* gene) sequence was not polymorphic within *G. intraradices* isolates BEG75 and BEG158 and *G. proliferum* isolate DAOM226389 but varied substantially between isolates (Raab et al. 2005). Thus, the mtLSU rDNA has been suggested to be an appropriate marker for studies of diversity in AMF populations (Peyret-Guzzon et al. 2016). The usefulness of mtLSU rDNA (*rnl* gene) has been demonstrated by Kiers et al. (2011) and Couillerot et al. (2013) where both studies have developed species-specific qPCR markers for *G. intraradices* (isolates 09 and JJ291), *G. aggregatum*, and *G. custos*. Therefore, targeting AMF mt genomes with qPCR probes is a promising avenue for developing more sensitive, accurate, and precise AMF quantitative assays.

The aim of this study is to develop and validate a qPCR assay to identify and quantify the spores of the model AMF *Rhizophagus irregularis* isolate DAOM-197198 in various spore formulations during industrial processes. Using the data from 14 complete AMF mitochondrial genomes, a novel hydrolysis probe-based qPCR assay targeting the *cox3-rnl* intergenic region was developed to detect and quantify spores of *R. irregularis* DAOM-197198. A rigorous validation protocol such as standard curve development and its robustness evaluation (tests on Master Mix, primers, and probe variation) of the qPCR assay has been applied to determine the number of mt genome copies per spore of *R. irregularis* DAOM-197198 and to assess the accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) of the method. We also performed tests for spore conservation and DNA stability during storage using our qPCR assay. The rational for choosing *R. irregularis* isolate DAOM-197198 in our investigation is because it has been recovered worldwide in agricultural and natural soils, it has been utilized as bioferilizer inoculant for two decades, and its nuclear and mitochondrial genomes have been published (Tisserant et al. 2013; Nadimi et al. 2012).

Materials and methods

Fungal strains and commercial formulations

Rhizophagus irregularis isolate DAOM-197198 (synonym *Glomus irregulare*; formerly *G. intraradices*) was cultivated in vitro with Ri T-DNA-transformed carrot (*Daucus carota* L.) roots in an industrial mycoreactor by Premier Tech Biotechnologies. The fungal material was provided in three different formulations: spores in liquid suspension (termed a simple suspension), spores mixed with fragmented carrot roots in liquid formulation (termed a complex liquid suspension), and spores mixed with root fragments in a dry formulation (kaolin-based substrate). Both formulations of spores and spores mixed with fragmented carrot roots in liquid suspension contain fragments of hyphae and cell debris. Primers

and probe specificity tests were performed on different AMF taxa including five different isolates of *R. irregularis* and six closely related species to *R. irregularis* (Table 1). All isolates listed in Table 1 (except isolate *R. irregularis* DAOM-197198) were cultivated in vitro with Ri T-DNA-transformed carrot roots in petri dishes containing M medium in 0.4 % Phytagel (Sigma-Aldrich, Oakville, ON, USA). Spores and hyphae were collected by dissolving Phytagel with citrate buffer as described in Hijri et al. (2007).

DNA extraction

DNA extraction, particularly for spore lysis steps, is crucial for quantification of R. *irregularis* DAOM-197198 using qPCR method. We have optimized two protocols: one protocol for DNA isolation from spores in liquid formulations and another for DNA isolation from spores in dry formulation using two different kits as described below. However, DNA of AMF isolates used for specificity tests (Table 1) was extracted using DNeasy Plant Mini Kit (Qiagen, Toronto, ON, USA) where spores and hyphae were crushed using pestle and a 1.5-ml microtube.

DNA isolation from spores in liquid formulations

Total genomic DNA (gDNA) of the isolate DAOM-197198 was isolated using the PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's instructions. However, the lysis of spores was optimized using the following procedure: spore suspensions were centrifuged at 11,000*g* for 10 min at 4 °C in a 1.5-ml tube. The supernatant was removed partially leaving approximately 100 μl of the sample solution at the bottom of the tube in order to not disturb the pellet. Four hundred microliters of buffer contained in the PowerBead tubes was transferred into the 1.5-ml tube. The final volume (500 μl) was then transferred in tubes containing ceramic bead tubes (1.4 mm diameter, Mo Bio, Carlsbad, CA, USA), 25 mg of Celite 545 (diatomaceous earth, Imerys Filtration Minerals, CA, USA), and 60 μl of C1 solution (C solutions are provided in the PowerSoil DNA isolation Kit). Samples were homogenized using a Precellys® 24 homogenizer (Bertin Technologies, France) with three runs performed at 5000 rpm for 15 s each, with a break of 5 s between runs. Five hundred sixty microliters of the solution was then transferred to a clean 2-ml collection tube containing 250 μl of Solution C2. Following incubation at 4 °C for 5 min, the tubes were centrifuged at 11,000*g* for 5 min and 750 μl of the solution was transferred into a clean 2-ml collection tube containing 200 μL of Solution C3. Following incubation at 4 °C for 5 min, the tubes were centrifuged at 11,000*g* for 5 min and the supernatant was transferred to a clean collection tube containing 1.2 ml of Solution C4. The remaining extraction steps followed the manufacturer's protocol.

