Final Report

Project title (Acronym)
Diagnostic methods for *Synchytrium endobioticum*, especially for pathotype identification (SENDO)
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2. Short project report

2.1 Executive summary

The main parts of the project consisted of testing new differential cultivars for pathotype identification (bioassays), and the generation of validation data for three molecular *S. endobioticum* detection and identification assays. As a bioassay, the partners in the project agreed upon the use of the Glynne-Lemmerzahl method to identify pathotypes. In the second year of the project (2013) a test performance study (TPS) was organised. Two different pathotypes were tested, pathotype 6(O1) and 18(T1), and five partners joined in. The cultivars Talent and Logo were chosen to replace cv Miriam in the actual set of differential cultivars (EPPO, 2004). In former research, cv Miriam proved to be less suitable for separating pathotypes 6(O1) and 18(T1), results were inconsistent. As a result, we concluded that cv Talent was the best replacement of cv Miriam in the actual set of differential cultivars. Also, the cultivar Gawin can be added to the set of differentials in a new version of the EPPO Diagnostic Protocol on *Synchytrium endobioticum.*

An international test performance study was organised to generate validation data for three molecular *S. endobioticum* detection and identification assays: Boogert *et al.* (2005), van Gent-Pelzer *et al.* (2010), and Bonants *et al.* (2015). Two TPS rounds were organised focussing on different test matrices: round 1: wart material, and round 2: winter spore suspensions. When using the assays for detection and identification of *S. endobioticum* in warted potato tissue, no significant differences were observed for diagnostic sensitivity, diagnostic specificity, overall accuracy, analytical sensitivity and robustness. After applying a Ct cut-off value for the Gent-Pelzer assay, all assays are regarded equal. When using the assays for detection and identification of *S. endobioticum* in winter spore suspensions, the Boogert and Gent-Pelzer assays significantly outperform the Bonants assay for diagnostic sensitivity and diagnostic specificity. For overall accuracy and analytical sensitivity, the Gent-Pelzer assay significantly outperforms the Boogert and Bonants assays and is regarded as the assay of choice when identifying *S. endobioticum* winter spores. The tests included in this TPS are regarded fit for purpose for routine testing of wart material and winter spore suspensions with ≥ 500 spores per sample.

2.2 Project aims

The project was structured into three work-packages (WP):

WP1 Development of a new differential set of potato cultivars for the identification of pathotypes of *Synchytrium endobioticum* (causal agent of potato wart disease)

The objective of the WP was to renew/update the current set of differential cultivars described in the EPPO Diagnostic protocol on *S. endobioticum*, and perform interlaboratory tests.

WP2 The idea was to compare results obtained using the two most widely applied methods in pathotype identification: Spieckermann method vs the Glynne-Lemmerzahl method. Due to time constraints and capacity problems activities in this WP were not started.

WP3 Test performance study (TPS) molecular biological assays and assay development

3a. An international test performance study to validate molecular tests for *Synchytrium endobioticum* detection and identification.

The objective was to organise an international test performance study to generate validation data for three molecular *S. endobioticum* detection and identification assays: Boogert *et al.* (2005), van Gent-Pelzer *et al.* (2010), and Bonants *et al.* (2015). Two TPS rounds were organised focussing on different test matrices: round 1: wart material, and round 2: winter spore suspensions.

3b. Generating full genome sequences of non-pathotype 1(D1) strains of *Synchytrium endobioticum*

2.3 Main activities

Work package 1

The partners in the project agreed upon the use of the Glynne-Lemmerzahl method (EPPO, 2004) to identify pathotypes. This method is used already for many years in Germany and Poland. In short the method works as follows:

Eye fields of potato (3 x 3 cm), cut out from tubers with sprouts 1–2 mm in length, were ringed with warm vaseline, using a syringe without needle. Then, the ring was filled with water and the sprout inoculated by placing fresh wart tissue inside the rings. Within the ring of vaseline, a water bridge between the inoculum and the sprout is essential to ensure movement of zoospores. Preferably, only the uncut surface of the warts/wart pieces should make contact with the water. After 48 h incubation at 10-12 °C, the wart tissue was removed. Then, the eye fields were moistened with water and immediately covered with a moist soil/peat mixture of about 2 cm thickness. This cover mixture was moistened with water every second or third day during the incubation period so as to promote wart formation. During the incubation period temperature was 16-18 °C and relative humidity about 60-70 %.

The reaction of the sprouts was evaluated after a period of 25 days. Before scoring the results, the sprouts were carefully cleaned of soil. Disease symptoms were scored according to the classification scheme described below (Table 1) using a stereo microscope at 40-80 x magnification.

Table 1. Scoring of reaction types in *Synchytrium endobioticum* tests

Reaction type	Classification	Description
1	Extremely resistant	Early defence necrosis; no visible sorus formation
2	Resistant	Late defence necrosis; sorus formation partially visible, sori immature or necrotic before maturity
3	Weakly resistant	Very late defence necrosis; single ripe sori or sorus fields developed, but completely surrounded by necrosis; defence reactions are dominant, but not always faster than sorus or sorus field maturation; scattered infections, up to five non-necrotic sori, clear necrosis in other zones of the same tuber piece, high degree of attack of the control cultivar (essential !).
4	Slightly susceptible	Scattered infections; sori or sorus fields non-necrotic, few in number; late necrosis can be present on other infection sites on the sprout; the sprout can be slightly malformed (thickened). At microscopic analysis of tissue: winter sporangia observed
5	Extremely susceptible	Dense infection fields, numerous ripe non-necrosed sori and sorus fields, fields with dense non-necrotic infection sites, predominant tumour formation

In the second year of the project (2013) a test performance study (TPS) was organised. Two different pathotypes were tested, pathotype 6(O1) and 18(T1), kindly provided by J. Przetakiewicz from IHAR-PIB, Poland. Initially, ten fresh warts per pathotype were sent to all participants, and then fresh warts were further multiplied in every lab participating. The following five partners joined this TPS: JKI, IHAR-PIB, ILVO, HLB and CLPQ. Each partner tested ten eye fields in three independent replications (3 x 10), the cultivars used are given in Table 2.

The cultivars Talent and Logo were chosen in order to replace cv Miriam in the actual set of differential cultivars (EPPO, 2004). In former research, Miriam proved to be less suitable for separating pathotypes 6(O1) and 18(T1), results were inconsistent (Flath *et al*., 2014). Also, the cultivars Ulme (inconsistent reactions) and Belita (very old one, not easily available) were destined to be replaced. For this the cultivars Transit and Gawin were tested.

Table 2. Set of differential cultivars used in the Test Performance Study with pathotypes 6(O1) and 18(T1)

Cultivar	Resistant to pathotype ..	Delivered in TPS by:	Remark
Deodara	none	NL	
Producent	1(D1)	NL	
Talent	1(D1), 2(G1), 6(O1)	DE	To replace cv Miriam
Logo	1(D1), 2(G1), 6(O1)	DE	To replace cv Miriam
Gawin	1(D1), 2(G1), 6(O1), 18(T1)	PL	To replace cv Ulme and Belita
Transit	1(D1), 2(G1), 6(O1), 18(T1)	DE	To replace cv Ulme/Belita

In the third year (2014), an attempt was made to test material of pathotype 8(F1). The same partners joined this TPS, but unfortunately only two out of five partners (JKI and IHAR-PIB) succeeded to multiply the initial inoculum.

