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Research

Dead or Alive, that Is the Question: Development and Assessment of Molecular *Synchytrium endobioticum* Viability Tests

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Abstract

Potato wart disease caused by the obligate biotrophic fungus *Synchytrium endobioticum* is a devastating disease that can result in significant crop losses. Resting spores of this pathogen can remain viable and infectious in soil for decades. The detection of viable resting spores using conventional methods such as bioassays and direct microscopic examination are challenging and time-consuming and require specific expertise and facilities. Molecular methods, such as real-time PCR, have been shown to be effective in detecting the presence of *S. endobioticum* DNA in soil samples but cannot differentiate between viable and nonviable spores. In this paper, we present three novel mRNA-based molecular tests to potentially detect viable *S. endobioticum* resting spores. The tests are specific to the transcribed mRNA and do not detect the genomic DNA of the target genes. We demonstrate the analytical sensitivity using synthetic constructs of the target mRNAs. The tests were found to be able to repeatedly detect 10 target copies per reaction. Soils and waste of potato processing industries free from *S. endobioticum* were used to assess the exclusivity of the tests. The biological relevance of mRNA detection was determined in the context of replicated bioassays. Applications of the tests to facilitate collection management, assessment of the effects of treatments on presumed viability of *S. endobioticum* resting spores, and the potential use in descheduling of previously infested plots are discussed.

Keywords: Chytridiomycota, molecular viability, mRNA, pathogen detection, potato wart disease, real-time RT-PCR

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Synchytrium endobioticum, the causal agent of potato wart disease, is an intracellular obligate biotrophic chytrid fungus that can cause significant crop losses in cultivated potato (*Solanum tuberosum* L.). Upon infection, hypertrophy and cell hyperplasia of meristematic tissue on tubers, stolons, eyes, and sprouts is induced by the pathogen. This results in the typical cauliflower malformation of affected tissue. As part of its life cycle, robust resting spores are formed in the malformed tissue. When the host decays,

these spores are released in the surrounding soil, where they can remain viable and infectious for decades (reviewed by van de Vossenberg et al. 2022b).

Molecular tests have been developed for the detection and identification of *S. endobioticum*, first by conventional PCR tests (Niepold and Stachewicz 2004; van den Boogert et al. 2005), followed by real-time PCRs (Smith et al. 2014; van Gent-Pelzer et al. 2010) resulting in increased sensitivity for pathogen detection (van de Vossenberg et al. 2018). In addition, a real-time PCR targeting a pathotype 1(D1)-specific SNP was developed representing an initial attempt toward molecular pathotype identification (Bonants et al. 2015) that was later shown to be impossible due to the complexity and the multiple independent mutation events leading to the loss of function of *AvrSen1* (van de Vossenberg et al. 2019a). These (real-time) PCR tests have proven to be highly valuable in research and plant health diagnostics. However, these DNA-based tests cannot differentiate between viable spores and nonviable spores as nonviable spores may also contain (fragmented) DNA remnants.

Currently, the viability of *S. endobioticum* is either determined visually by light microscopy examination or with bioassays. Determining the viability of resting spores by visual examination of the spore content as described in EPPO/OEPP (2017b) is complex, subjective, and often inconclusive as part of the spores remain unclassified. Determining spore viability with bioassays can be challenging, as these are time-consuming, require specific facilities, and also rely on specific conditions for infection to occur, such as the availability of susceptible potato varieties with their buds in the right physiological condition (out of dormancy) and availability of high numbers of resting spores. Even then, success rates have been reported varying from 27 to 90% infection (Baayen et al. 2005; Browning 1995). Despite the challenges associated with determining resting spore viability, recent European Union legislation specifically mentions absence of viable spores with direct examination as one of the requirements for complete or partial revocation of phytosanitary measures (Anonymous 2022).

Here, we describe the development and assessment of three molecular real-time reverse transcriptase (RT-)PCR tests to determine the presence of potentially viable resting spores. The molecular tests target mRNA of different single-copy nuclear protein-coding genes. Real-time RT-PCR viability tests for other plant-pathogenic fungi have been developed targeting specific mRNA transcripts (Chimento et al. 2012; Narusaka et al. 2010). In living cells, the presence of mRNA is an indicator of cellular viability, as mRNA is less stable and subject to degradation much faster than DNA. The performance characteristics of the tests were determined based on guidelines presented by the European and Mediterranean Plant Protection Organization (EPPO) (EPPO/OEPP 2021). The biological relevance of real-time RT-PCR results obtained was demonstrated in the context of the Spieckermann bioassay (EPPO/OEPP 2004). Application of the molecular mRNA detection tests, such as *S. endobioticum* spore collection management, effects of treatments on presumed viability of the pathogen, and molecular detection of potential viable spores in previously infested fields are discussed.

Materials and Methods

Real-time RT-PCR design

Three loci were selected for real-time RT-PCR design. First the *S. endobioticum* elongation factor 1 alpha gene (*Ef1*α) was selected because it is a single-copy gene, highly transcribed, and essential for protein synthesis. In addition, it has a high intron content, allowing for design of primers and probes spanning multiple exon–exon boundaries in the mature mRNA. Gene sequences were extracted from the two structurally and functionally annotated *S. endobioticum* reference genomes SeMB42 (assembly: GCA_006535955.1) and SeLEV6574 (GCA_006536045.1) and the related *Synchytrium microbalum* JEL517 (GCA_006535985.1). Coding sequences (CDSs) were extracted from the *Ef1*α gene sequences (MB42_g00306, LEV6574_g00664, and SmJEL517_g02074) in Geneious Prime 2019 (Biomatters, New Zealand) and aligned with MAFFT (Katoh and Standley 2013). Candidate primers and probe sequences were visually selected and optimized with the IDT PrimerQuest tool (Integrated DNA Technologies, IA). The two other loci, identified as transcripts D166 and D852, were selected from a de novo transcriptome assembly in CLC Genomics Workbench 11.0.1 (map reads back to contigs = on; length fraction = 0.8; similarity fraction = 0.8, minimum contig length = 200) with cDNA from resting spores of *S. endobioticum* isolate LEV6574 (SRA: SRR8068361-SRR8068376) (Smith and Singh 2017). Transcript D166 was identified as genes SeMB42_g04538 and SeLEV6574_g05356, and transcript D852 as genes SeMB42_g03161 and SeLEV6574_g01495, in the respective MB42 and LEV6574 reference genomes.