Table 1 Arbuscular mycorrhizal fungal strains included in the specificity tests

Species	Isolates	Origin
Rhizophagus irregularis	DAOM234179	Canada, Quebec
R. irregularis	DAOM240422	Canada, Ontario
R. irregularis	DAOM240415	Canada, Manitoba
R. irregularis	DAOM234328	Unknown
R. irregularis	DAOM240415	Canada, Manitoba
Rhizophagus sp.	DAOM229456	Unknown
Rhizophagus fasciculatus	DAOM240159	Canada, British Columbia
Rhizophagus clarus	DAOM240429	Canada, Ontario
Rhizophagus sp.	DAOM240422	Canada, Ontario
Rhizophagus clarus	DAOM234281	Cuba, Pinar del Rio
Glomus aggregatum[a]	DAOM240163	Tunisia

[a] *Glomus aggregatum* isolate DAOM240163 has been shown to be likely a *Rhizophagus irregularis* according to its complete mitochondrial genome (Nadimi et al. 2016)

DNA isolation from spores in dry formulation

The NucleoSpin® Soil isolation kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used to isolate the gDNA from spores contained in dry formulation samples, with the following modifications: 100 mg of samples was transferred to a NucleoSpin® Bead Tube containing the ceramic beads from the kit, 25 mg of Celite 545 (diatomaceous earth, Imerys Filtration Minerals, CA, USA), and 70 μL of Carnation® instant skim milk powder at 100 mg/ml (Takada-Hoshino and Matsumoto 2004). As recommended by the manufacturer's instruction, 700 μl of SL1 buffer and 150 μL of Enhancer SX buffer were added. Samples were homogenized using a Precellys® 24 homogenizer (Bertin Technologies, France) with three runs performed at 5000 rpm for 15 s each, with a break of 5 s between runs. The remaining extraction steps followed the manufacturer's protocol.

Primers and probe specific to *R. irregularis* DAOM-197198

The specific primer set 197198F (5 -CCCACCAG GGCAGATTAATC-3) and 197198R (5 -TGGCTTTG TACAGGCAACAG-3) and the minor groove binder (MGB) probe (*VIC*-CCCTGGAGTATCTG-*MGB-NFQ*) were designed on the mt genome of *R. irregularis* DAOM-197198 (accession number HQ189519, Fig. 1). Primers and the MGB probe labeled with VIC at 5 and *MGB-NFQ* quencher at 3 were synthesized and validated

