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ORIGINAL ARTICLE

Comparison of real-time PCR tests for the detection of *Synchytrium endobioticum* resting spores in soil and their potential application in descheduling of previously infested plots

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Abstract

Plots infested with *Synchytrium endobioticum*, the causal agent of potato wart disease, are scheduled, resulting in prohibition of potato cultivation of and cultivation of plants for planting. On account of robust resting spores that are present in infested soils, plots remain scheduled for 20 years. After this period, plots are intensively sampled and the presence of resting spores in soil is determined by direct examination. However, this method is very time-consuming and labour-intensive. In this paper, validation data for the molecular detection of resting spores in soil and its use to screen soils before direct examination are reported. In addition to the samples in the validation study, over 670 routine diagnostic samples were analysed by direct examination and molecular tests in parallel. Using an improved methodology, increased sensitivity was obtained relative to results reported from an interlaboratory comparison study in 2018, namely 7 spores per sample instead of 500 spores per sample. Molecular screening of the soil samples and direct examination of those samples testing positive was estimated to reduce the total hands-on time by half when compared to direct examination of all samples. We recommend inclusion of molecular detection in the update of EPPO PM 3/59 (3) and PM 7/28(2), and suggest that when all subsamples of a plot are negative, no additional direct examination or bio-assays are needed for descheduling (i.e. for releasing previously infested plots from official control).

Comparaison de tests PCR en temps réel pour la détection de spores dormantes de *Synchytrium endobioticum* dans le sol et leur application potentielle pour la déréglementation des parcelles précédemment infestés

Les parcelles infestés par *Synchytrium endobioticum*, l'agent causal de la galle verruqueuse de la pomme de terre, sont réglementés, avec notamment interdiction de la culture de la pomme de terre et de végétaux destinés à la plantation. En raison de la présence de spores dormantes robustes dans les sols infestés, les parcelles restent réglementés pendant 20 ans. Après ce lapse de temps, les parcelles sont échantillonnés de manière intensive et la présence de spores dormantes est évaluée

par examen direct du sol. Cette méthode est cependant chronophage et laborieuse. Cet article présente des données de validation pour la détection moléculaire des spores dormantes dans le sol et son utilisation pour un dépistage avant examen direct. En plus des échantillons de l'étude de validation, plus de 670 échantillons de diagnostic de routine ont été analysés par examen direct et par test moléculaire en parallèle. A l'aide d'une méthodologie améliorée, une sensibilité accrue a été obtenue par rapport aux résultats rapportés par une étude comparative inter-laboratoires réalisée en 2018, à savoir 7 spores par échantillon au lieu de 500 spores par échantillon. Il est estimé que le dépistage moléculaire des échantillons de sol et l'examen direct des seuls échantillons testés positifs ont permis de réduire de moitié le temps total de manipulation par rapport à l'examen direct de tous les échantillons. Nous recommandons d'inclure la détection moléculaire dans la mise à jour des normes PM 3/59 (3) et PM 7/28 (2) de l'OEPP et suggérons que, lorsque tous les sous-échantillons d'une parcelle sont négatifs, aucun examen direct du sol ou essai biologique complémentaire ne soit nécessaire pour la déréglementation d'une parcelle (c'est-à-dire, pour la levée des mesures de lutte officielle sur des parcelles précédemment infestées).

Сравнение тестов ПЦР в реальном времени для выявления покоящихся спор *Synchytrium endobioticum* в почве и их потенциальное применение для исключения из перечней ранее зараженных полей

Поля, зараженные *Synchytrium endobioticum*, возбудителем рака картофеля, подлежат внесению в списки заражённых полей, что приводит к запрету на выращивание на них картофеля, включая выращивание растений для посадки. Из-за жизнеспособных покоящихся спор, которые присутствуют в зараженной почве, эти поля остаются в этих списках в течение 20 лет. По истечении этого срока на этих полях интенсивно отбирают образцы, и наличие покоящихся спор в почве определяется путем прямого исследования. Однако этот метод очень трудоемкий и занимает много времени. В статье представлены данные по валидации молекулярного метода обнаружения покоящихся в почве спор и его использование для скрининга почв перед отчётом о прямом исследовании. В дополнение к образцам в валидационном исследовании, более 670 рутинных диагностических образцов были параллельно проанализированы прямым исследованием и молекулярными тестами. С помощью усовершенствованной методики была достигнута повышенная чувствительность по сравнению с результатами, представленными в межлабораторном сравнительном исследовании в 2018 году, а именно выявляемость 7 спор на образец вместо 500 спор на образец. Оценка молекулярного скрининга всех образцов почвы по сравнению с прямым исследованием тех образцов, которые дали положительный результат, показала сокращение общего времени работы в два раза. Мы рекомендуем включить молекулярное выявление в обновленные версии стандартов PM 3/59 (3) и PM 7/28(2) ЕОКЗР и предлагаем, чтобы при отрицательных результатах всех под-образцов одного поля не требовалось дополнительных прямых исследований или биоанализов для исключения из перечня заражённых полей (то есть для освобождения ранее заражённых участков от официальной борьбы).