Orthofinder results generated as part of a comparative genomics study of chytrid species (van de Vossenberg et al. 2019c) was used to identify orthologous genes that were included to test the in-silico specificity of the oligonucleotides. The oligonucleotides were tested using the "test saved primers" tool in Geneious Prime 2019 on the respective gene and CDS sequences (mismatches allowed = 3).

Public RNAseq data for pathotype 1(D1), 2(G1), 6(O1), and 18(T1) strains (Supplementary Table S1) were quality trimmed and mapped to the LEV6574 genome with the RNAseq-analysis tool (length fraction = 0.9; similarity fraction = 0.95, max hits per read = 10, library type = 3′ sequencing, ignore broken reads = on, expression value = TPM) incorporated into CLC genomics workbench 23.0.2. Gene transcripts per kilobase million (TPM) values were normalized within a dataset using the median expression level for that set to determine the relative expression of the target genes in the different RNA-seq data sets.

Fungal materials

The materials analyzed in this study include resting spores obtained from accessions in the NPPO-NL compost collection and resting spores extracted from tare soils and warted tubers obtained from Dutch potato wart findings in the municipalities Veendam and Westerwolde in 2021. Resting spores were extracted from composts and soils using either a zonal centrifuge following the methodology described by Wander et al. (2007) or by manual sieving with sieving method B as described in EPPO standard PM7/28(2) (EPPO/OEPP 2017b). Manual sieving resulted in a saturated $CaCl_2$ solution containing resting spores, which was subjected to filtration over a 20-μm nylon net sieve as described by van de Vossenberg et al. (2022a). For wart material, 5 to 10 small wart pieces were taken from infested tubers and transferred to a GrindoMix beaker with a disposable cutting knife (Retsch, Haan, Germany) and 100 ml of tap water. Warts were ground for 20 s at 10,000 rpm in a GM200 GrindoMix (Retsch), and 300 μl of homogenized material was used as input for RNA extraction. Alternatively, 300 mg of wart pieces were directly used as input for RNA extraction.

RNA and DNA extraction

RNA was extracted using one of two different methods: RNeasy plant mini kit (Qiagen, Germany) or AllPrep PowerFecal DNA/RNA kit (Qiagen). For the RNeasy plant mini kit, resting

spore suspensions, wart homogenate (up to 300 µl), and wart pieces (up to 300 mg) were collected in a 1.5-ml bead beat tube containing a single 4-mm stainless steel bead and 700 µl of GH+ buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate pH 5.2, 25 mM EDTA, and 2.5% PVP-10) (Botermans et al. 2013). Homogenization was performed with a Mixer Mill 200 (Retsch) for 2 min at 30 beats per second. RNA was further extracted following the protocol "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi" and eluted in 40 µl of molecular-grade water. For the AllPrep PowerFecal DNA/RNA kit, approximately 100 µl of resting spore suspensions was collected in a 2-ml bead beat tube containing pre-warmed (55°C) lysis buffer PM1, DTT, and two 3.2-mm stainless steel beads. Homogenization was performed with a Precellys Evolution Homogenizer (Bertin, France) for 3×20 s at 5,800 beats per minute. RNA and DNA were extracted in parallel following the manufacturer's instructions, and RNA and DNA were eluted separately respectively in 60-µl molecular-grade water and TE. DNA was extracted from wart pieces (up to 300 mg) and wart homogenate (up to 300 µl) using the PowerSoil Pro kit (Qiagen) following the manufacturer's instructions, and DNA was eluted in 100 µl of solution C6. RNA and DNA extracts were tested immediately or stored until use at −80 or −20°C, respectively.

Real-time RT-PCR reactions

The *EF1*α TaqMan real-time RT-PCR reactions were performed with the One Step PrimeScript RT-PCR Kit (Perfect Real Time) (Takara Bio Europe SAS, France) and consisted of 1× reaction buffer, 300 nM of primers EF1_F3m and EF1_R3m, 100 nM of probe EF1_P3m, 0.125 µl ROX reference dye II (when running the Quantstudio 12K Flex only), 2.5 U TaKaRa Ex Taq HS and 0.5 µl of PrimeScript RT enzyme Mix II. Molecular-grade water and 1 µl of RNA template were added to reach a final volume of 25 µl. Reactions were performed in a CFX96 Touch (BioRad, CA, U.S.A.) or a Quantstudio 12K Flex (Thermo Fisher Scientific, MA, U.S.A.) with the thermocycler protocol 10 min at 42°C, 10 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Real-time RT-PCR reactions were regarded as positive when exponential amplification curves and quantification cycle (Cq) o cycle threshold (Ct) values under 40 were obtained. The D166 and D852 SYBR real-time RT-PCR reactions were performed with the One-Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) (Takara Bio Europe SAS) and consisted of 1× reaction buffer, 400 nM of primers 166F2 and 166R2 or 852F2 and 852R2, 0.125 µL ROX reference dye II (when running the Quantstudio 12K Flex only), 1 µl of PrimeScript 1 step Enzyme Mix 2. Molecular-grade water and 1 µl of RNA template were added to reach a final volume of 25 µl. Reactions were performed in a CFX96 Touch (BioRad) or a Quantstudio 12K Flex (Thermo Fisher Scientific) with thermocycler protocol 10 min at 42°C, 10 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C and a melt curve analysis in the CFX96 Touch: hold 1 min at 60°C, 0.5°C increment steps 60 to 95°C (5 s/step + plate read), or a melt curve analysis in the Quantstudio 12K Flex: starting 15 s at 95°C, hold 1 min at 60°C, 0.05°C increment steps 60 to 95°C + plate read. Real-time RT-PCR reactions were regarded as positive when exponential amplification curves, Cq values under 40, and a melt peak at 83.5 ± 1.0°C (test D166) or 81.0 ± 1.0°C (test D852) were obtained. To quantify *S. endobioticum* DNA in the nucleic acid extracts, *S. endobioticum* specific real-time PCRs described by van Gent-Pelzer et al. (2010) and/or described by Smith et al. (2014) were performed using the Premix Ex Taq DNA Polymerase (Perfect Real Time) (Takara Bio Europe SAS) or Maxima Probe qPCR Master Mix (2×), with separate ROX vial (Thermo Fisher Scientific) reagents, respectively. Reactions were performed in a CFX96 Touch (BioRad) or Quantstudio 12K flex (Thermo Fisher Scientific) using a thermocycler program at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers and probes used in this study are listed in Table 1.