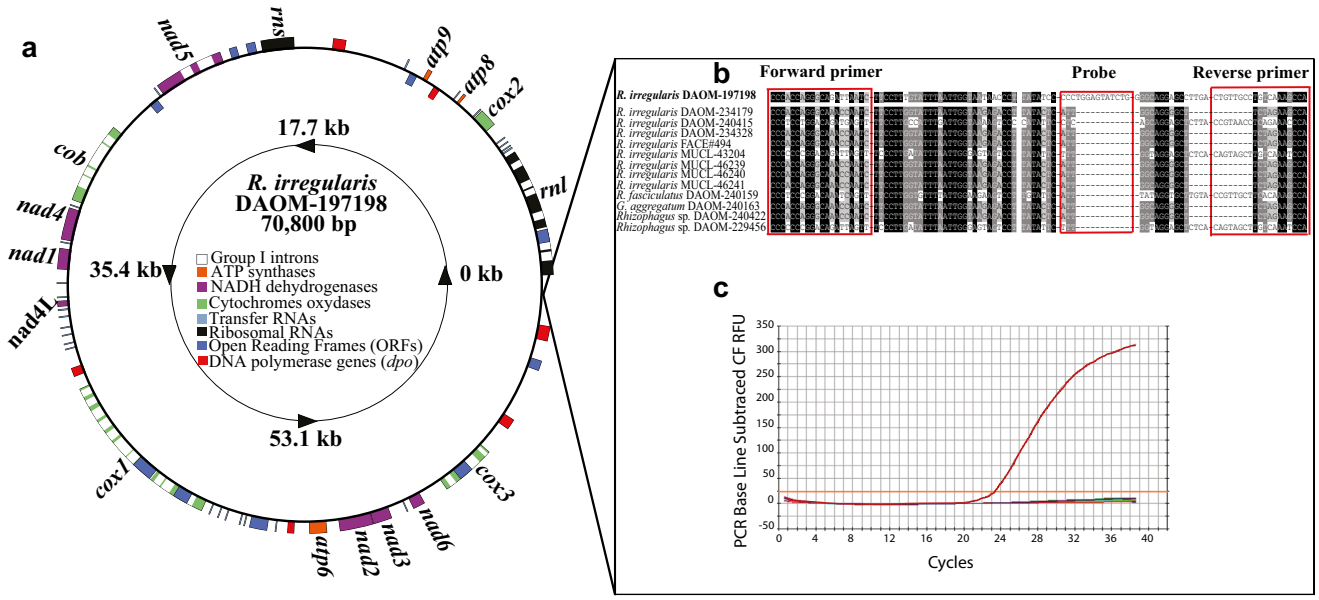

Fig. 1 **a** The mitochondrial circular-mapped genome of *Rhizophagus irregularis* DAOM-197198. Genes on the *outer* and *inner circumference* are transcribed in a clockwise and counterclockwise direction, respectively. Genes and corresponding product names are *atp6, 8, 9,* ATP synthase subunit 6; *cob*, apocytochrome b; *cox1–3*, cytochrome c oxidase subunits; *nad1–4, 4L, 5–6*, NADH dehydrogenase subunits; *rnl, rns*, large and small subunit rRNAs; A-W, tRNAs, the *letter* corresponding to the amino acid specified by the particular tRNA followed by their anticodon. Open reading frames smaller than 100 amino acids are not shown. **b** Alignment of a small portion of the *cox3-rnl* intergenic region where the *R. irregularis* DAOM-197198 specific marker was designed. The alignment was performed using four distinct *R. irregularis* isolates, along with three closely related species. The *red boxes* correspond to the forward primer, fluorescent probe, and reverse primer, respectively. The resulting marker was also tested for specificity on NCBI nucleotide blast against all published mt genomes. **c** *Graphical view* of qPCR amplifications using the *R. irregularis* DAOM-197198 specific marker. The specificity was tested on DNA from *R. irregularis* isolate DAOM-234179, DAOM-240415, DAOM-234328, DAOM-2207225, and DAOM-46328, along with the closely related species *Rhizophagus sp*. DAOM-229456, *R. fasciculatus* DAOM-240159, *R. clarus* DAOM-240429 and DAOM-234281, *Rhizophagus sp*. DAOM-240422, *G. aggregatum* DAOM-240163, and a negative control. Only *R. irregularis* DAOM-197198 was successfully amplified with the marker (color figure online)

by Applied Biosystems (Thermo Fisher, Burlington, ON, USA). Their specificity was first assessed by checking in silico the absence of significant similarity of our primers and probe with other known DNA sequences or with the recently published nuclear genome of *R. irregularis* DAOM-197198 (accession no. AUPC00000000.1, Tisserant et al. (2013)) using BLAST (Altschul et al. 1990) in the NCBI GenBank public database. Tests of specificity were performed in qPCR using the gDNA of various AMF taxa as templates, either in separate reactions or mixed together in the same reaction with the gDNA of *R. irregularis*. We mixed the DNA of isolate DAOM-197198 to the DNA of the other isolates in order to simulate AMF community in roots in nature. Amplifications were performed in volumes of 30 µl containing 15 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Thermo Fisher, Burlington, ON, USA), 660 nM of each primer, 83 nM of TaqMan MGB Probe, and 4 µl of template DNA. The reactions were carried out on an iCycler thermal cycler (Bio-Rad) and consisted of an initial denaturation step at 95 °C for 8 min and 30 s followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Standard curve development, validation, and robustness evaluation of the qPCR assay

Absolute quantification of the number of copies of mt genomes per spore was done by standardization with respect to tenfold serial dilutions of purified plasmids containing the PCR product of *cox3-rnl* intergene from *R. irregularis* DAOM-197198. The PCR product was ligated into the StrataClone™ PCR cloning vector pSC-A and transformed in the StrataClone™ SoloPack® competent cells (Stratagene, La Jolla, CA, USA). Minipreps of isolated plasmid DNA were prepared using the illustra plasmidPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK). Plasmid concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher, Burlington, ON, USA) as well as Qubit fluorometric quantification using Qubit dsDNA HS Assay Kit (Life Technologies, Thermo Fisher, Burlington, ON, USA) following the manufacturer's instructions. The purified plasmid solution was diluted to prepare a serial tenfold standard curve ranging from 5×10^7 to 5×10^2 copies in a volume of 4 µl.

The standard curve was validated as per the International Conference on Harmonization guideline (ICH, 1995) and the

Center for Drug Evaluation and Research (CDER, 2001). Three different tenfold serial dilutions were performed and tested over 3 days. On day 1, tenfold series of six dilutions each were performed from the stock of plasmid DNA and six replications for each dilution were done in qPCR. On day 2, new tenfold serial dilutions were performed using the stock of plasmid DNA and three replications for each dilution were done in qPCR. This was repeated on day 3. The standard curve was validated if the qPCR efficiency was between 90 and 110 % and the correlation coefficient greater than 0.995 for each real-time PCR performed and if the interassay coefficient of variation was lower than 7.5 %. The amplification efficiency of a qPCR reaction is calculated based on the slope of the standard curve according to the following formula: $\text{Efficiency} = -1 + 10^{(-1/\text{slope})}$.

The robustness of a qPCR assay is a measure of its capability to remain unaffected by small but deliberate variations during the procedure, which highlights its reliability during routine usage. The influence of a 10 % variation of Master Mix, primers, and probe in the final reaction volume was analyzed to assess the robustness of the qPCR assay.

Determination of mt genome copy number per spore and its validation

To assess the number of mt genome copies per spore in simple liquid suspension, we used six (6) qPCR assays on five known concentrations of spores that were manually counted: 50, 100, 200, 400, and 800 spores. Six qPCR experiments were performed over 2 days. To validate the determined mt copy number per spore, three different experiments were performed across 3 days with known concentrations of spores: day 1, eight replicates of 100, 200, and 400 spores; days 2 and 3, four replicates each, totaling 16 qPCR assays. gDNA was isolated from each spore suspension of *R. irregularis* DAOM-197198 using the PowerSoil DNA Isolation Kit, according to the protocol described above. Three technical replicates for each sample were performed in qPCR.

Limit of detection and limit of quantification

The method proposed by Shrivastava and Gupta (2011) was used to calculate the LOD and LOQ of the qPCR assay. For a linear regression, LOD and LOQ can be expressed as $\text{LOD} = 3S_a/b$ and $\text{LOQ} = 10S_a/b$, where S_a is the standard deviation of y-intercepts of regression lines and b is the slope of the calibration curve. LOD and LOQ were calculated based on the analysis of 0, 5, 25, and 50 spores in triplicate.