Interpretation of the results.
Key goal was to supply European /EPPO labs with stable cultivars for pathotype identification, reacting with clearly distinguishable symptoms. Therefore, we decided to rate the candidate differentials in two classes: '+' and '-', indicating :

+ = clear wart formation visible (= reaction type 5, Table 1)
- = no wart formation (reaction types 1 up to 4, Table 1)

Work package 3a

The cosmopolitan soil-borne obligate parasitic fungus *Synchytrium endobioticum* (Schilb.) Perc. is the causal agent of potato wart disease and is considered one of the most important quarantine organisms of cultivated potatoes (Smith *et al*., 1997, Ejikeme Obidiegwu *et al*., 2014). Upon infection, *S. endobioticum* induces tumor-like growth (galls or warts) in host tissues of susceptible potato cultivars resulting in yield losses up to 100% (Hampson, 1993). Robust

resting spores are formed in the warted tissue and are released into the surrounding soil when host tissue decays. These resting spores, together with the lack of successful chemical control agents (Hampson, 1993), present a major impact on potato cultivation as they remain viable and infectious over 40 years in undisturbed soil (Laidlaw, 1985; Przetakiewicz, 2015).

At present, more than 30 pathotypes of the fungus have been described (Baayen *et al.*, 2006) and phytosanitary measures heavily rely on pathotype identification. The main focus of the current version of the EPPO *S. endobioticum* standard PM7/28 (EPPO, 2004) lies with pathotype identification using different bioassays, and no molecular tests for pathogen detection or identification are included. It was the aim of the Sendo project to fill this gap by generating validation data for three molecular assays in an international test performance study (TPS). Tests that are regarded to be fit for purpose will be added to the update of PM7/28 together with the performance criteria determined in this study.

Initially, a TPS on only one test matrix (i.e. wart tissue) was foreseen. During the course of the TPS, the wish for expanding the scope to winter spore suspensions was expressed by several participants. A second TPS round was organised focussing specifically on the test matrix winter spore suspensions. TPS results and additionally generated data were used to determine the performance criteria analytical sensitivity, analytical specificity, diagnostic sensitivity, diagnostic specificity, repeatability and robustness following EPPO standard PM7/98 (EPPO, 2014). A draft version of the EPPO standard on the organisation of interlaboratory comparison studies (EPPO, 2014) was used for guidance during the TPS design.

Materials and Methods

Participant and test selection

An official invitation was circulated in the EUPHRESCO II framework for collaboration. Fifteen laboratories working on *S. endobioticum* detection or *S. endobioticum* resistance in potato cultivars, agreed to participate in WP3a of the project (table 3). Laboratory acronyms were randomised and each partner was assigned a laboratory code to suit data analysis, evaluation and communication of results.

During the TPS design phase, three tests for *S. endobioticum* detection were described in literature; two conventional PCRs described by Niepold and Stachewicz (2004) and Boogert *et al.* (2005), and a real-time PCR described by Van Gent-Pelzer *et al.* (2010). Tests described by Boogert *et al*. and Van Gent-Pelzer *et al*. were most frequently used in the EU-region, and were therefore selected for the TPS. A recently developed real-time PCR test for *S. endobioticum* pathotype 1(D1) identification (Bonants *et al.* 2015), and unpublished at the time of TPS preparation, was also included. This test, targeting a pathotype 1(D1) associated single nucleotide polymorphism (SNP), is the first example of molecular *S. endobioticum* pathotype identification using real-time PCR. The recently described real-time PCR test described by Smith and colleagues (2014) was not yet available at the time of the TPS design phase. In addition to the specific tests, a TaqMan test targeting the plant COI gene was used as an internal control (Mumford *et al.* 2004). In this report, these tests will be referred to as ‘Boogert’, ‘Gent-Pelzer’, ‘Bonants’ and ‘COX’ respectively.

Table 3. Participants test performance study

Acronym	Institute	Country
BG-PPS	Central Laboratory for Plant Quarantine	Bulgaria
FERA	Food and Environmental Research Agency	United Kingdom
HLB	Hilbrands Laboratorium voor Bodemziekten	the Netherlands
IE-DAFF	Plant Health Laboratory	Ireland
IHAR	State Plant Health and Seed Inspection Service	Poland
ILVO	Institute for Agricultural and Fisheries research	Belgium
JKI	Julius Kühn Institut	Germany
LT-MOA	State Plant Service	Lithuania
NAAS	Institute of Plant Protection	Ukraine
NAK	Nederlandse Algemene Keuringsdienst	the Netherlands
NVWA	Netherlands Food and Consumer Product Safety Authority	the Netherlands
PRI	Plant Research International BV	the Netherlands
SASA	Science and Advice for Scottish Agriculture	United Kingdom
VIZR	All-Russia Institute of Plant Protection	Russian Federation
VNIIKR	All-Russian Plant Quarantine Center	Russian Federation

TPS standard operating procedures

To have an honest evaluation of the performance of protocols proposed for EPPO PM 7/28 (2), test descriptions were provided according to latest instructions to authors for EPPO diagnostic protocols (Appendices 1-3).

Sample set preparation

In both TPS rounds, participants were provided with positive and negative amplification controls (PAC and NAC), positive and negative isolation controls (PIC and NIC), and ten unknown samples (table 4). The control samples were included to allow participants to determine if the tests were performed successfully. When unclear or contradictory results were obtained, a back-up sample set could be used to repeat the tests.

Warted potato tissue was used as starting material for DNA extraction in the first TPS round. The sample set consisted of randomised healthy potatoes pieces and pieces of warts taken from *S. endobioticum* pathotypes 1(D1), 2(G1), 6(O1), 18(T1), and 38(Nevsehir) infected potatoes (table 4). Healthy potatoes “Eersteling” and warts were cut in portions of approximately 100 mg, added to 2 mL lyophilisation ampoules (VWR, Radnor, USA) and frozen 16 hours at -80 °C prior to lyophilisation with a BenchTop 4K BTXL-75 freeze-dryer (VirTis, Warminster, USA). Ampoules were closed under vacuum and topped off with a tear-away crimp cap (VWR, Radnor, USA).

In the second TPS round, winter spore suspensions were provided. The unknown sample set consisted of molecular grade water (MGW) used for winter spore suspension preparation, and undiluted (approximately $5.0*10^5$ spores*mL^{-1}) and two 10-fold dilutions of *S. endobioticum* pathotype 1(D1) and 6(O1) winter spore suspensions (table 4). Winter spores were isolated from fresh warts using stacked sieves with 75 µm and 25 µm screens. A heat treatment (15 min

at 95 °C) was performed on the winter spore suspension stocks to render the winter spores non-viable. Preliminary tests performed on heat treated and non-heat treated winter spore suspensions showed that the heat treatment had no effect on PCR success (data not shown). For each sample, 10 µL heat treated winter spore suspensions or MGW was added to a 1.5 mL screw cap tube (VWR, Radnor, USA).

A workshop with training session was organised before the start of the first TPS round to familiarise the participants with the TPS set-up and the tests included.