Analytical sensitivity

The limit of detection (LOD) for the molecular vitality tests was determined by testing serial dilutions with gBlock gene fragments (Integrated DNA Technologies, IA, U.S.A.) representing the CDSs of target genes and containing 100,000 to 1 copies/µl in triplicate. The LOD was determined as the lowest amount of gBlock copies that produce positive results in all dilution ranges tested. In addition, dilutions containing presumed viable resting spores from a 6-month-old compost prepared for inoculation experiments were tested. Samples consisted of 250, 100, 50, 10, 5, 2, or 1 individually counted resting spore(s) in molecular-grade water and were prepared in triplicate or five-fold.

Template specificity

The ability of the tests to specifically amplify the extracted mRNA transcript and not the nuclear-encoded gene was analyzed by testing gBlock fragments of gene sequences and the CDSs of the target genes. Furthermore, RNA extracted from wart material and wart homogenate was tested with and without RNase treatment (RNAse A [Qiagen], 4 µl = 28 Units, 30 min incubation at 37°C). RNA extracts were used to test both the mRNA-based

TABLE 1

Oligonucleotides used in this study

Test	Name	Sequence (5′–3′) and modifications[a]	Reference
*EF1*α TaqMan real-time RT-PCR	EF1_F3m	ACGATCCAGCCAAAGAAGC	This study
	EF1_R3m	GGACACATCTTGACGATAGCAG	This study
	EF1_P3m	FAM-ATTGCCTGC/ZEN/AAGTTTGCCGAGTTG-Iowa Black FQ	This study
D166 SYBR real-time RT-PCR	166F2	TCCATACGCGGCTTGAGA	This study
	166R2	GCAAAGATACCGCCTCATCA	This study
D852 SYBR real-time RT-PCR	852F2	TATTATGCGAAAGCCAACTC	This study
	852R2	TCTTTTCTATCCAGGAATATTTTGAC	This study
ITS2 TaqMan real-time PCR	SendoITS2F	TTTTTACGCTCACTTTTTTTAGAATGTT	van Gent-Pelzer et al. 2010
	SendoITS2R	CTGCCTCACACACCACATACA	van Gent-Pelzer et al. 2010
	Sendo probe2	FAM-AATTCGAGT/ZEN/TTGTCAAAAGGTGTTTGTTGTGG-EDQ	van Gent-Pelzer et al. 2010
18S TaqMan real-time PCR	Se18S_RTF1	CTCTGGTTGAGCTCCATTTAC	Smith et al. 2014
	Se18S_RTR2	CCTATTCTATTATTCCATGCTGTA	Smith et al. 2014
	Se18S_TM1	FAM-TATCCTGGTTCCCCACAGGCACTC-BHQ1	Smith et al. 2014

[a] ZEN is an internal quencher used to reduce background signals.

real-time RT-PCR tests and the DNA-based real-time PCR detections tests. Results for the DNA-based real-time PCR tests obtained with extracted RNA were compared with those obtained from DNA extracts of the same materials with the PowerSoil Pro kit (Qiagen) to determine the potential of DNA extraction with the RNeasy plant mini kit (Qiagen).

Analytical specificity

RNA extracted from soils ($n = 15$) and starch potato processing waste ($n = 4$) free from *S. endobioticum* were analyzed to demonstrate the exclusivity of the mRNA detection tests. The materials were determined to be free from the target pathogen following direct microscopic examination as described in EPPO standard PM7/28 (2) (EPPO/OEPP 2017b). Three main soil types for growing potatoes in the Netherlands were analyzed (five subsamples per soil type) (i.e., clay, sandy, and 'dal' soil; the latter being a mixture of the topsoil and the sandy subsoil after removal of the intermediate peat).

Biological relevance

The biological relevance of the obtained real-time RT-PCR results was determined by subjecting selected composts from the NPPO-NL spore collection to a Spieckermann bioassay. Composts with Cq values for the DNA-based ITS2 real-time PCR between 19.2 and 28.0, and Cq values for the mRNA-based *EF1*α real-time RT-PCR ranging from 25.3 to 39.2, were selected for testing. The DNA-based ITS2 and RNA-based *EF1*α, D166, and D852 tests were performed on DNA and RNA extracts obtained with the AllPrep PowerFecal DNA/RNA kit (Qiagen). Bioassays were performed in 2020 and 2021 according to PM7/28 (1) (EPPO/OEPP 2004). In 2020, 14 composts were tested in susceptible potato variety Deodara for pathotype 1(D1) and 18(T1) samples or Producent for pathotype 6(O1) and 8(F1) samples (14 to 24 tuber blocks per test), and bioassays were scored 41 to 62 days postinoculation (dpi). In 2021, 41 composts were tested using susceptible potato variety Deodara (22 to 24 tuber blocks per test), and bioassays were scored 48 to 55 dpi (Supplementary Table S2) to ensure not to miss potential late reactions. Compost 42012763 (Pot-ID_217) containing known viable pathotype 38(Nevşehir) *S. endobioticum* resting spores was included as a positive control in the biological relevance experiments. A subsample of this material was autoclaved for 15 min at 121°C, and spores were extracted seven days after treatment and analyzed with the mRNA real-time RT-PCR tests and in the Spieckermann bioassay.