Assessing the precision of the qPCR assay

The precision of the qPCR assay was assessed using spores in complex liquid formulation and in dry formulation. For spores in complex liquid formulation, total DNA was isolated from three samples containing 100, 200, and 400 spores per milliliter of *R. irregularis* DAOM-197198 using the PowerSoil DNA Isolation Kit, as described above. Each spore solution was sampled on three different days with respectively eight, four, and four replicates. Three technical replicates were performed in qPCR. The qPCR reactions were run independently for each assay. The quantification calculation for the three assays was based on the same standard curve. This experimental design was analyzed with a repeated measures ANOVA.

The same experimental design was applied to assess the precision of the qPCR assay on spores in dry formulation, except that total DNA was isolated from two samples containing 3200 and 35,000 spores per gram of *R. irregularis* DAOM-197198 using the NucleoSpin® Soil Isolation Kit, as described above.

Finally, the quantitative values obtained with the qPCR assay on 20 blind samples (these samples were provided by Premier Tech and their concentration was kept secret) from complex liquid formulations and 20 samples from dry formulations were compared with the concentration of viable and total spore number determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric assay (Mosmann, 1983). DNA isolation was performed in triplicate for each sample, and three technical replicates were used in qPCR. For both formulations, MTT and qPCR results were compared using a *t* test for dependent samples. The null hypothesis consists in the mean of the differences from the paired samples being equal to zero.

In order to determine the number of viable and total spores using MTT staining of liquid formulations, 0.5 mL of spore suspensions of 400 spores per milliliter was collected and mixed with 0.5 mL of MTT solution (0.5 %) and incubated prior to the stereomicroscope observation, while 5 g of solid formulations (PS3) was weighed and passed through a wet sieve using a 38-μm opening sieve. Spores were then collected and resuspended in water to obtain a spore suspension of 400 spores per milliliter followed by MTT staining. A determined fraction of MTT-stained spores was manually counted under a stereomicroscope. MTT-stained spores (red color) were considered to be viable while non-stained spores were considered to be dead spores.

Intermediate precision

According to the ICH (2005), the intermediate precision covers the various influences within a laboratory and gives a first indication of the future transferability of an analytical method. In the present study, two different laboratory operators conducted intermediate precision on three different days. Every day, each operator took three samples from the same batch of spores in complex liquid formulation and subsequently extracted DNA and performed qPCR using the same

procedures described above. Data were analyzed through a factorial design ANOVA.

Tests for spore conservation and DNA stability using qPCR assay

In order to evaluate the potential of the qPCR assay for quality control, we performed additional tests in which we simulated the impact of the transport conditions on the stability of spores. We used three different batches of spores in complex liquid formulation that were kept at three temperatures: 4, 22, and 32 °C. Samples of 200 spores were collected at different time points, 0, 24, 72, and 168 h, and they were subjected to DNA extraction followed by qPCR assay in three replicates as described above. To assess spore storage conditions, we used a time-frame of 6 months during which 18 aliquots of 200 spores each were stored at −20 °C. At each time point (0, 1, 3, and 6 months), six aliquots were taken from the freezer and subjected to DNA extraction and qPCR in three replicates. We also used three fresh samples as a control.

For DNA stability, the effect of time and temperature (4 and −20 °C) on the qPCR assay was analyzed. DNA of six samples of 200 spores was extracted. Two extracted DNA samples were pooled resulting in three different DNA pools. Each DNA pool was divided in eight aliquots. Four aliquots were kept at 4 °C while the remaining four aliquots were stored at −20 °C for each DNA pool. At different time points, 0, 24, 48, and 72 h, three aliquots of each DNA pool were taken and subjected to qPCR assay in three replicates each.

Data from the transport condition experiment and stability of extracted DNA were analyzed through repeated measures ANOVA, whereas long-term storage of frozen spores was analyzed with a one-way ANOVA.

Statistical analysis

Statistical tests described above were performed with Prism version 5 (GraphPad software, California) and Statistica version 7 (StatSoft).

Results and discussion

Rhizophagus irregularis DAOM-197198 specific real-time PCR marker

The comparative analyses of mt genomes between closely related isolates and species to *R. irregularis* DAOM-197198 (Formey et al. 2012; Beaudet et al. 2013a, 2013b, Nadimi et al. 2015) showed that most of the differences observed are caused by the presence of a large number of mobile elements of different lengths, orientations, and degrees of erosion such as plasmid-related DNA polymerase, small inverted repeats, and homing endonuclease genes. Based on the alignment of 14 mitochondrial genomes, including numerous *R. irregularis* isolates and closely related species, primers and probe specific to *R. irregularis* DAOM-197198 were designed to target the hyper-variable *cox3-rnl* intergene of the mt genome, which is a large region comprising substantial sequence divergence (Fig. 1a, b). The primer sets 197198F and 197198R amplified a fragment of 106 bp, and their specificity was assessed in silico using NCBI BLAST on all mt genomes published so far. No cross-specificity was observed with other *R. irregularis* isolates or its closely related species (Table 1), thus confirming its usefulness in detecting and quantifying *R. irregularis* DAOM-197198. The primers and probe specificity were also experimentally validated by a quantitative real-time PCR assay (Fig. 1c). DNA of transformed carrot roots was also used in our specificity tests where no amplification has been observed. Since mt genomes in AMF are assumed to be homoplasmic (Lee and Young 2009; Formey et al. 2012; Beaudet et al. 2013a, 2013b; la Providencia et al. 2013), a TaqMan assay targeting single-copy mt intergenic regions is expected to be more accurate and precise than quantification based on polymorphic and putative tandem-repeat rDNA sequences (Sanders et al. 1995; Clapp et al. 1999; 2001) in potentially heterogeneous nuclear genomes.