1 | BACKGROUND

Potato wart disease is an important potato disease caused by the obligate biotrophic chytrid fungus *Synchytrium endobioticum*. On infection of susceptible potato varieties, typical cauliflower malformations are formed, rendering the crop unmarketable (Obidiegwu et al., 2014). Complete yield loss has been reported as a result of infestation (Hampson, 1993). As part of its life cycle, the fungus produces robust resting spores that can remain viable and infectious in infested soils for decades (Przetakiewicz, 2015b). Isolates of the fungus are further characterized as pathotypes based on the ability to form warts on a differential set of potato cultivars (EPPO, 2017b). Currently over 40 pathotypes have been described (Baayen et al., 2006; Çakır et al., 2009; Przetakiewicz, 2015a). As there are no successful treatments or control agents known to eradicate the fungus (Hampson, 1988), prohibiting potato cultivation and restricting cultivation to resistant varieties in buffer zones surrounding infested plots are the only ways to prevent the spread of the pathogen. Following initial detection of the pathogen, infested plots are 'scheduled' and potato cultivation is prohibited for 20 years. After that, the plot is intensively sampled to determine if the pathogen is still present. Presence of the pathogen can be determined by direct examination after sieving and washing of soil samples in combination with bio-assays or field trials (EPPO, 2017a). Typically, direct examination is performed first as this is much quicker compared to the biological tests. When the pathogen is no longer found to be present, plots may be descheduled (Anonymous, 1969).

As part of the Euphresco Sendo project, an interlaboratory test performance study (TPS) was performed to compare several molecular tests for the detection of *S. endobioticum* spores in warted tubers and soil suspensions (Van de Vossenberg et al., 2018). The tests included were an *S. endobioticum*-specific conventional PCR (Van den Boogert et al., 2005), an *S. endobioticum*-specific real-time PCR (van Gent-Pelzer et al., 2010), and a pathotype 1(D1) specific real-time PCR (Bonants et al., 2015). In combination with the DNeasy Plant Mini kit these tests performed well on warted potato tissues, but for the detection of spores in soil the analytical sensitivity was inadequate, with a limit of detection of 500 spores per sample.

Here we report on the comparison of the performance of two *S. endobioticum*-specific real-time PCR tests for the detection of resting spores in soils. These were the ITS2 test by Van Gent-Pelzer et al. (2010) and the 18S test by Smith et al. (2014) in combination with a filtration set-up for $CaCl_2$ purified soil suspensions and the Qiagen DNeasy PowerSoil kit. In addition to the samples in the validation study, over 670 subsamples from annual descheduling surveys performed by the Dutch National Plant Protection Organization (NPPO-NL) were analysed by direct examination and with the two molecular detection tests. Following the results of this comparison, recommendations are made on the use of the molecular tests in descheduling tests of previously infested plots.

2 | MATERIAL AND METHODS

2.1 | Preliminary tests

To determine the effect of DNA extraction kits on the performance of the real-time PCR tests, a preliminary test was performed using three series of freeze-dried warted (sample MB08, pathotype 2(G1)) and healthy potato material (variety Eersteling) samples from the Euphresco Sendo project, and a range of 500, 50, 10, 5 and 1 extracted spores (sample MB19, pathotype 18(T1)). DNA was extracted from these materials as described below, but in addition these materials were processed with the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) as described in EPPO PM7/28(2) and the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) using a vortex for 10 min at maximum speed following the manufacturer's instructions.

2.2 | DNA extraction

Samples consisting of 200 g of soil or compost were used whole or as subsampled 25-g portions. These (sub) samples were processed using two stacked sieves with mesh sizes of 75 μm for the upper sieve and 25 μm for the lower sieve according to sieving method B described in the *S. endobioticum* EPPO standard PM7/28(2). The 25-g subsamples were sieved manually whereas the 200-g soil samples were subjected to automated sieving using a Analysette 18 heavy duty sieve shaker (Fritsch, Idar-Oberstein, Germany) with 200-μm, 71-μm and 25-μm sieves. After automated or manual sieving and purification, 40–50 mL of soil/spore suspension in a saturated $CaCl_2$ solution was obtained. For practical reasons, the soil/spore suspensions obtained from the 200-g samples were divided over two 50-mL tubes. Given the uneven distribution of spore (fragments) in the saturated $CaCl_2$ solution these two 50-mL tubes were not regarded as biological duplicates. The soil/spore suspension is subjected to a filtration set-up containing a filtration beaker fitted with a 20-μm nylon woven mesh filter. The filtration set-up is connected to a vacuum pump (Figure S1). When using this particular combination, the cellulose nitrate filter provided with the filtration beaker had to be removed before applying the nylon filter. The supernatant of the saturated $CaCl_2$ solution was transferred to the filtration beaker without disrupting the pellet, and a vacuum was applied to the set-up. The filtration beaker was