Results

Test development

S. endobioticum genes SeMB42_g00306 and SeLEV6574_g00664 were identified as the genes encoding elongation factor 1 alpha, which belong to the cluster of orthologous genes (COG) OG0000504 as defined by van de Vossenberg et al. (2019c). Forward and reverse primers were designed to target the fifth and seventh exon–exon boundaries, and the TaqMan probe was designed to target the sixth exon–exon boundary of the mature mRNA, resulting in a 226-bp amplicon. From this COG, gene SmJEL517_g02074 was identified as the *S. microbalum* ortholog of which the CDS shares 84.2% sequence homology with the *S. endobioticum* CDS sequences.

The sequenced cDNA libraries from resting spore mRNA resulted in 7,285 transcripts representing 6,663 loci. Only 283 loci were expressed in resting spores at 500 TPM or higher, and two candidates, D166 and D852, were selected as targets for test development. For transcript D166, identified as SeMB42_g04538 and SeLEV6574_g05356 belonging to OG0006196, no *S. microbalum* homolog was identified, and Interproscan analysis did not result in any predicted function. The forward primer was placed in the fifth exon, and the reverse primer was designed to target the fourth exon–exon boundary, resulting in a 161-bp amplicon. The target gene of transcript D852, identified as SeMB42_g03161 and SeLEV6574_g01495 and belonging to OG0001112, was predicted to encode ribosomal protein S7e. The CDS sequence of the orthologous *S. microbalum* gene (SmJEL517_g05346) shares 74.4% sequence homology with the *S. endobioticum* CDS sequences. The forward primer was placed in the fourth exon, and the reverse primer was designed to target the fourth exon–exon boundary, resulting in a 183-bp amplicon (Fig. 1).

Expression of target genes

RNAseq datasets obtained from resting spores ($n = 18$) and aboveground infected plant parts ($n = 9$) showed an average expression of 88% of genes annotated in the LEV6574 genome. The median TPM per dataset that was used to normalize the coverage of real-time RT-PCR target genes ranged from 19 to 55. The normalized TPM values for the target gene of test D852 (average $TPM_{normalized} = 18$) are significantly lower (ANOVA + Tukey Contrasts: $P < 0.001$) compared with those of tests *EF1*α and D166 (average $TPM_{normalized} = 32$ and 38 for the respective tests). For the gene targeted by test D852, expression across the 27 datasets is more or less similar (StDev = 5), whereas for *EF1*α and the gene targeted by test D166, a much broader range of expression is observed (StDev = 15 and 16 for the respective tests). The *S. endobioticum EF1*α gene shows a higher normalized TPM ($mean_{plant} = 42$; $mean_{spores} = 26$) in material obtained from aboveground infected plant compared with those obtained from resting spores. In contrast, the gene targeted by test D166 shows a lower normalized TPM ($mean_{plant} = 45$; $mean_{spores} = 25$) in material obtained from aboveground infected plant compared with those obtained from resting spores. For the gene targeted by assay D852, in both cases, the normalized TPM is similar ($mean_{plant} = 13$; $mean_{spores} = 20$) (Fig. 2). In general, the normalized expression levels are, on average, 18 to 38 times higher than the median expression.

Analytical sensitivity

The LOD for the three real-time RT-PCRs was determined by testing dilution series of gBlock constructs of the target gene CDS sequence in triplicate. At 100,000 gBlock copies, 22.4 ± 0.1, 19.2 ± 0.2, and 20.8 ± 0.2 are the mean Cq values for the *EF1*α, D166, and D852 tests, respectively. The lowest amount that could be detected in all replicates was 10 gBlock copies for all tests at Cq values 37.5 ± 1.1 (*EF1*α), 34.4 ± 0.5 (D166), and 35.2 ± 0.7 (D852). The LOD was set at 10 gBlock copies for all three tests, but test D852 results in Cq values that are on average 3.1 cycles lower than the *EF1*α test. For the test D166, Cq values are on average 4.5 cycles lower than the *EF1*α test. Analysis of the gBlock dilutions resulted in an R^2 of more than 0.98 for all three tests, indicating linearity between the Cq values and the log value of the gBlock (Fig. 3; Supplementary Table S3). Testing accessions from the NPPO-NL compost collection reveal that the real-time RT-PCR tests are highly correlated (Supplementary Figs. S1 and S2; Supplementary Table S4).

Samples containing either 100 or 250 presumed viable spores, extracted from a 6-month-old compost, tested positive, with Cq values ranging from 34.7 to 38.8 for the EF1α test, 30.5 to 35.0 for test D166, and 31.4 to 35.0 for test D852. When testing lower amounts of presumed viable spores, the positive agreement (PA) varied between the tests (i.e., 50 spores: $PA_{EF1\alpha} = 60\%$, $PA_{D166} = 80\%$, $PA_{D852} = 40\%$; 10 spores: $PA_{EF1\alpha} = 20\%$, $PA_{D166} = 60\%$,