It has been shown that mitochondrial DNA copies (numts) can be present in the nuclear genome of AMF (Lee and Young 2009; Beaudet et al. 2015), so we performed numt searches—no numt of the *cox3-rnl* region were found in the nuclear genome of *R. irregularis* DAOM-197198 (Lin et al. 2014; Beaudet et al. 2015). Furthermore, it has been shown that compatible AMF isolates can perform anastomosis and exchange genetic material, including mitochondrial DNA (de la Providencia et al. 2013), giving rise to a heteroplasmic state where recombination can occur (Beaudet et al. 2013a, 2013b). This process could hamper the detection of the rightful isolate in natural or agricultural condition if hybrids are created through this process, but this heteroplasmic state has been shown to be rapidly lost in vitro through subcultivation (Daubois et al. 2016). The isolate-specific mitochondrial quantification assay presented in this study not only paves the way to use this methodology in a broad range of species but will also allow targeting of agriculturally important AMF families such as the *Glomeraceae* by using conserved mt protein-coding genes.

Validation and robustness of standard curve and copy number determination of mt genome per spore

Standard curve validation was successful since each amplification of the standard curve had an efficiency ranging from 98.32 to 105.74 % and a correlation coefficient of 0.999, and the interassay coefficient of variation was lower

Table 2 Validation of the standard curve performed across three days on series of six dilutions of tenfold each

Validation test	Number of replicates	Correlation coefficient	Slope	Intercept	PCR efficiency	Coefficient of variation (%)
Day 1	6	0.998	−3.353	40.590	98.7	0.79
Day 2	3	0.999	−3.327	40.920	99.8	0.63
Day 3	3	0.999	−3.192	40.309	105.7	0.92

than 7.5 % for each point (Table 2). Robustness tests on Master Mix, primers, and probe variation (±10 %) did not significantly influence the variation of slopes (linear regression, $p = 0.225$, $p = 0.118$, and $p = 0.054$, respectively, for Master Mix, primers, and probe, Table S1). Because the *cox3-rnl* intergene is present within the mt genome as a single copy and no numt has been observed in the nuclear genome, the validated standard curve allowed determination of the number of mitochondrial genomes per spore using the following known numbers of spores in suspensions: 50, 100, 200, 400, and 800 spores. Results from the qPCR assays showed that the average number of copies per spore was 3172 (±342 SE, $n = 6$, Table S2). The copy number per spore was validated using known spore concentrations of 100, 200, and 400 spores with a maximum coefficient of variation of 9 % (Table S3). The LOD and the LOQ were 5 and 17 spores, respectively (Fig. 2). Our study reports the first absolute quantification of mitochondrial DNA copy number in Glomeromycota. Traditionally, mitochondrial copy number has been determined using optical microscopy and fluorescent probes such as MitoTarcker (Lang and Hijri 2009). Mitochondria vary substantially in number (ranging from a single mitochondrion per cell in some unicellular microorganisms, to thousands of mitochondria per cell in animal cells) and location depending on cell type and organisms (das Neves et al. 2010). However, caution should be made for correlation between mitochondrial number per cell and mitochondrial genome (mtDNA) copy number per cell, because each mitochondrion could harbor many copies of mtDNA (e.g., 20 to 50 copies of mtDNA per mitochondrion in budding yeast, Hausner (2003)).

Numerous studies have attempted to develop a qPCR assay to quantify AMF propagules using both TaqMan or SYBR Green assays. Most of these assays relied on the nuclear large ribosomal subunit gene and ITS regions (Filion et al. 2003; Alkan et al. 2006; Jansa et al. 2008; Gamper et al. 2008; Thonar et al. 2012), although some studies have developed and used qPCR assays based on the mitochondrial large subunit rDNA (mtLSU rDNA, also known as *rnl* gene) in some AMF species (Kiers et al. 2011; Krak et al. 2012; Couillerot et al. 2013). Krak et al. (2012) have compared mtDNA-based quantification method to that of nuclear rDNA, and they reported that the ratio of these two assays varied consistently between two isolates of *G. intraradices*. However, none of these studies applied a validation protocol for their standard curves and clearly linked the amount of DNA to the spore number. The determination of spore number is crucial in the context of the commercial production of AMF-based biofertilizers in order to provide the industry with reliable molecular tools for quality control. These biofertilizers mostly contain spores along with other propagules such as hyphae, vesicles, and root fragments as well as other microbes. Gamper et al. (2008) showed that the spore number was a good predictor of the amount of DNA recorded using qPCR while hyphae contribution was limited. This could be explained by the high nuclear content (and likely mitochondria content) found in AMF spores (Marleau et al. 2011).