rinsed with large amounts of tap water to collect all the soil and spore material on the nylon filer. The filter was transferred from the beaker to a clean 50-mL tube and soil/spore material was rinsed off using tap water. The 50-mL tube was centrifuged for 5 min at 5300 × *g* to concentrate all soil/spore material in a pellet. The pellet was resuspended in 60 µL of C1 buffer from the DNeasy PowerSoil kit (Qiagen) and transferred to a 2-mL tube containing the kit's PowerBeads. The material was disrupted using a Mixer Mill 200 (Retsch, Haan, Germany) for 10 min at 30 beats per min. The resulting homogenate was further processed according to the manufacturer's instructions. DNA eluted in 100 µL of C6 solution was used immediately or stored at −20°C until use. Per DNA extraction round, a positive (resting spore solution) and negative isolation control (molecular grade water) was included to determine the efficiency of DNA extraction.

2.3 | Real-time PCR

For both real-time PCR tests, the methodology described in EPPO Standard pm7/28(2) was followed. In short, reaction mixes for the Van Gent-Pelzer et al. (2010) ITS2 TaqMan were based on the Premix Ex Taq Master Mix (Takara Bio, Kusatsu, Japan) and contained 250 nM of primers Sendo ITS2F and ITS2R, and 83 nM of Sendo probe 2. Genomic DNA (3 µL) and molecular-grade water were added to reach a final volume of 30 µL. Reaction mixes for the Smith et al. (2014) 18S TaqMan were based on the Maxima qPCR master mix (Thermo Fisher, Waltham, USA) and contained 500 nM of primers Se18S_RTF1 and SE18S_RTR2, and 200 nM of probe Se18S_TM1. Genomic DNA (2 µL) and molecular-grade water were added to reach a final volume of 20 µL. Apart from the *S. endobioticum* specific real-time PCR tests, a generic plant cox1 test was performed in a separate reaction. Reaction mixes of this internal control were based on the Premix Ex Taq Master Mix (Takara Bio) and contained 200 nM of primers COX F and COX RW, and 100 nM of probe COX SOL 1511T (Weller et al., 2000; Mumford et al., 2004). Genomic DNA (3 µL) and molecular-grade water were added to reach a final volume of 30 µL. Amplification for all tests was performed in a CFX96 touch (BioRad, Hercules, USA) using thermocyler program 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Baseline and threshold settings were determined automatically and amplification curves were assessed visually. Where needed, the baseline was set between cycles 2 and 15 for the ITS2 assay to correct for background signals that were not corrected automatically. Tests were considered positive when they produced exponential amplification curves and a Cq value below 40. Positive and negative amplification controls were included during each real-time PCR run.

2.4 | Analytical sensitivity

Analytical sensitivity was determined based on five series of a range containing 10, 5, 2 and 1 extracted spores (sample 5998981, pathotype 1(D1)) in MGW. The limit of detection was expressed in number of spores and determined using the following formula:

$$\mathrm{LOD} = \frac{x_1 + x_2 + x_n}{n_{\mathrm{series}}} + 3 \times \mathrm{StDev}$$

In which LOD is the limit of detection (number of spores), $X_{1,2,n}$ is the lowest number of spores that could be detected (Cq < 40 and exponential curves) and StDev is the standard deviation.

2.5 | Selectivity

To determine the effect of the soil suspension in which the spores have to be detected, 200 g of clean soils of three main soil types for growing potatoes in the Netherlands (i.e. clay, sandy and 'dal' soil, the latter being an artificial soil, a mix of the topsoil and the sandy subsoil after removal of the intermediate peat) were spiked with 250, 50 and 10 extracted spores (sample 5998981, pathotype 1(D1)) and processed as described above. Results from the same number of spores obtained in MGW were compared to those from the spiked samples to determine if a negative inhibitory effect was observed from the different soil types. DNA extractions were performed on spore suspensions with and without including the soil pellet from the $CaCl_2$ saturated solution. The R^2 of each real-time test was determined using the combined results from the analytical sensitivity samples and the selectivity samples without the soil pellet. A general analysis of variance (ANOVA) was performed in GenStat v21.1 (VSN International, Hemel Hempstead, United Kingdom) on the selectivity samples. The effect of the different factors (i.e. real-time PCR test, soil types and DNA extraction with or without the soil pellet) and their interaction were tested, and their means were compared with the Bonferroni method to identify statistical significant groups.