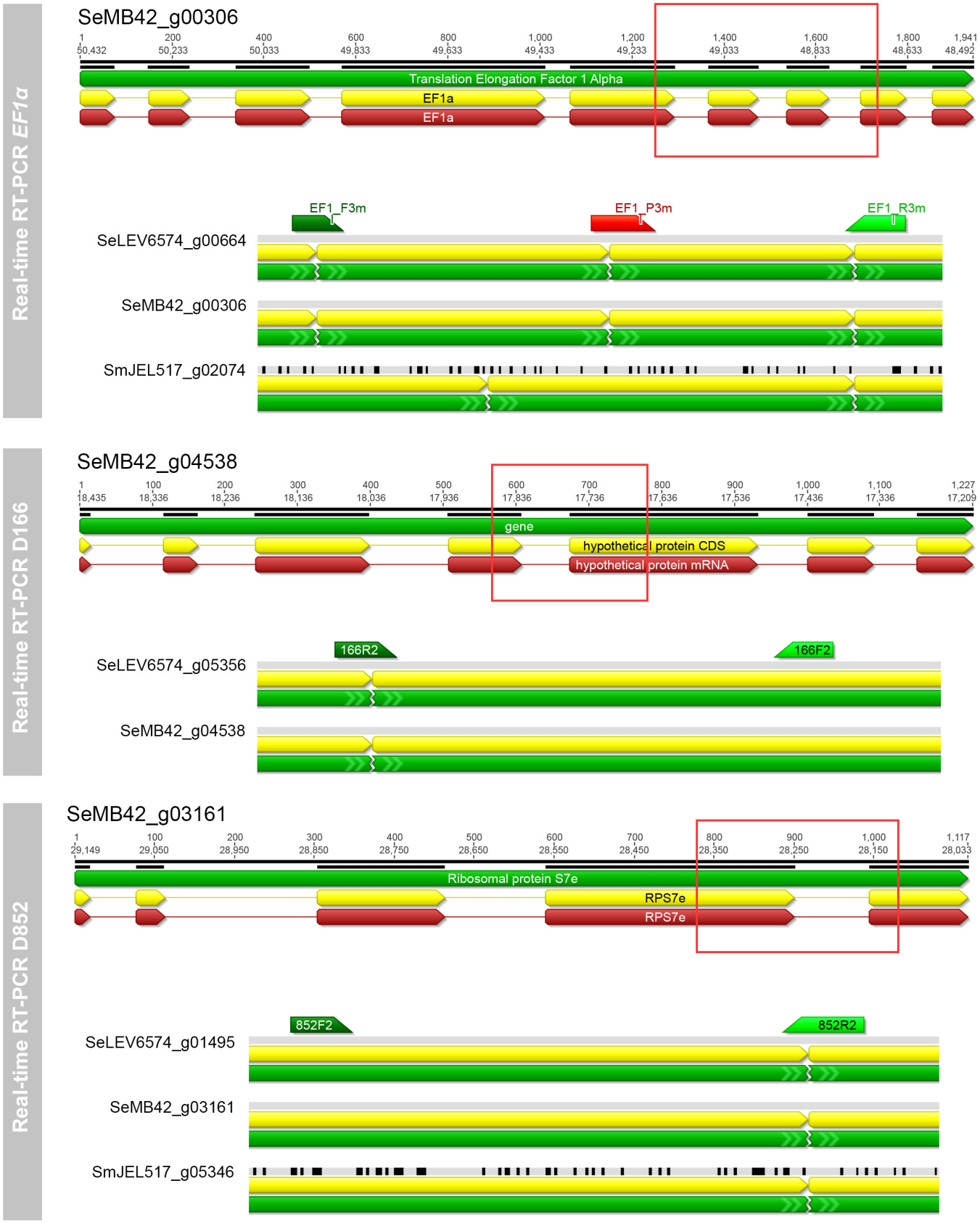

FIGURE 1

Oligonucleotide design. Target genes of the three real-time RT-PCR tests are shown with the gene annotation in green, mRNA annotation in red, and coding sequences (CDSs) in yellow. Red boxes indicate the regions targeted by the oligonucleotides. MAFFT alignments of CDSs are shown with sequences from the two *Synchytrium endobioticum* reference genomes, SeMB42 and SeLEV6574. When an orthologous gene was identified in the *S. microbalum* genome JEL517, this was included in the alignment. Oligonucleotides are annotated on the CDS obtained from the SeLEV6574 genome. Nucleotide polymorphisms in the *S. microbalum* CDS are highlighted in black.

$PA_{D852} = 60\%$). For samples containing 5 or 2 spores, only test D166 produced positive results, with PA of 40 and 20%, respectively (Fig. 4; Supplementary Table S5), corroborating the higher TPM values found for the gene targeted by test D166.

Template specificity

When testing gBlock constructs of the target gene sequences, no amplification was obtained. This demonstrated that the template specificity of the real-time RT-PCR tests the target gene mRNA and not the nuclear-encoded gene sequence itself. Furthermore, template specificity was demonstrated with RNase-treated RNA extract (Supplementary Table S6). Before treatment, RNA extracted from fresh warts and wart homogenate produced average Cq values before treatment of 27.4 ± 1.6, 23.5 ± 2.6, and 23.8 ± 1.8 for *EF1α*, D166, and D852, respectively. After RNase treatment, no amplification was observed for the *EF1α* test. In tests D166 and D852, two samples produced late Cq values ranging from 37.0 to 39.1.

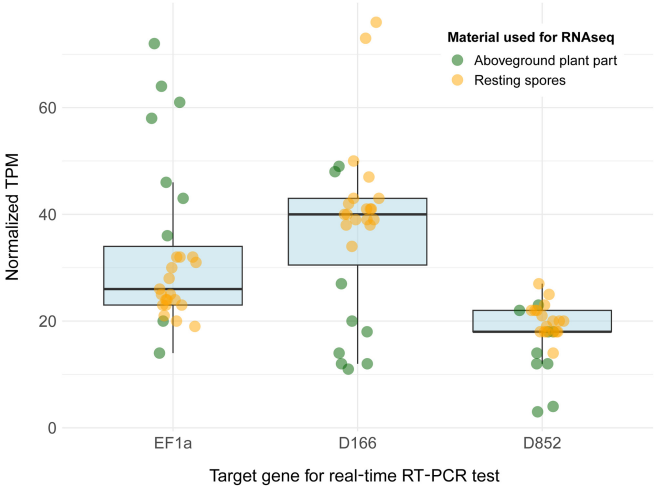

FIGURE 2
Normalized expression levels of real-time RT-PCR target genes. Transcripts per kilobase million (TPM) values are normalized relative to the median expression TPM value per dataset. Boxplots show the distribution of normalized TPM values using public *Synchytrium endobioticum* RNAseq data. Dots shown in green are obtained from RNAseq data generated from aboveground infected plant material, whereas yellow dots represent values obtained from RNAseq data generated from resting spores.