Participants were provided with most items needed for TPS participation to minimise factors that could influence test performance. A TPS package was prepared for each participant containing 10 samples, positive and negative isolation controls, aliquots of the DNeasy Plant mini kit (Qiagen, Hilden, Germany), positive amplification controls, return sample, primers and probes, 15 mL MGW for reaction mix preparation, transport documents for the return sample (aliquot of sample wart 2(G1), round 1 only) and an instruction booklet (supplemental file 1). Prior to shipment (express service) of TPS packages, aliquoted samples, primers, probes and extraction kits were tested for homogeneity and usability. Sample set homogeneity was determined by analysing 10 aliquots per sample using all selected tests (table 4). Reagents and extraction kits used for the homogeneity tests were taken from the same batch as was provided to the participants. Aliquots were stored at room temperature until shipment of the TPS package.

Upon receipt of the TPS package, partners had to send the return sample to the TPS organisers who extracted DNA from the samples and analysed them using the Gent-Pelzer test. Ct values obtained from the return samples were used to determine if sample shipment influenced the TPS results.

For each sample analysed, participants were asked to provide qualitative test results, gel-images (Boogert assay only) and Ct values (Gent-Pelzer and Bonants assays). In addition, participants had to state: 1. if the protocols were strictly followed, 2. which grinding procedure was used (mechanical versus manual), 3. if the back-up sample set was used using an alternative DNA extraction method, 4. thermocyclers used, and 5. if tests were performed with alternative reagents.

Performance criteria

Using the data generated by TPS participants, diagnostic sensitivity, diagnostic specificity, accuracy, repeatability and robustness were determined for each test-matrix combination. Data from partners that failed to produce correct results for the provided controls were excluded from the analysis. Positive agreement (PA), negative agreement (NA), positive deviation (PD) and negative deviation (ND) (EPPO 2014) of results provided by TPS participants relative to the assigned values were calculated. Diagnostic sensitivity (PA/(PA+ND), diagnostic specificity (NA/(NA+PD) and accuracy ((PA+NA)/(PA+NA+PD+ND)) are expressed in percentages and provide insight in false negative results, false positive results and the overall performance of a test matrix combination, respectively. Each TPS participant received multiple aliquots of the same original sample (e.g. PIC, sample 2 and sample 11 from TPS round 1: *S. endobioticum* 1(D1) wart material). Qualitative results of these biological duplicates and triplicates were used to calculate the repeatability per TPS partner and the overall repeatability per test-matrix

combination. Variations to the protocols were inventoried and analysed to determine if they influenced the accuracy of a test-matrix combination (i.e. robustness).

Table 4. Samples provided in TPS and their assigned qualitative values based on homogeneity test results

TPS round	Control/ sample	Material	pathotype/ cultivar	Strain	Amount	Assigned values		
						Boogert	Gent-Pelzer	Bonants
1 & 2	NAC	MGW	-	-	15 mL[a]	-	-	-
1 & 2	PAC1 P1	Wart DNA	1(D1)	MB42	30 µL	+	+	P1
1 & 2	PAC2 P1	10^{-2} PAC1 P1	1(D1)	MB42	30 µL	+	+	P1
1 & 2	PAC1 non-P1	Wart DNA	2(G1)	MB08	30 µL	+	+	Non-P1
1 & 2	PAC2 non-P1	10^{-2} PAC1 non-P1	2(G1)	MB08	30 µL	+	+	Non-P1
1	Sample 1	wart	2(G1)	MB08	100 mg	+	+	Non-P1
	Sample 2	wart	1(D1)	MB42	100 mg	+	+	P1
	Sample 3	wart	18(T1)	MB86	100 mg	+	+	Non-P1
	Sample 4	wart	38(Nevsehir)	MB56	100 mg	+	+	Non-P1
	Sample 5	healthy potato	Eersteling	-	100 mg	-	-	-
	Sample 6	wart	6(O1)	MB10	100 mg	+	+	Non-P1
	Sample 7	wart	18(T1)	MB86	100 mg	+	+	Non-P1
	Sample 8	wart	6(O1)	MB10	100 mg	+	+	Non-P1
	Sample 9	wart	2(G1)	MB08	100 mg	+	+	Non-P1
	Sample 10	wart	38(Nevsehir)	MB56	100 mg	+	+	Non-P1
	Sample 11	wart	1(D1)	MB42	100 mg	+	+	P1
	Sample 12	healthy potato	Eersteling	-	100 mg	-	-	-
	NIC	healthy potato	Eersteling	-	100 mg	-	-	-
	PIC	wart	1(D1)	MB42	100 mg	+	+	P1
2	Sample 1	WSS	1(D1)	MB42	5 sps[b]	int[c]	int	P1

Sample 2	WSS	6(O1)	MB10	5000 sps	+	+	Non-P1
Sample 3	MGW	-	-	10 µL	-	-	-
Sample 4	WSS	6(O1)	MB10	50 sps	int	int	Non-P1b
Sample 5	WSS	6(O1)	MB10	5 sps	int	int	Non-P1b
Sample 6	WSS	1(D1)	MB42	500 sps	+	+	P1b
Sample 7	MGW	-	-	10 µL	-	-	-
Sample 8	WSS	6(O1)	MB10	500 sps	+	+	Non-P1
Sample 9	WSS	1(D1)	MB42	50 sps	int	int	P1
Sample 10	WSS	1(D1)	MB42	5000 sps	+	+	P1
NIC	MGW	-	-	10 µL	-	-	-
PIC	WSS	1(D1)	MB42	5000 sps	+	+	P1

a. molecular grade water used for reaction mix preparation, b. winter spores per sample (10 µL molecular grade water), c. intermediate: samples failed to produce expected results for all aliquots tested: 5000 and 500 sps were used to determine the performance criteria.

Additional to the performance criteria analysed using the TPS data, analytical sensitivity and analytical specificity were analysed in addition by NPPO-NL. Analytical sensitivity was determined for both tests matrices: wart material (determined using seven samples covering five pathotypes), and winter spore suspensions (determined using five samples covering two pathotypes). The analytical sensitivity for wart material is expressed in a relative infection rate as the presence of the non-culturable pathogen cannot be quantified from wart material. A naturally infected potato wart is regarded to have a relative infection rate of 100%. For winter spore suspensions, the amount of winter spores per sample is used to express the limit of detection (LOD). Wart material of fifteen *S. endobioticum* strains covering five different pathotypes were analysed to determine the analytical specificity of the different tests.

2.4 Main results

Work package 1

The infection results for the cultivars Producent and Deodara were as expected, many warts developed in a consistent way on all eye fields tested. Specific numbers of infection of the other cultivars are shown in Table 5. Here the number of eye fields showing wart formation (reaction type 5, Table 1) in the three replicates are individually given, so to show clearly the variation observed between replicates.

The results of the Test Performance Study with pathotypes 6(O1) and 18(T1) are shown in Table 5.