2.6 | Routine samples for descheduling previously infested plots and associated controls

Soil samples taken as part of the regular 2019 and 2020 NPPO-NL surveys to determine the presence of *S. endobioticum* spores in scheduled plots were included in this study. In 2019, 55 samples were taken, resulting in 439 25-g subsamples, and in 2020, 114 samples were taken, resulting in eight 25-g and 226 100-g subsamples. The resulting 673 subsamples were processed as usual, meaning that the fraction floating on the $CaCl_2$ saturated solution was transferred to a counting chamber

and visually inspected under a microscope. After direct examination, the content of the counting chamber was transferred back to the original tube containing the $CaCl_2$ saturated solution. These tubes were further processed as described above for DNA extraction and subsequent testing with the *S. endobioticum* ITS2 and 18S tests and the plant cox1 real-time PCR test. Apart from the survey samples, known positive (composts and clean 'dal', sand and clay soils spiked with 50 resting spores, $n = 90$) and negative ($n = 51$) samples were included that were processed and assessed both visually and molecularly. Technical duplicates were tested for the survey samples and controls, and when both replicates produced positive results the pathogen was regarded as detected. Results from the visual assessment were used to define true positive and true negative samples. Positive agreement (PA), negative agreement (NA), positive deviation (PD) and negative deviation (ND) of real-time PCR results were determined, allowing calculation of the diagnostic sensitivity (PA/(PA + ND), diagnostic specificity (NA/(NA + PD) and accuracy ((PA + NA)/(PA + NA + PD + ND)). These performance criteria were expressed in percentages, providing insights into the true-positive rate and the false-positive rate, and the overall performance of the tests. The significance of the observed differences was determined using a two-sample binomial test incorporated in the GenStat v21.1 software.

3 | RESULTS

3.1 | Preliminary tests

When testing the infected and healthy freeze-dried tuber material the expected qualitative results were obtained for all three DNA extraction methods (Table S1). When comparing the results obtained from the Euphresco TPS samples with the different DNA extraction methods, the DNeasy Plant Mini kit and the DNeasy PowerSoil kit (bead-beating) significantly (two-sided *T*-test, $p < 0.05$) outperformed the DNeasy PowerSoil kit (vortex) for both the ITS2 and the 18S tests ($mean_{Plant\,Mini} = 19.5 \pm 0.03$, $mean_{PowerSoil\,beads} = 19.9 \pm 0.93$, $mean_{PowerSoil\,vortex} = 23.3 \pm 1.12$). The DNeasy Plant Mini kit and the DNeasy PowerSoil kit (bead-beating) performed equally well on the freeze-dried samples for both tests (two-sided T-test: ITS2 $p = 0.51$, 18S $p = 0.62$). Results obtained for the Euphresco TPS samples with the DNeasy Plant Mini kit and the DNeasy PowerSoil kit (bead-beating) were within the range obtained for these samples in the Euphresco TPS, i.e. $mean_{Van\,Gent\text{-}Pelzer} = 19.6 \pm 2.2$. The real-time PCR test targeting the plant cox1 produced positive results for all tuber-derived samples, i.e. mean = 21.8 ± 2.1.

When comparing the results obtained for the extracted spores with the different DNA extraction methods, significant qualitative differences were observed. Where both the ITS2 and 18S tests managed to detect 500 spores or more in combination with the DNeasy plant mini kit, 10 and 5 spores per sample could be detected using the PowerSoil kit with vortex or bead-beater, respectively (Table S1). When considering all the results obtained with resting spores, no significant (two-sided *T*-test, $p = 0.86$) differences were observed for the ITS2 (mean = 33.6 ± 2.8) or 18S (mean = 33.4 ± 2.9) tests. As the extracted spores contain little to no plant DNA, the real-time PCR test targeting the plant cox1 did not produce positive results for the spore samples except in a single case.

Based on these results, the DNeasy PowerSoil DNA extraction kit in combination with bead-beating for sample disruption was selected and used with both *S. endobioticum* real-time PCRs for further comparison and determination of performance characteristics.

3.2 | Analytical sensitivity

When analysing DNA extracted from resting spore series, both real-time PCR tests managed to detect the pathogen in all five subsamples when testing 10 or 5 spores per sample. For the ITS2 test, mean Cq values of 35.1 ± 0.4 and 37.1 ± 1.4 were obtained for 10 and 5 spores, respectively. The 18S test produced significantly lower (two-sided paired *T*-test: $p < 0.001$) mean Cq values for the same samples, i.e. 32.7 ± 0.7 and 35.2 ± 0.8 for 10 and 5 spores, respectively. When considering the subsamples with 2 spores or 1 spore per sample, four out of five tested positive for both tests (Table S2). When applying the formula to determine the limit of detection (LOD) at the 99.7% confidence level, both tests performed equally well and had an LOD at 7 resting spores per reaction.