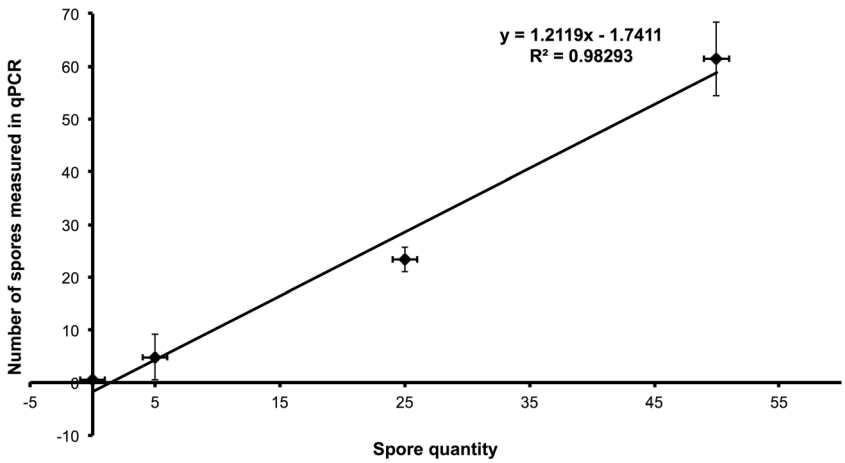

Fig. 2 Determination of limit of detection and the limit of quantification using known number of spores (5, 25, and 50 spores, $n = 3$ for each)

Table 3 Precision results for liquid formulations obtained from repeated measures ANOVA

		Day 1			Day 2			Day 3				
Liquid formulation: level of factor	N	Mean (spores/mL)	Std. dev. (spores/mL)	CV%	Mean (spores/mL)	Std. dev. (spores/mL)	CV%	Mean (spores/mL)	Std. dev. (spores/mL)	CV%	Mean per factor level	Std. dev over the 3 days
100	4	119	16	13	98	24	25	101	19	19	106	20
200	4	180	19	10	195	43	22	175	37	21	183	33
400	3	389	51	13	361	41	11	353	48	14	368	48

Precision and intermediate precision of the qPCR assay

The quantification of complex liquid formulation containing 100, 200, and 400 spores per milliliter were respectively 106 ± 20, 183 ± 33, and 368 ± 48 (Table 3). According to the repeated measures ANOVA, DNA quantification per spore level did not significantly ($p = 0.46$) change between the 3 days of repetition (Table 4). The quantification of dry formulation containing 3200 and 35,000 spores per gram recovered 2212 ± 370 and 26,142 ± 3015 spores per gram, respectively (Table 5). Contrarily to the liquid formulations, DNA extraction from dry formulation was less reproducible with significant differences ($p = 0.003$) between days of extraction (Table S4). The number of spores in dry formulations seemed also to be underestimated. This could be the result of the difficulty of sampling homogenous aliquots of 0.1 g in dry formulations containing different sizes of aggregates of spores and root fragment.

We performed the so-called Intermediate precision evaluation with two different laboratory operators who have used the same experimental procedure on the same complex liquid formulation over 3 days. The results did not show any significant variation between the operators ($p = 0.56$) nor was there any interaction between the operators and the day of analysis ($p = 0.78$) (Table S5). Both operators obtained similar DNA quantification with means of 303 and 290 spores per milliliter, with similar coefficients of variation in the range of 6 to 24 % (Table S6).

Additional tests such as transport conditions (4, 22, and 32 °C from 0 to 168 h), frozen (−20 °C) spore storage (0, 1, 3, and 6 months), and DNA extract stability on different temperatures (4 and −20 °C) were also performed. Conclusions from the "transport conditions experiment" are not clear (Fig. S1). Results from the repeated measures ANOVA show significant effects of exposure time ($p = 0.005$) and of the interaction "temperature × exposure time" ($p = 0.02$). However, the Tukey test indicates that the worst conditions tested (at 32 °C) were not those having the significant effects. Although the interpretation of these results remains ambiguous, the results suggest that spores in liquid formulations can be sensitive to temperature higher than 4 °C. Care should be taken to keep samples in cold conditions for transportation.

Medium-term storage of samples could be very useful for a commercial laboratory. We tested spore stability at −20 °C for a period of 6 months (Table S7). Results indicate that whole spores can be frozen and kept 6 months without affecting the DNA quantification ($p = 0.53$).

DNA extract stability would also be a useful property for commercial laboratory operations. Although Fig. S2 suggests that DNA extracts are stable at 4 and −20 °C up to 72 h, the repeated measures ANOVA indicated a significant effect ($p = 0.01$) (Table S8) of time with a small decrease after 24 h for both temperatures.

Spore quantification in samples with unknown concentration

Blind tests performed on various samples of unknown spore concentration in complex liquid and dry formulations are

Table 4 Repeated measures analysis of variance on liquid formulations

	SS	Degree of freedom	MS	F	*p* value
Intercept	1553672	1	1553672	2177.71	0
Liquid formulation	361723	2	180861	253.505	0
Error	5708	8	713		
DAYS	2260	2	1130	0.824	0.456363
DAYS × liquid formulation	2231	4	558	0.407	0.801058
Error	21934	16	1371		

Table 5 Precision results for dry formulations obtained from repeated measures ANOVA