Table 5. Number of eye fields showing wart formation[a)] in three consecutive tests (n=10 for each replicate), cultivars tested in five different laboratories[b)]

Pathotype 6(O1)

	laboratory	IHAR-PIB	HLB	JKI	ILVO	CLPQ
cultivar						
Talent		0/0/0	0/0/1	0/0/0	0/0/0	0/0/0
Logo		0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
Gawin		0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
Transit		0/0/0	0/0/0	0/0/0	0/0/0	0/0/0

Pathotype 18(T1)

	laboratory	IHAR-PIB	HLB	JKI	ILVO	CLPQ
cultivar						
Talent		9/10/10	2/3/8	9/7/7	7/4/3	5/8/3
Logo		0/0/0	0/0/0	0/0/0	0/0/0	2/m.v./0
Gawin		0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
Transit		0/0/0	0/0/0	0/0/0	0/0/0	0/0/0

a) reaction type 5, extremely susceptible (see Table 1)

b) only data for the discriminative cultivars are shown, results for cvs Deodara and Producent not shown
m.v. = missing value

The data in Table 5 clearly show that cv Talent is the preferable differential cultivar to discriminate between pathotype 6(O1) and pathotype 18(T1), this in comparison with cv Logo. In all test laboratories and in all replicate experiments, cv Talent showed wart formation when infected with pathotype 18(T1), while this cultivar did not show any wart formation (except one wart scored in laboratory HLB) when infected with pathotype 6(O1). The results for cvs Gawin and Transit were similar, no wart formation observed neither with pathotype 6(O1) nor with 18(T1). As cv Transit showed slightly susceptible (class 4, Table 1) reactions in two laboratories with pathotype 18(T1), and cv Gawin did not (data not shown), the preferable differential with resistance to all main pathotypes is cv Gawin.

We propose to replace cv Miriam by cv Talent in the actual set of differential cultivars (EPPO, 2004). Also, the cultivar Gawin can be added to the set of differentials in a new version of the EPPO DP on *Synchytrium endobioticum.*

Work package 3a

Homogeneity and stability results

TPS samples were regarded as suitable when resulting in the expected qualitative results and producing Ct values (Gent-Pelzer, Bonants and COX tests only) with standard deviations <3.3 (equivalent to a 10x dilution). Potato wart sample sets used in TPS round 1 produced homogenous test results for all samples in all tests (table 6). Mean Ct values ranged from 15.5 to 19.6 with standard deviations ranging from 1.0 to 2.2. Winter spore suspensions produced homogenous test results with the Gent-Pelzer assay for the undiluted and 10x diluted (5000 and

500 spores per sample) 1(D1) and 6(O1) samples, and with the Bonants assay for undiluted 6(O1) samples. Other sample-test combinations failed to produce the expected qualitative results for all aliquots tested. In the analysis of the results, samples with 5000 and 500 winter spores per sample were regarded positive for all tests and used to determine the performance criteria of the tests.

To determine if sample shipment influenced the TPS results, partners had to send a return sample to the organisers upon receipt of the TPS package. Returned samples were stored at room temperature until all samples were returned to the organisers. DNA extraction and analysis of the samples using the Gent-Pelzer assay was performed according to the protocol sent to the TPS participants. Analysis of the return samples produced Ct values similar to the Ct values obtained for the homogeneity tests (HT) ($Ct_{mean\ HT}$: 20.7, $Ct_{mean\ return}$: 19.6, p-value Students T-test: 0.284).

Table 6. Homogeneity results test performance study samples rounds 1 (wart material) and 2 (winter spore suspensions). Mean qualitative test results are provided for the conventional PCR test (Boogert), whereas mean Ct values and standard deviations are provided for the Gent-Pelzer and Bonants assays.

	Boogert	Gent-Pelzer	Bonants		COX
			P1-probe	non-P1 probe	
TPS round 1 – warts					
1(D1)	+	16.3 (1.0[b])	25.9 (1.6)	26.8 (2.0)	19.1 (1.6)
2 (G1)	+	19.6 (2.2)	-	28.4 (2.2)	19.9 (1.6)
6(O1)	+	15.5 (1.2)	-	24.6 (1.1)	18.3 (0.9)
18(T1)	+	18.1 (1.7)	-	26.9 (1.6)	19.5 (0.9)
38(Nevsehir)	+	18.7 (1.3)	-	27.5 (1.4)	20.4 (0.9)
Healthy potato	-	-	-	-	19.9 (0.6)
TPS round 2 – winter spores					
1(D1) 5000 sps[a]	+[c]	30.2 (0.5)	37.6 (0.9)[c]	37.5 (0.7)[c]	N.A.
500 sps	+[c]	33.8 (0.4)	-	-	N.A.

	50 sps	-	36.2 (0.5)[c]	-	-	N.A.
	5 sps	-	38.4 [c]	-	-	N.A.
6(O1)	5000 sps	+ [c]	30.3 (0.5)	-	35.8 (0.6)	N.A.
	500 sps	+ [c]	33.4 (1)	-	38.0 [c]	N.A.
	50 sps	-	36.3 (0.3) [c]	-	-	N.A.
	5 sps	-	38.9 [c]	-	-	N.A.
MGW		-	-	-	-	-

a. winter spores per sample (10 µL molecular grade water), b. standard deviation, c. results inconsistent for aliquots tested

Gent-Pelzer test: Ct cut-off value

Ct values obtained by TPS participants in TPS round 1 (wart material) were consistent with results found during the homogeneity tests: only a few outliers relative to the homogeneity results ($Ct_{partner}$ > mean $Ct_{homogeneity\ test}$ + 3 StDev) were found (Fig. 1). However, preliminary analysis of TPS round 1 (warts) data showed that the Gent-Pelzer test suffered from false positive results. Twenty of the forty-four healthy potato samples tested resulted in false positive results in late cycles. A Ct cut-off value was determined as the false positive results could be easily distinguished from truly positive samples for this test-matrix combination. Qualitative data provided by TPS participants and homogeneity results were used to calculate the mean false-positive Ct value and corresponding standard deviation (StDev). Three StDevs were subtracted from the mean false positive Ct value resulting, after rounding down to the nearest natural number, in a Ct cut-off value of 30. Performance criteria were determined for both a Ct cut-off value of 40 and 30. A Ct cut-off value is not needed when testing winter spores suspensions.

Diagnostic sensitivity, diagnostic specificity and accuracy

When using wart material for *S. endobioticum* detection, diagnostic sensitivity (the percentage of sample with presence of the target that test positive) values ranging from 95.4% to 97.2% are obtained (table 7). For diagnostic specificity (the percentage of samples with absence of the target that test negative) both the Boogert and Bonants assay yield 100% correct results. Almost half of the healthy plant samples analysed by the TPS participants resulted in positive (late) Ct values in the Gent-Pelzer assay resulting in a diagnostic specificity of 54.5% for that test-matrix combination. Applying the Ct cut-off value of 30 (see above), 100% diagnostic sensitivity was obtained. When applying a Ct cut-off value of 30 to the Gent-Pelzer assay, no significant differences (2-sample binomial tests) for diagnostic sensitivity ($p \geq 0.344$), diagnostic specificity ($p = 1.0$) and accuracy ($p \geq 0.461$) are found between the different tests when testing wart material.

For the test matrix winter spores (5000 and 500 winter spores per sample), the best values are obtained for the Gent-Pelzer assay with 76.7% diagnostic sensitivity, 100% diagnostic specificity, and 85.4% overall accuracy (table 7). The Gent-Pelzer and Boogert assay significantly (2-sample binomial tests) outperform the Bonants assay for diagnostic sensitivity ($p \leq 0.05$) and diagnostic specificity ($p = 0.003$) when testing winter spore suspensions. The Gent-

Pelzer assay significantly outperforms the Boogert and Bonants assays for overall accuracy ($p < 0.030$).