3.3 | Selectivity

When spiking different numbers of resting spores to $CaCl_2$ saturated solutions obtained from clay, sandy soils and 'dal' soil free from *S. endobioticum* spores, no significant differences were observed between the ITS2 and the 18S real-time tests (general ANOVA: $p = 0.566$). Mean Cq values of 29.0 and 30.2 were obtained for samples without the soil pellet, and 34.6 and 34.8 for samples with the soil pellet for the ITS2 and 18S tests, respectively. A significant negative effect (general ANOVA: $p < 0.001$) was observed when comparing the DNA extraction of the spiked samples with the soil pellet relative to the samples extracted without the soil pellet (Figure 1a). When comparing spore samples spiked to different soil types, no significant differences (ANOVA: $p = 0.816$) were found (Figure 1b). Mean Cq values of 29.3, 29.7 and 29.8 were obtained for 'dal' soil, sandy soil and clay samples when extracted without the soil pellet. Again, when including the soil pellet in the DNA extraction, significant higher

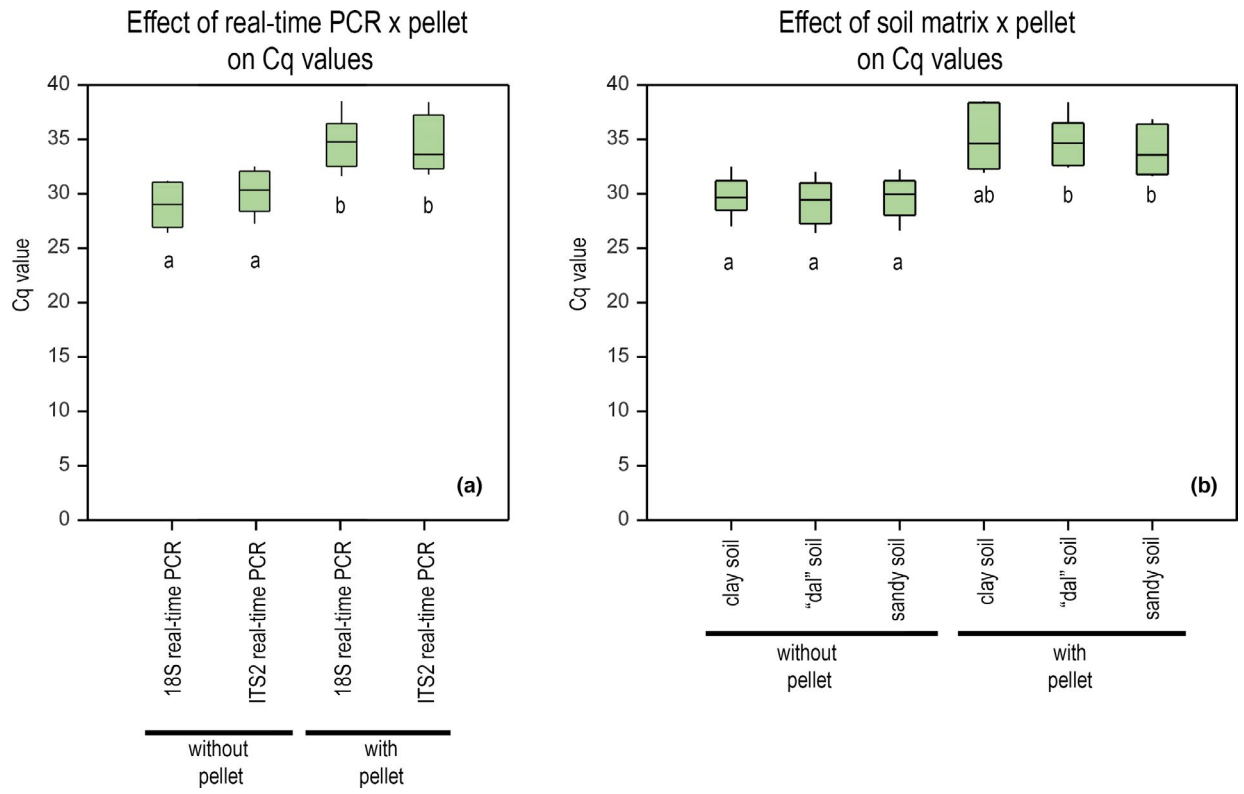

FIGURE 1 Boxplots of Cq values obtained from the selectivity samples. Statistically significant groups are indicted with Latin letters. In both plots a negative effect on the Cq value is observed when including the soil pellet in the DNA extraction. (a) Samples from different soil types and extracted with or without the soil pellet are grouped by test performed. (b) Results from both real-time PCRs on samples extracted with or without the pellet are grouped by soil type