Total nucleic acid extraction

Performing the DNA-based ITS2 real-time PCR test on RNA extracted from fresh warts and wart homogenate showed that the test performed equally well (Student's *t* test $P = 0.577$) as when using DNA extracted from the same materials (i.e., average $Cq_{ITS2\ RNA\text{-}extract} = 18.5 \pm 1.3$; $Cq_{ITS2\ DNA\text{-}extract} = 18.3 \pm 1.1$). For the 18S real-time PCR test, the extracted RNA significantly outperformed the DNA extracted from the same materials (Student's *t* test $P < 0.001$; i.e., $Cq_{18S\ RNA\text{-}extract} = 15.2 \pm 1.4$; $Cq_{I18S\ DNA\text{-}extract} = 16.9 \pm 1.2$).

Analytical specificity

To assess the exclusivity of the real-time RT-PCR tests, soils and starch potato processing waste free from *S. endobioticum* were tested. No false positive results were obtained when analyzing sandy soils, clay soil, or dal soil (Supplementary Table S7). Also, analysis of the organic waste from starch potato processing industries did not result in false positive results.

Biological relevance

In total, 49 composts from the NPPO-NL compost collection were selected for testing with the Spieckermann bioassay, based on ITS2 real-time PCR and *EF1α* real-time RT-PCR results (Fig. 5). Of the selected composts, two were tested in two rounds in the same year, and for four composts, testing was repeated over the course of 2 years. Composts with Cq values of 30.0 or less for the *EF1α* real-time RT-PCR ($n = 33$) resulted in wart formation

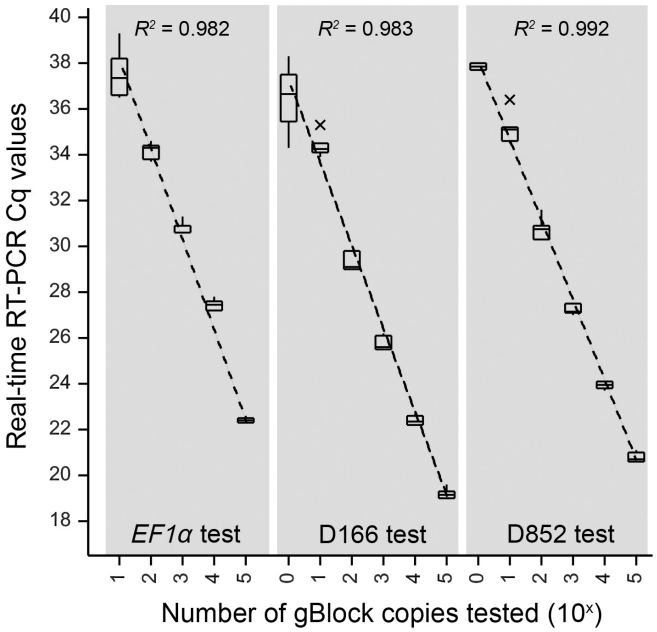

FIGURE 3
Analytical sensitivity expressed in gBlock copies of the target coding sequence.

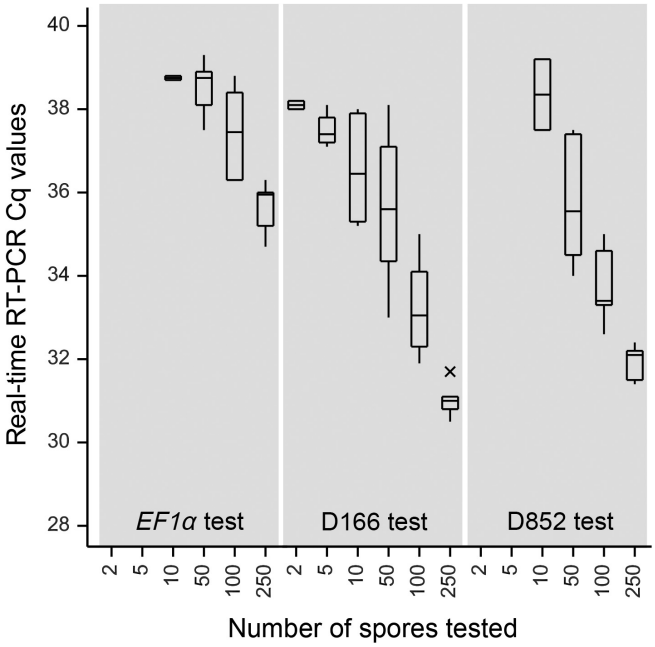

FIGURE 4
Analytical sensitivity determined with suspensions with known amounts of presumed viable spores.

in 91% of all cases (Supplementary Table S2). The percentage of warted tuber blocks for these samples varied from 4 to 100%, but no correlation was observed between Cq value and percentage of infected tuber blocks. For the remaining 22 composts, no wart formation was observed in 86% of the bioassays performed. For the composts that were tested in duplicate within the same year, the same qualitative results were obtained. The number of infected blocks per test varied strongly, with 59 and 93% for compost Pot-ID_037 ($Cq_{EF1\alpha} = 26.0$) and 8 and 43% for compost Pot-ID_055 ($Cq_{EF1\alpha} = 25.7$). The same was observed for composts tested in two consecutive years. When considering the results of the D166 and D852 real-time RT-PCR results (Supplementary Figs. S3 and S4), similar trends are observed, although the spread of Cq values is lower compared with the *EF1*α test ($\Delta Cq_{Ef1\alpha} = 13.9$, $\Delta Cq_{D166} = 10.8$, $\Delta Cq_{D852} = 12.6$). Control sample 42012763 (Pot-ID_217) resulted in wart formation and produced mean Cq values of 26.7, 22.4, and 23.0 for the *Ef1*α, D166, and D852 real-time RT-PCR tests, respectively. After autoclaving the positive control compost, no wart formation was obtained in the bioassay, and no mRNA was detected in any of the three real-time RT-PCR tests.

Discussion

One of the key risk elements of potato wart disease is that the resting spores of causal agent *Synchytrium endobioticum* can remain viable and infectious in soil for decades. This makes the assessment of *S. endobioticum* resting spore viability highly relevant for descheduling of fields that were previously found infected and restricted. In addition, determination of spore viability could be important to assess the quality of inoculum used for resistance testing on potato and in the management of maintaining viable collections for future reference. Finally, determination of resting spore viability facilitates determining the effect of treatments on spore viability. Currently, resting spore viability is determined with bioassays or by direct microscopic examination.