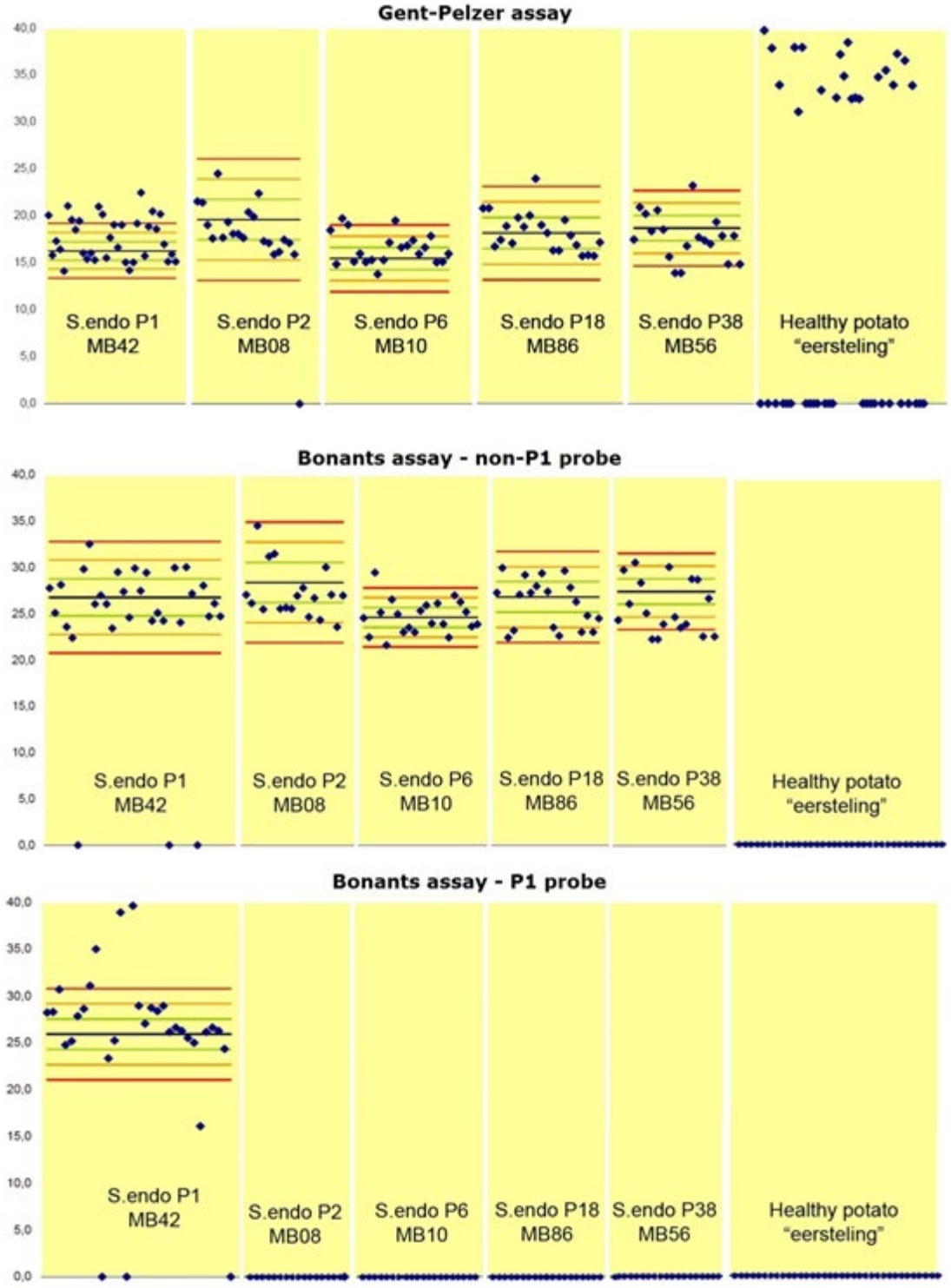

Figure 1. TPS round 1 (warts) Ct values obtained by TPS participants (♦). Horizontal blue lines represent the mean Ct values obtained from the homogeneity tests. Corresponding ±1 SD, ±2 SD and ±3 SD values are represented by green, orange and red horizontal lines, respectively. Negative samples are assigned the value "0".

Table 7. Diagnostic sensitivity, diagnostic specificity and overall accuracy values obtained using TPS results. For TPS round 2, samples containing 5000 and 500 winter spores per sample were used to determine the performance criteria.

TPS round	Test		DSens[a] (%)	DSpec[b] (%)	Acc[c] (%)
1 (wart material)	Boogert		96.3 %	100 %	97.2 %
	Gent-Pelzer	cut-off: 40	97.2 %	54.5 %	85.4 %
		cut-off: 30	97.2 %	100 %	97.9 %
	Bonants		95.4 %	100 %	96.5 %
2 (winter spores)	Boogert		68.0 %	100 %	73.9 %
	Gent-Pelzer	cut-off: 40	76.7 %	100 %	85.4 %
	Bonants		45.7 %	77.1 %	61.4 %

a. diagnostic sensitivity, b. diagnostic specificity, c. accuracy

Repeatability

Biological duplicates and triplicates tested by TPS partners were used to calculate the overall repeatability of the different tests using the test matrices warts and winter spore suspensions (table 8). For TPS round 1, results of 13 partners were taken into account, and a total of 52 repeatability samples were analysed. For the second TPS round, 14 to 28 repeatability samples tested for the Boogert assay (14 partners), Gent-Pelzer assay (12 partners), and Bonants assay (7 partners). When applying the Ct cut-off value of 40 for the Gent-Pelzer assay when testing wart material, no significant differences are obtained. With an overall repeatability of 98%, the tests have demonstrated to yield repeatable results when analysing wart material. When testing winter spore suspensions with 5000 spores per sample, the Gent-Pelzer assay and Boogert assay significantly (2-sample binomial test) outperform the Bonants assay ($p \leq 0.029$) with 83% repeatability. The repeatability of the Boogert assay and the Bonants assay is poor when analysing winter spore suspensions (respectively 64% and 29%).

Table 8. Repeatability

TPS round	Boogert	Gent-Pelzer	Bonants
1 (wart material)	94% (52)	83% (52)/*94% (52)*[a]	98% (52)
2 (winter spores)	64% (28)	83% (24)	29% (14)

a. repeatability based on a Ct cut-off value of 30, and 40 (italics)

Robustness

In TPS round 1, eleven participants indicated which disruption method they used (i.e. manual versus mechanical). Two partners manually grinded samples preceding the DNA extraction, whereas nine partners indicated they used a mechanical disruption method. Samples tested as biological duplicate or triplicate (i.e. wart 1(D1), and healthy potato) were used to determine the qualitative and quantitative (Gent-Pelzer and Bonants assay only) influence of the disruption method applied. Table 9 gives an overview of the samples analysed, the qualitative success rate of the different methods used, and corresponding p-values obtained using a 2-sample binomial test (qualitative results) or Students T-test (quantitative). No significant differences are found based on qualitative results obtained with the different disruption methods for all assays (table 9). Samples which were manually disrupted yielded higher Ct values for all real-time tests (Gent Pelzer: +3.1, Bonants P1 probe: +3.4, Bonants non-P1 probe: +0.9). These differences are only significant for the Gent-Pelzer assay as the spread of values is much larger for the Bonants P1 probe. Data obtained in the second TPS round could not be used to determine the robustness when using the tests with winter spores as starting material.