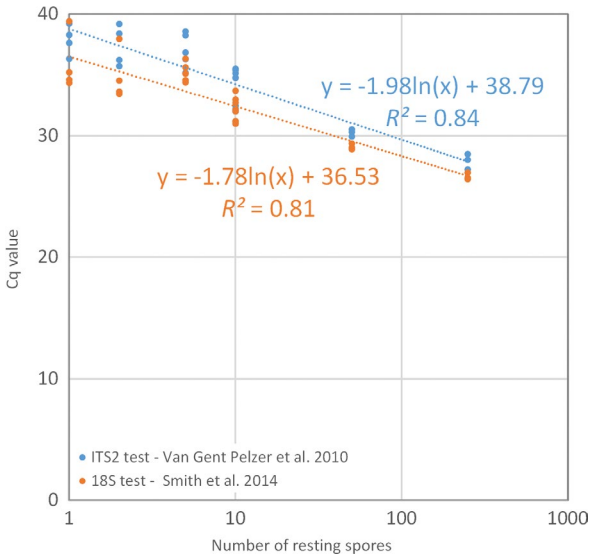

FIGURE 2 The coefficient of determination (R^2) for *Synchytrium endobioticum* real-time PCR detection tests. Analytical sensitivity samples and selectivity samples without the soil pellet were used to calculate the R^2 for both the ITS2 and the 18S real-time PCR tests

Cq values (general ANOVA: $p < 0.001$) were obtained for all three matrices, i.e. 34.0, 34.9 and 35.2 for 'dal' soil, sandy soil and clay samples, respectively (Table S3).

When combining the analytical sensitivity samples and selectivity samples without the soil pellet to calculate the R^2, both tests produced similar values ($R^2_{\text{ITS2 test}} = 0.84$ and $R^2_{\text{18S test}} = 0.81$). With these samples the ITS2 test produced on average Cq values that were 1.8 cycles higher than the 18S test. When lower number of spores was tested, a broader range of Cq values was obtained compared to testing higher numbers of spores (Figure 2).

3.4 | Routine samples for descheduling previously infested plots and associated controls

3.4.1 | Control samples

Of the 90 samples that were initially considered to be 'positive', resting spores were visually detected in 74 samples. These samples were regarded as true positive and spore numbers ranging from 1 to ~30 000–50 000 were estimated. Using the real-time PCR tests, 60 and 70 of these samples produced positive results for the ITS2 and 18S tests, respectively (Table S4). In the remaining 16 'positive' samples, no spores could be visually detected and they were regarded as true negative. In 13 of these samples negative results were obtained with both tests, but in three subsamples positive results were obtained

TABLE 1 Agreement and deviation of real-time PCR results relative to the visual assessment of control and survey samples

Real-time PCR test	Positive agreement	Positive deviation	Negative agreement	Negative deviation
Control samples				
ITS2 test	60	1	66	14
18S test	70	2	65	4
Survey samples				
ITS2 test	0	19	654	0
18S test	0	19	654	0
Total				
ITS2 test	60	20	720	14
18S test	70	21	719	4

with mean Cq values of 33.5 and 32.5 for the ITS2 and 18S tests, respectively. When applying the formula determined under analytical sensitivity and sensitivity, this results in an estimation of 14 and 10 spores per reaction for the respective tests.

Of the 51 samples initially regarded as 'negative', no spores were visually detected in any of the samples and they were regarded as true negative. Both real-time PCR tests produced negative results for the majority of the true negative samples. One negative control sample produced positive results for both the ITS2 (Cq_{mean} = 34.5) and 18S (Cq_{mean} = 34.8) tests, and one negative control sample tested positive for the 18S test only (Cq_{mean} = 37.3).

3.4.2 | Routine samples

In the 673 subsamples obtained from the 169 routine samples, taken as part of the 2019 and 2020 descheduling survey, no resting spores were visually detected and therefore all samples were regarded as true negative. The ITS2 and 18S tests produced negative results for 654 of the 673 subsamples. Positive results were obtained with the real-time PCR tests for 20 subsamples belonging to 13 diagnostic samples. For four of these diagnostic samples more than one subsample produced positive results (Table S5). The obtained Cq values ranged from 33.3 to 37.5 for the ITS2 test and 32.1 to 39.4 for the 18S test, representing an estimation of 2 to 16 and <1 to 12 spores per reaction for the respective tests. Direct examination of new subsamples taken from the samples in which positive results were obtained with the real-time PCR tests did not result in the detection of resting spores.