		Day 1			Day 2			Day 3				
Dry formulation: level of factor	*N*	Mean (spores/mL)	Std. dev. (spores/mL)	CV%	Mean (spores/mL)	Std. dev. (spores/mL)	CV%	Mean (spores/mL)	Std. dev. (spores/mL)	CV%	Mean per level factor over the 3 days	Std. dev over the 3 days
P3200	4	2119	400	19	2482	492	20	2035	234	11	2212	370
P35000	4	27224	3428	13	27198	3772	14	24006	2184	9	26142	3015

shown in Fig. 3. For quantification performed in complex liquid formulation, spore estimates based on qPCR were in general superior by 7 % to the viable spore estimates based on MTT staining ($p < 0.05$) (Fig. 3a). However, they were not significantly different ($p = 0.31$) from the total spore estimates (Table S9). Estimates based on qPCR were more variable than those based on MTT staining. The relative standard deviation for estimates based on MTT staining ranged between 0 and 10 % and between 2 and 30 % for qPCR. For quantification performed in dry formulation, spore estimates based on qPCR were in general superior by 33 % to the viable spore and by 26 % to the total spore estimate based on MTT staining ($p < 0.05$) (Fig. 3b, Table S10)). The relative standard deviation for estimates based on MTT staining ranged between 0 and 15 % with an average of 4.3 % and between 7 and 38 % for qPCR with an average of 11 %. It is noteworthy to mention that the quantification of viable spores using the MTT method in dry formulations could be challenging because of the large

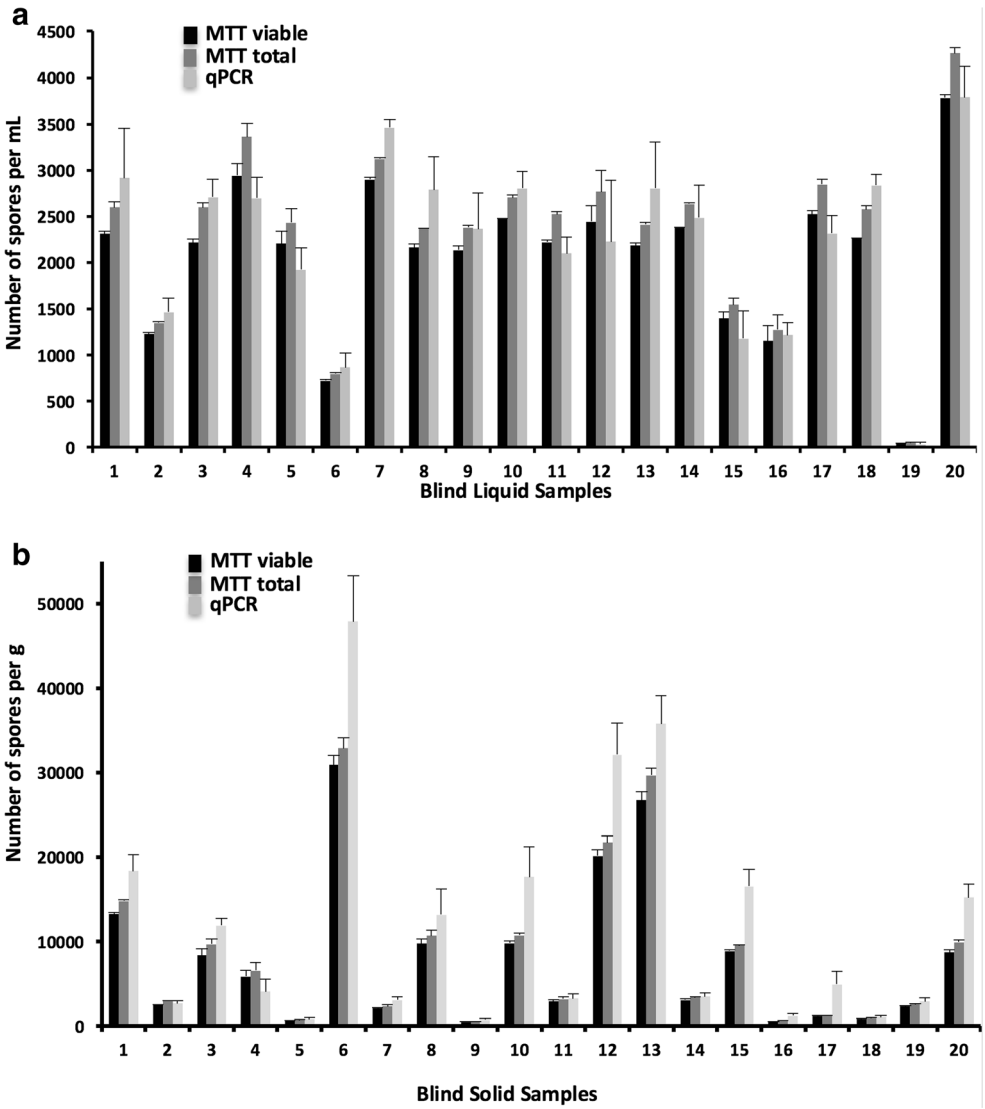

Fig. 3 Comparison of spore concentration in blind samples between qPCR analyses and (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric assay (MTT). Panel **a** shows blind liquid samples; panel **b** shows blind solid formulation. MTT viable means positively stained spores (those spores that showed *red-purple color*), while MTT total means the total number of spores counted regardless of the color (color figure online)

Table 6 Workflow and experimental procedure used to develop and validate the qPCR assay for the absolute quantification of spores of the isolate *R. irregularis* DAOM-197198