Table 9. Robustness – disruption methods

Qualitative		**Boogert**			**Gent Pelzer cut-off: 40**			**Bonants**		
sample	disruption method	samples tested	success rate	p value	samples tested	success rate	p value	samples tested	success rate	p value
wart 1(D1)	manual	6	1.00	0.603	5	1.00	0.642	6	1.00	0.271
	Mechanical	23	0.96		24	0.96		22	0.86	
healthy potato	manual	6	1.00	1.000	6	0.67	0.185	6	1.00	1.000
	Mechanical	26	1.00		26	0.50		26	1.00	
Quantitative		**Gent Pelzer cut-off: 40**			**Bonants P1 probe**			**Bonants non-P1 probe**		
sample	disruption method	samples tested	mean Ct	p value	samples tested	mean Ct	p value	samples tested	mean Ct	p value
wart 1(D1)	manual	6	20.1	0.002	6	30.7	0.162	6	27.6	0.515
	Mechanical	22	17.0		23	27.3		23	26.7	

Conventional PCR tests in the first TPS round have successfully been performed on the following thermocyclers: Peltier PTC-200 (MJ research), GeneAmp PCR System 9700 (Applied

Biosystems), GeneAmp PCR System 2720 (Applied Biosystems), Mastercycler personal (Eppendorf), C1000 (Bio-Rad), Veriti 96well thermalcycler (Applied Biosystems). Real-time PCR tests in the first TPS round have successfully been performed on the following thermocyclers: 7300 Real-Time PCR System (Applied Biosystems), 7900HT Fast real-time PCR system (Applied Biosystems), ABI 7500 Real time PCR system (Applied Biosystems), CFX96 (Bio-Rad), Eppendorf Mastercycler® ep realplex, Stratagene Mx3005P.

Table 10. Analytical sensitivity results for the three different tests using both test matrices.

	Boogert		Gent-Pelzer		Bonants P1 samples			Bonants non-P1-samples		
Sample material	success rate (samples)	Amplicon	success rate (samples)	mean Ct (StDev)	success rate (samples)	mean Ct (StDev)		success rate (samples)	mean Ct (StDev)	
						P1 probe	non-P1 probe		P1 probe	non-P1 probe
Potato wart material										
100%[a]	100% (7)	+	100% (7)	20.3 (2.0)	100% (3)	28.3 (1.7)	29.4 (2.4)	100% (4)	-	29.5 (1.1)
10%	100% (7)	+	100% (7)	23.7 (1.9)	100% (3)	31.8 (1.6)	32.8 (2.3)	100% (4)	-	32.9 (1.1)
1%	100% (7)	+	100% (7)	27.1 (2.0)	100% (3)	35.0 (2.2)	36.3 (3.2)	100% (4)	-	37.0 (2.2)
$1{\cdot}10^{-1}$ %	57% (7)	w+[c]	43% (7), *100%(7)*[d]	30.4 (1.9)	33% (3)	37.7	37.0	50% (4)	-	37.4 (0.3)
$1{\cdot}10^{-2}$ %	14% (7)	w+	0 (7), *100%(7*	34.0 (2.3)	33% (3)	37.5	39.5	0 (4)	-	-
$1{\cdot}10^{-3}$ %	0 (7)	-	0 (7), *86% (7)*	37.0 (2.0)	0 (3)	-	-	0 (4)	-	-
$1{\cdot}10^{-4}$ %	0 (7)	-	0 (7), *29% (7)*	37.5 (1.4)	0 (3)	-	-	0 (4)	-	-
$1{\cdot}10^{-5}$ %	0 (7)	-	0 (7), *14%(7)*	38.6	0 (3)	-	-	0 (4)	-	-
$1{\cdot}10^{-6}$ %	0 (7)	-	0 (7)	-	0 (3)	-	-	0 (4)	-	-
Winter spore suspensions										
5000[b]	82% (11)	+	100% (11)	29.8 (0.6)	40% (6)	37.6 (0.9)	37.5 (0.7)	100% (5)	-	35.8 (0.6)
500	80% (10)	w+	100% (10)	33.5 (0.8)	0 (5)	-	-	20% (5)	-	38.0
50	40% (10)	w+	90% (10)	35.8 (0.5)	0 (5)	-	-	0 (5)	-	-
5	0 (10)	-	40% (10)	38.2 (0.6)	0 (5)	-	-	0 (5)	-	-

a. relative infection rate: undiluted naturally infected wart material is regarded as 100% infected, b. winter spores per sample (10 µL molecular grade water), c. weak positive amplicon, d. success rate based on a Ct cut-off value of 30, and 40 (italics)

Analytical sensitivity

LODs are determined for both test matrices: wart material and winter spore suspensions (table 10). For the test matrix wart material, a Ct cut-off value of 30 is used to avoid false positive results. This however has an adverse effect on the LOD. The lowest relative infection rate at which all wart sample produce a positive result is regarded as the LOD. Under these criteria, all tests Boogert, Gent-Pelzer, Bonants for pathotype 1(D1) samples, and Bonants for non-pathotype 1(D1) samples have a LOD at a relative infection rate of 1% (i.e. a 100x dilution of a naturally infected wart). For the test matrix winter spore suspensions, only the Gent-Pelzer test, and the Bonants test for non-pathotype 1(D1) samples produced consistent results for all subsamples at a given amount of winter spores. The Gent-Pelzer assay produced all positive results up to 500 winter spores per 10 µL sample, whereas the Bonants test for non-pathotype 1(D1) samples produced all positive results for 5000 winter spores per 10 µL sample. The Boogert assay, and the Bonants assay for pathotype 1(D1) samples were not sensitive enough to detect the pathogen in all subsamples with 5000 winter spores per 10 µL sample (success rate of 82% and 40% respectively). The LOD for the latter two tests lies higher than 5000 spores per 10 µL sample.

Analytical specificity

Wart material of 15 strains was analysed to determine their reaction in the different tests (table 11). For the Boogert and Gent-Pelzer assays all strains produced results as expected. For the Bonants assay however, one pathotype 1(D1) sample originating from Sweden produced a result consistent with a non-pathotype 1(D1) sample. Other samples produced results as expected in the Bonants assay.

Table 11. Analytical specificity

Strain	Pathotype	Origin	Boogert	Gent-Pelzer	Bonants		COI
					P1	non-P1	
MB42	1(D1)	Netherlands	+	17,4	26,0	27,8	18,5
MB69	1(D1)	Sweden	+	21,8	-	31,4	21,5
5022364	1(D1)	Netherlands	+	15,2	23,1	26,6	18,7
MB81	1(D1)	Ireland	+	19,9	32,1	29,0	21,1
MB08	2(G1)	Netherlands	+	20,1	-	28,4	18,1
MB10	6(O1)	Netherlands	+	17,6	-	26,6	18,4
MB14	18(T1)	Germany	+	20,1	-	28,5	21,0
MB55	18(T1)	Sweden	+	21,5	-	29,9	21,2

4112001	18(T1)	Germany	+	28,1	-	36,9	29,8
39.9.2893	18(T1)	Sweden	+	22,1	-	30,7	21,4
MB85	18(T1)	Greece	+	20,5	-	29,5	21,0
MB82	18(T1)	Germany	+	16,9	-	26,2	22,8
MB15	18(T1)	Germany	+	18,3	-	27,1	19,4
MB86	18(T1)	Greece	+	24,6	-	32,9	19,8
MB56	38(Nevsehir)	Turkey	+	18,5	-	27,1	20,2

Discussion and Conclusions (work package 3a)

Three assays were selected for the detection and identification of *Synchytrium endobioticum*, the causal agent of potato wart disease. Tests were validated in an international TPS with fifteen participants for two test matrices: warted potato tissue, and winter spore suspensions. Not all partners acted correctly when faced with incorrect results of control samples. In the TPS instructions, partners were asked to repeat the tests with the back-up sample set in such cases, but this was not always done. Datasets with incorrect control results were excluded from the analysis. Especially for the second TPS round (winter spore suspensions), this limited the number of datasets that could be included in the analysis. Performing a second TPS round focusing on winter spore suspensions was decided after completion of the first round and limited time was available to prepare the second TPS round. Because of that, winter spore suspensions with several concentrations were included and sent to TPS partners before the homogeneity tests could be finalised. This resulted in including winter spore amounts that were below the limit of detection. Only samples with 5000 and 500 spores per 10 µL sample could be used in analysis of the TPS results.