For the 375 samples on which the cox1 plant internal control test was performed, positive results were obtained for only 48% of the samples, with a mean Cq of 35.7.

Combining the results from the control samples and the routine samples the agreement and deviation of real-time PCR results relative to the results obtained from the visual assessment could be determined (Table 1). Both tests perform equally well considering the diagnostic specificity (= true negative rate), i.e. 97% with a corresponding false-positive rate of 3%. The 18S real-time PCR test significantly (two-sample binomial test: $p < 0.05$) outperforms the ITS2 test in terms of diagnostic sensitivity (= true positive rate), i.e. 95% and 81% with corresponding false-negative rates of 5% and 19% for the respective tests. The accuracy for the 18S and ITS2 real-time PCR tests relative to the visual assessment were found to be 97% and 96%, respectively.

Overall, both real-time PCR tests produced consistent results. Of the 814 samples analysed (i.e. 141 controls and 673 diagnostic samples), both tests produced the same qualitative result in 799 cases (consistency = 98%). When considering the 80 samples that tested positive for both real-time PCRs, the 18S test produced Cq values that were on average 0.2 ± 1.26 cycles lower. Both tests performed equally well on this subset of the samples as the observed difference was not significant (two-sided paired *T*-test: $p = 0.133$). However, it was observed that the ITS2 test produced high background signals. In 44% of the 1624 ITS2 real-time PCR reactions these produced Cq values which could be identified as non-specific after visual assessment (Figure S2).

4 | DISCUSSION AND CONCLUSION

Whenever warted potato tubers are found, visual verification of *S. endobioticum* infestation is usually fairly straightforward. Typical summer or winter spores can be observed in the warted material. In the Netherlands, plots in which warted tubers are found are scheduled and potato cultivation or planting plants for planting is prohibited for at least 20 years. After this period, samples are taken from the scheduled plots to determine if the pathogen is still present. If so, the scheduled period is prolonged for another 3 years. With the uneven distribution of spores in infested plots a large number of samples (60 subsamples per 0.33 ha) is required to reliably determine resting spore absence (EPPO, 2017a). However, with large numbers of subsamples taken, the direct

examination of soils for resting spore presence is very labour-intensive. Furthermore, it requires expertise to distinguish structures resembling *S. endobioticum* resting spores from true resting spores of the fungus. Based on long-term experience, it is known that a vast majority of the samples taken 20 years after initial infestation of a plot will test negative for the presence of *S. endobioticum* resting spores when using the sieving and purification method. With this method only a limited number of samples can be processed daily as it is laborious and time consuming. Using a sensitive and specific molecular test to pre-screen all samples could be a way to save labour and time, and still result in reliable outcomes. In addition, the specific real-time PCR tests will prevent false-positive results as result of mis-identification of structures resembling resting spores such as conifer pollen. Only those samples producing positive results in the molecular test could subsequently be selected for further visual inspection.

In this study we compared two *S. endobioticum* specific real-time PCR tests for the detection of spores from suspensions obtained after sieving and $CaCl_2$ purification as described in EPPO PM7/28(2). Recovery of resting spores from soil with the sieving methods was not part of this study. *S. endobioticum* specific real-time PCR tests included in EPPO PM 7/28(2) were selected for validation. One of those, the ITS2 test described by Van Gent-Pelzer et al. (2010), was part of the Euphresco TPS. In combination with the DNeasy Plant Mini kit, this real-time PCR was not fit for the detection of spores in soil (Van de Vossenberg et al., 2018). With the introduction of the filtration set-up to concentrate the spores from the saturated $CaCl_2$ solution and the DNeasy PowerSoil kit with the bead-beater (this study), an increased sensitivity was obtained for the ITS2 real-time PCR, i.e. 7 spores per sample instead of 500 spores per sample. Using this set-up, the same sensitivity was obtained for the 18S rDNA real-time test described by Smith et al. (2014). As a soil sample typically consists of 25 g (manual sieving) or 200 g (automatic sieving), this is far more sensitive than the recommended density threshold of 5 spores per gram needed for partial descheduling (EPPO, 2017a). When preparing samples for DNA extraction, subjecting the entire $CaCl_2$ fraction with the soil pellet to the filtration set-up should be avoided as a negative inhibitory effect was observed when including the soil. When using the supernatant without disturbing the pellet such a negative effect was not observed. Apart from being an efficient low-tech solution, an added benefit of the filtration set-up is that the entire saturated $CaCl_2$ solution can be used for DNA extraction and molecular detection whereas only the flotation fraction is used for direct examination. This increases the chance of detecting spores that are present in the sample but that are not buoyant. This potential increased chance of detecting spores could explain the 3% false-positive rate observed from the samples from routine diagnostics. Based on the obtained Cq values it was estimated that these low numbers of apparent 'false positives' contained ~1 to 20 spores, and it is likely that they represent true-positive samples. These low number of spores could have been missed in the direct examination as they might have been broken or fragmented and were therefore not buoyant or by microscopist error, as suggested previously by Smith et al. (2014).