Workflow	Experimental procedure and variables	Measurement	Pass criterion
Development and specificity tests of qPCR assay of *R. irregularis* DAOM-197198			
In silico analysis of mtDNA sequences	Alignment of publically available mtDNA of AMF; identification of polymorphic regions; design of primers and probe; comparison and BLAST searches of sequence similarity in public databases	Percentage of nucleotide sequence similarity	Confirmation of primers and probe specificity in silico
Specificity tests using conventional PCR and qPCR of individual samples	11 non-target isolates closely related to *R. irregularis* DAOM-197198 listed in Table 1, and DNA from carrot roots	PCR and qPCR signals	No detectable signal
Specificity tests in mixed reactions (multiplexes reactions) in qPCR	DNA from 11 non-target AMF (Table 1) and carrot roots mixed and used as a template in qPCR	qPCR signal	No detectable signal
Standard curve development and evaluation			
Validation	Three qPCR assays performed during 3 days (one qPCR assay per day)	qPCR efficiency Correlation coefficient Interassay coefficient of variation	90–110 % ≤0.995 ≤10 %
Robustness	10 % variation of Master Mix, primers, and probe in the final reaction volume	qPCR efficiency Correlation coefficient Interassay coefficient of variation	90–110 % ≤0.995 Not significant
Spores quantification			
Determination of mt genome copy number per spore	Five known concentrations of spores (manually counted) Six qPCR assays performed during 2 days	Average number of copies per spore	Not applicable
Validation of the copy number of mt genome per spore	Three known concentrations of spores (theoretical concentration, i.e., obtained by dilution) Sixteen qPCR assays performed during 3 days	Interassay coefficient of variation	≤10 %
Performance of spore quantification			
Limit of detection (LOD) and limit of quantification (LOQ)	Four known concentrations of spores (manually counted)	Method proposed by Shrivastava and Gupta (2011)	Not applicable
Precision in liquid formulation	Three known concentrations of spores (theoretical concentration, i.e., obtained by dilution) Sixteen qPCR assays performed during 3 days	Intra-assay and interassay coefficient of variation	Not significant ($p < 0.05$)
Precision in dry formulation	Two known concentrations of spores (theoretical concentration, i.e., obtained by dilution) Sixteen qPCR assays performed during 3 days	Intra-assay and interassay coefficient of variation	Not significant ($p < 0.05$)
Intermediate precision	Two different operators qPCR assays performed during three different days	Interoperator coefficient of variation	Not significant ($p < 0.05$)
Exactitude in liquid and dry formulations	Twenty blind samples from each formulation were tested	Comparison of spore estimates with the MTT staining method	Not applicable
Stability of analyzed samples in industrial process			
Resilience of spores	Three temperatures were tested; four time points; six replicates	Intra-assay and interassay coefficient of variation	Ambiguous results
Conservation of spores at −20 °C	Four time points over 6 months; six replicates	Intra-assay and interassay coefficient of variation	Not significant ($p = 0.53$)
DNA stability	Two temperatures; four time points; three samples replicates	Intra-assay and interassay coefficient of variation	Significant ($p = 0.01$)

amount of root debris and aggregates. Spores are found to cluster in the roots, and this can cause significant variations between estimates from different operators depending on their level of experience. Because of the small amount of material used for DNA quantification (0.1 g), the qPCR assay would also be affected by spore clustering in the roots. Variation of the estimates of qPCR is most likely caused by the difficulty of obtaining consistent weight of 0.1 g per sample of a potentially not entirely homogenous material versus 5 g samples used in MTT staining. In addition, solid formulations can result in the formation of aggregates of propagules, which can introduce bias.

Together, all these tests (summarized in Table 6) demonstrate that our qPCR assay is relatively robust and has the potential to be used routinely. Commercial laboratories interested to offer the qPCR service for end-users have to validate the qPCR assay according to their equipment and operators. We are confident that qPCR assay could replace the conventional MTT staining method at least when the volume of samples is important, the principal advantage of qPCR being automation of the process and time (qPCR assay can be done in less than 4 h). Of course, conventional methods of numerating AMF propagules in liquid formulations based on MTT staining and microscopy can give precise results with well-trained operators. But when comes numeration in solid formulations, it becomes fastidious, time consuming, and less precise.

Conclusion

We report a new qPCR assay specifically designed to detect and quantify spores of the model AMF *R. irregularis* isolate DAOM-197198. This strain is of major importance as it has been used worldwide in commercial inoculants since the 1990s. We also demonstrate the usefulness of the mitochondrial genomes in developing efficient biomarkers in AMF. Although repetitions are needed in order to confirm the different results, we performed numerous validation tests and showed that our qPCR assay is robust and reliable. qPCR assays, compared with conventional microscopic estimation of AMF spores, have the advantage of quickly and efficiently identifying the isolate of interest even if mixed with other AMF spores or found in a complex matrix, as well as rigorously quantifying AMF abundance in a large number of samples. The former advantage is of interest for quality control of commercial products using *R. irregularis* spores. Regarding environmental studies focusing on presence and abundance of AMF in soil samples and plant roots, the assay could be very attractive although more work is required to demonstrate the absence of interferences in the extraction of DNA soil samples. Since DNA quantification requires very small samples, the question of the representativeness would also arise quickly. Caution should also be made to interpret the results of spore quantification of inoculants in which spores could germinate, if not stored adequately because their germination may influence mtDNA copy number per propagule. Further experiments are needed to correlate AMF biomass, function, and mtDNA copy number in order to efficiently trace AMF inoculants in crop roots and agricultural soils. Nonetheless, we believe that our experimental procedure and validation protocol can be applied to a wide range of AMF taxa as well as to other crop beneficial microorganisms if additional isolate-specific primers and probes were developed.

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