When using the assays for detection and identification of *S. endobioticum* in warted potato tissue (with the Cq cut-off value for the Gent-Pelzer assay), no significant differences were observed for diagnostic sensitivity, diagnostic specificity and overall accuracy and the assays are regarded equal. Also, the assays show an equal performance in terms of analytical sensitivity using this test matrix. For the Bonants assay, this includes the correct identification of pathotype 1(D1) samples versus non-pathotype 1(D1) samples. All tests were found to be robust for the disruption method used.

Several TPS partners generated late Ct values for some of the healthy potato samples tested. A Ct cut-off value had to be determined for the Gent-Pelzer assay to eliminate late Ct values without risking the introduction of false negative results. These late Ct values could be the result of contamination or non-specific annealing of primers and probe. We tried to assess the most likely steps where contamination could have occurred, and concluded that it is likely the first steps of the DNA extraction are the source of contamination. In the first TPS round, healthy and warted potato tissues were freeze-dried in 2 mL ampoules and closed under vacuum. The different samples were prepared and tested for homogeneity on different days to prevent contamination during either preparation or DNA extraction. During the

preparation of the TPS, late Ct values in healthy potato tissue were observed only once. Opening the vacuum sealed ampoules under the TPS conditions (all at once) might have introduced contamination of the healthy potato samples by the heavily infected warted potato samples. In case of contamination this remained undetected in the Boogert and Bonants assay since they are less sensitive compared to the Gent-Pelzer assay. Since there are no frequent *S. endobioticum* outbreaks, no large scale testing of warted potato tissue is performed using the assay described in this report and little experience exists in regard to contamination in large scale routine testing. For individual outbreaks contamination is not expected to play a role. However, we propose to include the cut-off value in the EPPO standard since the contamination during extraction was found to be repeatable, and no false negative results were obtained when using the cut-off value. Laboratories implementing the Gent-Pelzer assay have to determine the need of a Ct cut-off value for their diagnostic workflow through the process of verification (PM7/98(2)). After the EUPHRESCO Sendo project, ILVO compared the Gent-Pelzer test with the Smith test on extracts obtained with zonal centrifugation. The Gent-Pelzer test produced late Cq values for some truly negative samples, which could not be reproduced with the Smith test. With the Smith test producing slightly lower Cq values in general, this rather hints towards non-specific annealing of primers and probes in the Gent-Pelzer test rather than a contamination effect (personal communication Kurt Heungens, ILVO, Belgium).

The second TPS round proved to be more challenging than the first since winter spore suspensions close or below the limit of detection were provided to TPS partners. To illustrate this for a pathotype 1(D1) sample below the LOD: analysing a sample of 500 spores per 10 µL sample, the DNA equivalent of 10 spores in the conventional PCR assay, and 30 spores in the real-time PCR assays is used (DNA extraction in 50 µL, 1 µL or 3 µL DNA extract template in reaction mix respectively). Optimisation of the amount of template used in the different reaction mixes was not part of the TPS. When using the assays for detection and identification of *S. endobioticum* in winter spore suspensions, the Boogert and Gent-Pelzer assays significantly outperformed the Bonants assay for diagnostic sensitivity and diagnostic specificity. For overall accuracy, the Gent-Pelzer assay significantly outperforms the Boogert and Bonants assays. Using the Bonants assay for pathotype 1(D1) identification at low levels of the target proved to be difficult. Under the conditions used in the TPS we would recommend to use caution when testing winter spore suspensions below 5000 spores per sample with the Bonants assay.

In general, the Bonants assay was found difficult to interpret as, apart from Ct values, participants had to identify pathotype 1(D1) and non-pathotype 1(D1) specific amplification plots, also described by Bonants *et al.* (2015). This was found to be particularly true for winter spore suspension samples with low amount of target. Some TPS partners indicated that, under their conditions, Cq values were as expected but that the obtained amplification plots were different compared to the expected reactions. The TPS organisers and some TPS partners also found slight differences in the amplification plots for some *S. endobioticum* collection items compared to the results published by Bonants and colleagues. Real-time PCR machines, and in particular the ramp rate of the thermal block, might have an influence on the amplification efficiency and the corresponding shape of the real-time PCR amplification curve. This aspect has not been further investigated under the TPS. In addition, the added value of the non-P1 reaction was questioned as it was found to be confusing.

Improved user-friendliness of the Bonants assay can be achieved by using the P1 reaction without the non-P1 reaction in conjunction with one of the generic *S. endobioticum* tests.

For the determination of analytical specificity, samples used were limited to different *S. endobioticum* pathotypes and healthy potato as no other *Synchytrium* spp. were available to us. It is not likely that the symptoms caused by potato wart disease are also induced by closely related *Synchytrium* species as they are highly specialised for certain hosts. Wart disease symptoms could be confused with pseudo-wart: a proliferation of eyes that may be a physiological response, a varietal response, or could be induced by chemical factors. In essence, potatoes with pseudo-wart are healthy potatoes. False non-pathotype 1(D1) results were obtained with Swedish pathotype 1(D1) isolate (MB69) using the Bonants assay. Bonants *et al*. (2015) obtained similar results with some pathotype 1(D1) isolates originating from outside the Netherlands and Germany. The pathotype 1(D1) associated SNP that lies at the basis of the Bonants assay design was identified using mainly Dutch and German 1(D1) isolates. This means that for diagnostic purposes, only pathotype 1(D1) positive results produced by the Bonants assay can be used for molecular 1(D1) identification. Strains identified as pathotype 1(D1) using a bioassay can produce non-pathotype 1(D1) results.

As goes for all potato wart disease studies, having access to sufficient and well characterised isolates covering a broad geographical range is problematic. Obtaining this material is problematic because of the low outbreak frequency for this pest, the fact that it is difficult to maintain in collections, and the different pathotyping methods used in different laboratories. However, having sufficient and well characterised isolates covering a broad geographical range is paramount for reliable development and validation of diagnostic tests. The *Synchytrium endobioticum* community would strongly benefit from a centralised repository for collection material that maintains the material and keeps track of its “genealogy”. Euphresco could play a role in addressing this when new potato wart disease initiatives are launched within this research framework. For future wart disease projects, recently published tests for molecular *S. endobioticum* detection (Smith *et al*. 2014), and pathotype identification (Gagnon *et al*. 2016) could be considered.