In 98% of all DNA extracts tested both real-time PCR tests produced consistent qualitative results and for the samples testing consistently positive similar Cq values were obtained for both tests. Nevertheless, the true-positive rate was significantly lower for the ITS2 test compared to the 18S test. This difference was caused by samples in which fewer than 10 spores per sample were observed. For the 18S test, four samples with fewer than 5 spores per sample tested negative. Given the performance of this test on the other samples with the same number of spores it is possible that they tested negative as a result of loss of spores when the content of the counting chamber was transferred back to the saturated $CaCl_2$ solution before filtration. Alternatively, loss of the low number of spores could have occurred during filtration.

Molecular screening of the soil samples and direct examination of those samples testing positive was estimated to reduce the total hands-on time by half when compared to direct examination of all samples. In addition, with the predominantly negative sample set being converted to a much smaller potentially positive sample set, the level of attention for microscopists is raised, increasing the chance of resting spore detection. It should be noted that taking a new subsample for direct examination following positive real-time PCR results, results in two different samples. Given the uneven distribution of spores, these could lead to different qualitative results when low numbers of spores are present.

From the validation experiments there was no need for the introduction of a Cq cut-off value, and all reactions with exponential amplification curves and Cq values below 40 were regarded positive. The fact that in only 20 of the 673 subsamples from routine diagnostics positive results were obtained indicates that the test matrix does not introduce false-positive late Cq values. It was observed that the ITS2 test produced high background fluorescent signals which resulted in Cq values in 44% of all real-time PCR reactions. After visual inspection of the amplification curves these could be clearly identified as non-specific background signals, but this hampers the user-friendliness of the test. The high background signals are believed to be the result of the length of the FAM-labelled Sendo probe 2. This oligo is 32 bases long, which results in inefficient quenching of the fluorescent signal as the fluorophore and the quencher are too far apart. This problem could be overcome by introducing a ZEN™ Double Quenched Probe. This internal quencher is placed internally between the 9th and 10th bases from the fluorophore on the 5′ end of a TaqMan probe,

resulting in decreased background fluorescence and an increased signal to noise ratio (Wilson et al., 2011).

When using real-time PCR for the detection of resting spores in soil samples, the internal plant cox1 control real-time PCR reaction is of no added value. This underlines the need for an external positive DNA isolation control to be included during each round of DNA extraction. For laboratories that have no access to resting spore suspensions, this could be problematic. Typically, the need for *S. endobioticum* reference material is fulfilled by individual institutes with *S. endobioticum* compost collections, such as the Dutch NPPO and the Polish Plant Breeding and Acclimatization Institute. However, a more coordinated approach would be preferable, and the European Union Reference Laboratory (EURL) for pests on plants for fungi and oomycetes hosted by the French Agency for Food, Environmental and Occupational Health & Safety could play a role in this by assigning delegated laboratories for potato wart research and collections under the EURL framework.

Molecular viability real-time RT-PCR tests targeting the pathogen mRNA are currently being developed by Wageningen University, the Canadian Food Inspection Agency and NPPO-NL. These tests could take the molecular descheduling testing strategy one step further. With the DNA-based real-time PCR tests, the presence of (fragmented) *S. endobioticum* resting spores can be established. Instead of using labour-intensive and error-prone bio-assays to establish the viability of the detected spores, molecular detection of the expression of one or more *S. endobioticum* genes could be used to determine viability. Moving towards molecular detection of *S. endobioticum* resting spores and subsequent molecular verification of their viability will allow more laboratories to test for this important pathogen.

Even though the 18S test outperforms the ITS2 tests, both are regarded fit for purpose for the detection of *S. endobioticum* resting spores in soil samples considering the recommended density threshold of 5 spores per gram needed for partial descheduling. The authors recommend considering the inclusion of molecular detection in the update of EPPO PM3/59 (3) and PM 7/28 (2), and suggest that when all subsamples of a plot test negative in this test, no additional direct examination or bio-assays are needed for descheduling. Additionally, it is suggested that when subsamples test positive new subsamples are taken for direct examination, and only when spores are detected microscopically is the sample regarded as positive. This would greatly reduce greenhouse capacity and hands-on time without affecting the reliability of the descheduling test strategy.

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