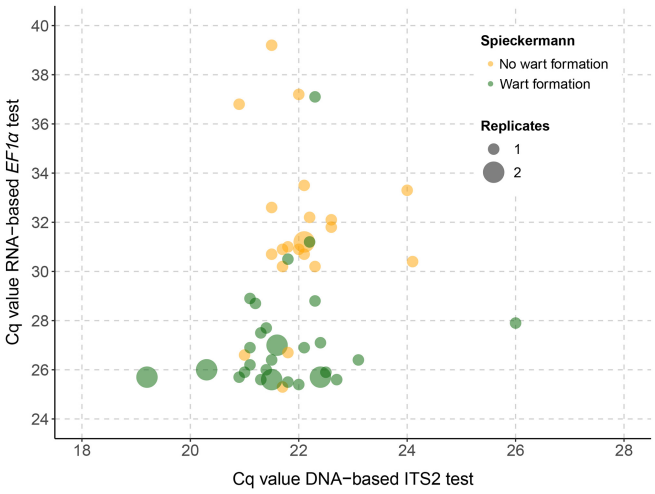

FIGURE 5
Biological relevance of *EF1*α Cq values determined with the Spieckermann bioassay. Cq values of the DNA-based ITS test are shown on the *x*-axis and values of the *EF1*α test are shown on the *y*-axis. Forty-nine composts from the NPPO-NL compost collection were tested with the molecular tests and subjected to a Spieckermann bioassay. Dot size reflects whether the bioassay was performed once or twice (within the same year or spanning two years) and dot color indicates whether wart formation was observed (green) or not (yellow) in the bioassay.

Both methods have their challenges and depend on the availability of specific facilities and/or the expertise of staff. Molecular determination of viability was likely achieved by mRNA detection. The rational for using a mRNA-based test for viability is that mRNA is relatively unstable and is quickly degraded when cells are no longer viable. The half-life of eukaryotic mRNA can vary from hours to days, and mRNA can still be detected after cell death. In the oomycete *Phytophthora ramorum* and the ascomycete *Grosmannia clavigera*, mRNA could be detected several hours after heat treatment inactivating the target organisms. Two to seven days after treatment, the target mRNA could not be detected anymore for the respective pathogens (Wong et al. 2020). The specific pattern and timing of *S. endobioticum* target mRNA is not known, but from the biological relevance experiments, trends could be observed.

The structurally and functionally annotated reference genomes together with associated RNAseq data (van de Vossenberg et al. 2019b, c) facilitated the design of a molecular viability test based on mRNA. Selection of candidate target genes based on known function and/or high abundance mRNA levels resulted in three target loci for real-time RT-PCR test design based on mRNA detection.

The real-time RT-PCR tests were designed so that at least one oligonucleotide would target an exon–exon boundary in the mature mRNA. In the nuclear-encoded gene sequence, an intron-exon boundary is present for those sites preventing annealing of the oligo(s) and subsequent amplification of the target ensuring mRNA template specificity. Template specificity was demonstrated by testing gBlock constructs of both the gene sequence with intronic sequences and the CDS that does not contain the intronic sequences. Only the CDS gBlock constructs resulted in amplification of the target. Additionally, template specificity was demonstrated by treating RNA extracts with RNase. In a few cases, late Cq values (≥37.0) were obtained for the D166 or D852 test. These late Cq values are attributed to incomplete RNA degradation rather than a lack of template specificity.

Access to the NPPO-NL *S. endobioticum* compost collection, containing viable resting spores of different pathotypes and geographical origins, allowed for demonstrating the inclusivity of the real-time RT-PCR tests. For the determination of exclusivity, the options to test nontargets were limited, as viable nontarget *Synchytrium* species were not available to us. Frequently used test host matrices were previously shown to contain an abundance of nontarget eukaryotic, bacterial, or viral species (van de Vossenberg et al. 2019b). To ensure these nontargets did not cross-react with the mRNA tests, soils and starch potato processing waste free from *S. endobioticum* were tested, and no false positive results were obtained. Ensuring the validity of molecular tests is an ongoing process, and when new materials of relevant viable nontarget species become available, these should be included in test evaluation.

The real-time RT-PCRs were demonstrated to be highly sensitive, and all three tests were able to detect 10 gBlock copies of the target. A direct translation between the number of gBlock copies and the number of mRNA transcripts of the target genes cannot be made due the unknown efficiency of the RT step (Lindén et al. 2012; Schwaber et al. 2019). Furthermore, difficulties in RNA extraction from the robust resting spores could hamper the detection of viable spores, particularly when low numbers of (viable) spores are available. This could explain why in only a few cases positive results were obtained when testing low amounts (≤10) of presumed viable spores. To partly undercut these difficulties, multiple genes with relatively high abundance in resting spores were chosen for test design. Where the target gene for test D852 is more or less expressed similarly in materials obtained from

composts and aboveground infected plant parts, *EF1*α and the target gene of test D166 are differentially expressed when considering the different biological materials used to generate RNAseq. For *EF1*α, higher expression was observed in aboveground infected plant parts while the target gene of D166 was higher expressed in resting spores obtained from composts. This underlines how these tests complement each other and could be used in parallel to determine if there is an optimal test for specific uses.

When testing spores (estimated at 30,000 spores based on ITS2 Cq values) extracted from wart material and wart macerate, which are regarded in optima forma in terms of viability, the lowest Cq values obtained ranged from 20.1 to 25.5 for the different tests. Similarly, in composts from the NPPO-NL collection, the lowest Cq values ranged from 20.8 to 25.4 for the tests. It is likely that with the current test setup (i.e., input material, RNA extraction methods, and reagents used), lower Cq values are not to be expected.

For compost samples with Cq values lower than 30.0 with the *EF1*α test, wart formation was observed with the Spieckermann bioassay in 91% of all cases. For the D166 and D582 tests, this threshold is slightly lower, with 24.0 and 26.0 for the respective tests. However, the range of Cq values for these tests are not as wide as the *EF1*α test, making it more difficult to establish a clear cutoff. In conclusion, this demonstrates that if significant amounts of mRNA are detected, the resting spores are likely to be viable. In this study, we identified samples with low amounts of mRNA that still resulted in wart formation in the bioassay. For all composts with Cq values above the threshold values tested, 14 to 18% produced warts in the Spieckermann bioassay. Possibly, this can be attributed to the nonhomogeneous presence of the (viable) spores and subsequent subsampling. Also, biological replications of selected composts show that the percentage of infected tuber blocks per test greatly differs. For instance, where 6 of the 14 tuber blocks showed wart formation for MB42 compost pot_ID-055, only 2 of 24 blocks were infected with the same compost in a repeated experiment. This highlights the variability in the bioassays, where many factors influence successful wart formation. As an alternative to subsampling variation, samples with low amounts of mRNA that still resulted in wart formation in the bioassay could indicate that a low number of viable resting spores (below the detection limit; <10) could still result in wart formation in the bioassay. Wart formation with a low number of spores has been reported (e.g., 2 to 5% disease incidence in Spieckermann when using inoculum with 1 to 5 spores per gram of soil) (Baayen et al. 2005). This sends out a clear warning for a pathogen that can remain viable and infectious for decades. Even with low levels of viable spores, infection can take place under the right conditions. Autoclaving was shown to be effective to inactivate *S. endobioticum* resting spores (Hinrichs-Berger and Zegermacher 2022), and in the current study, autoclaved composts did not produce wart formation in the Spieckermann bioassay, whereas the untreated material resulted in infection. Where the untreated material resulted in Cq values ranging from 22.4 to 26.7 for the different real-time RT-PCR tests, no mRNA could be detected in the treated materials 7 days after treatment. Application for *S. endobioticum* mRNA detection in collection management and detection of presumed viable spores in scheduled fields are typically related to much larger time scales (i.e., years instead of days). Composts included in the biological relevance experiments were 2 to 34 years old at the time of analysis with the mRNA tests and Spieckermann bioassay. The timing and pattern of mRNA degradation in dead *S. endobioticum* spores in composts is not known, but in materials that were stored 2 to 10 years in collection, the mRNA content appears to be rather stable, and all but one accession resulted in wart formation. After 15 years of storage, the Cq range of detected mRNA increases, and the number of composts resulting in wart formation in the bioassay decreases (Supplementary Fig. S5). When implementing the SYBR real-time RT-PCR tests, it was noted that the melt peak obtained differed between the different laboratories. A reaction mix based on sensiMix II (Bioline, U.K.) and Evagreen (Biotium, CA, U.S.A.) reagents resulted in melt peaks for tests D166 and D852 of 86.2 and 84.2°C, respectively. These temperatures are approximately three degrees higher compared with those obtained with the One-Step Takara TB GreenMix reagents (Takara). A known drawback of SYBR melt curve analysis is that the temperature is dependent on the reagents, dye concentration, and DNA concentration (Gudnason et al. 2007). Similarly, RNA extraction methods that worked well in one lab were not necessarily the best methods for other labs. This highlights the importance of test verification when implementing tests in a diagnostic laboratory as indicated in EPPO standard PM7/98 (EPPO/OEPP 2021).

The three developed and validated molecular tests for the detection of *S. endobioticum* mRNA are sensitive and are specific to the mRNA target. The use of nucleic acid extracted with bespoke total nucleic acid extraction kits or RNA extraction kits without using DNase treatments enable detection of *S. endobioticum* spores with DNA-based tests and the detection of mRNA to determine viability from the same biological material. This is an important feature, as spore homogeneity is always uncertain, particularly in compost samples. By performing both the DNA- and RNA-based tests on nucleic acids extracted from a single biological sample, a direct link can be made between spore presence and suspected viability.

Determining *S. endobioticum* spore viability is important in collection management. Selecting composts containing resting spores for future research is problematic, as viability of (old) composts is not known. Currently, a bioassay has to be performed to determine viability. With only two or three rounds of bioassays that can be performed successfully annually, this greatly reduces the capacity in research programs. By prescreening composts to determine if a compost is likely to result in wart formation, compost with Cq values higher than the cutoff determined in this paper could initially be rejected.

Also in risk mitigation studies, in which treatments could be easily assessed for their efficacy in killing *S. endobioticum* resting spores, the molecular viability tests can play an important role. With the molecular tests, more treatments can be studied than would be possible when classical bioassays are used to determine the treatment effect. Molecular testing could be used to rapidly exclude ineffective treatments considering the mRNA stability and possible detection of mRNA directly after treatment. With the difficulties associated with bioassay and the increased sensitivity obtained with the molecular tests, it is to be expected that bioassays produce false negative results while spores are still viable and could, under the right conditions, result in infection. When treatment effectiveness and risk assessment is based on bioassays alone, this could result in a too-optimistic view of the treatment under evaluation. In addition, a real-time RT-PCR approach could enhance the numbers and statistical power of differentiating between the different treatments.

Another application for the molecular viability tests is the screening of previously infested fields. The application of DNA-based real-time PCR tests in screening samples as part of the official descheduling testing has been highlighted recently (van de Vossenberg et al. 2022a). Under the current EPPO testing scheme in PM3/59 (EPPO/OEPP 2017a), descheduling of previously infected fields can be performed after direct microscopic examination and a bioassay, or after performing two bioassays.

When molecular viability tests are integrated into the procedures, possibly (part) of the labor-intensive and resource- and time-consuming tests, could be eliminated, resulting in a reduction of hands-on time and greenhouse capacity. This would be particularly the case for all samples that show significant amounts of mRNA and are likely to contain viable spores. Additional test validation for this specific purpose would be needed to ensure that the test is fit for purpose from time of sampling to molecular testing. Validating the molecular tests in the context of the bioassay pot test, which is currently mentioned as a recommended bioassay in EPPO standard PM3/59, is highly recommended, as it is to be expected that the molecular tests will be more sensitive and repeatable than the pot test.

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