2.5 Conclusions and recommendations to policy makers

- As goes for all potato wart disease studies, having access to sufficient and well characterised isolates covering a broad geographical range is problematic. Obtaining this material is problematic because of the low outbreak frequency for this pest, the fact that it is difficult to maintain in collections, and the different pathotyping methods used in different laboratories. However, having sufficient and well characterised isolates covering a broad geographical range is paramount for reliable development and validation of diagnostic tests. The *Synchytrium endobioticum* community would strongly benefit from a centralised repository for collection material that maintains the material and keeps track of its “genealogy”. Euphresco could play a role in addressing this when new potato wart disease initiatives are launched within this research framework.
- For the bio-assay to identify pathotypes, we propose to implement and use a sole method, the Glynne-Lemmerzahl method. In the actual set of differential cultivars cv Miriam should be replaced by cv Talent (EPPO, 2004). Also, the cultivar Gawin can be added to the set of differentials in a new version of the EPPO DP on *Synchytrium endobioticum.*

- Policy makers should encourage the implementation of a uniform, standardised set of differential cultivars to identify pathotypes as described in the EPPO DP.
- Using the generic assays for detection and identification of *S. endobioticum* in **warted potato tissue**, no significant differences were observed for diagnostic sensitivity, diagnostic specificity and overall accuracy and the assays are regarded equal. Also, the assays show an equal performance in terms of analytical sensitivity using this test matrix. For the Bonants assay, this includes the correct identification of pathotype 1(D1) samples versus non-pathotype 1(D1) samples. All tests were found to be robust for the disruption method used.
- When using the assays for detection and identification of *S. endobioticum* in **winter spore suspensions**, the Boogert and Gent-Pelzer assays significantly outperformed the Bonants assay for diagnostic sensitivity and diagnostic specificity. For overall accuracy, the Gent-Pelzer assay significantly outperforms the Boogert and Bonants assays. Using the Bonants assay for pathotype 1(D1) identification at low levels of the target proved to be difficult. Under the conditions used in the TPS we would recommend to use caution when testing winter spore suspensions below 5000 spores per sample with the Bonants assay.

2.6 Benefits from trans-national cooperation

- Successful merging of every partner ‘s specific skills. In the bio-assays (subproject 1) we relied on the experience of partner 3 in producing starting material in large quantities (fresh warts). In selecting new differentials, the personal experience of partners with variety testing in their respective country was successfully exploited.
- Strengthening effort to speed up harmonisation in applying methods, and use of the same differential cultivars to identify pathotypes.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None

3.2. Article for publication in the EPPO Reporting Service

None

3.3. Article(s) for publication in other journals

PJM Bonants, MPE van Gent-Pelzer, GCM van Leeuwen & TAJ van der Lee, 2015. A real-time Taqman PCR assay to discriminate between pathotype 1(D1) and non-pathotype 1(D1) isolates of *Synchytrium endobioticum.* European Journal of Plant Pathology 143, p 495-506 (see Appendix)

4. Open Euphresco data

No data has been opened by the consortium.

5. References

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Bonants PJM, van Gent-Pelzer MPE, van Leeuwen GCM, van der Lee TAJ (2015) A real-time TaqMan PCR assay to discriminate between pathotype 1 (D1) and non-pathotype 1 (D1) isolates of *Synchytrium endobioticum*, Eur J Plant Pathol, 143: 495

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Instruction booklet for Test Performance Study (TPS) participants

Diagnostic methods for *Synchytrium endobioticum*, especially for pathotype identification (SENDO)

Subproject 3a: Test performance study molecular biological tests

Version 1.0
28 May 2013

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Table of content

1 Introduction

This instruction booklet will guide you through the EUPHRESCO *Synchytrium endobioticum* subproject 3a Test Performance Study (TPS). Subproject 3a focuses on the molecular tests described in the draft revision of EPPO standard PM7/28 (2): one conventional PCR (Levesque *et al.*, 2001) and one real-time PCR (van Gent-Pelzer *et al.*, 2010) for the detection of *S. endobioticum*, and one real-time PCR to distinguish between pathotype 1 and non-pathotype 1 strains (Bonants *et al.*, unpublished) in potato wart material. Using the TPS set-up, several performance criteria will be determined for the three different tests (analytical specificity, diagnostic specificity, diagnostic selectivity, repeatability, reproducibility and robustness). Table 1 gives an overview of the partners included in the TPS.

Table 1. Overview of ring test partners

Partner #	Institute	Country
1	Science and Advice for Scottish Agriculture (SASA)	United Kingdom
2	Food and Environmental Research Agency (FERA)	United Kingdom
3	Instituut voor Landbouw- en Visserijonderzoek, Eenheid Plant - Gewasbescherming (ILVO)	Belgium
4	The State Plant Service under the Ministry of Agriculture of the Republic of Lithuania (LT-MOA)	Lithuania
5	Central Laboratory for Plant Quarantine (BG-PPS)	Bulgaria
6	All-Russian Plant Quarantine Center, Ramensky region, Bykovo	Russian Federation
7	Julius Kühn – Institut, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland (JKI)	Germany
8	Plant Health Laboratory, Pesticides, Plant Health & Seed Testing Laboratories (IE-DAFF)	Ireland
9	The Central Laboratory of the State Plant Health and Seed Inspection Service (IHAR)	Poland
10	All-Russia Institute of Plant Protection (VIZR)	Russian Federation
11	National Plant Protection Organization (NPPO-NL)	The Netherlands
12	Hilbrands Laboratorium voor Bodemziekten (HLB)	The Netherlands
13	Plant Research International (PRI)	The Netherlands
14	Institute of Plant Protection (NAAS)	Ukraine
15	Nederlandse Algemene Keuringsdienst (NAK)	The Netherlands

A checklist is provided in appendix 4 which will help you to make sure that all the necessary steps have been performed.

2 Dates and Deadlines

The wrap-up meeting in St. Petersburg represents a very definite deadline for the TPS and we have to present the TPS results and evaluation at that stage. This results in the following time table:

- Sending TPS packages (round 1): 3 June 2013 (for labs that provided transport documents in time)
- Sending TPS packages (round 2), only when additional labs are able to provide transport documents: end of June 2013
- TPS experiments performed by participants: mid-June - end of August
- Deadline reporting TPS results: 30 August 2013
- TPS data evaluation (NPPO-NL): September 2013
- Wrap-up meeting St. Petersburg: 1 - 4 October 2013

Please respect the dates and deadlines above. Should you have problems with keeping the deadlines, please let us know as soon as possible.

3 How to get started

- When you provided the relevant transport documents in time, your TPS package will be sent in the first week of June 2013.
- Check if all items that should have been provided are actually there. See also section 5 "ring test material provided". When items are missing or broken, contact Bart van de Vossenberg (b.t.l.h.van.de.vossenberg@minlnv.nl, +316 4635 2244) or Marcel Westenberg (m.westenberg@minlnv.nl, +316 4620 0605).
- Store the different items provided at the correct storage temperature.
- Read this instruction booklet carefully and familiarise yourself with the set-up of the TPS.
- Order reagents and consumables not provided, see also section 6 "ring test material to be provided by participant".
- Plan your experiments sensibly in order to report the results in time. Participants have until the 30th of August 2013 to report the data.
- Provide an endorsement stamp for the transport document of the return sample as soon as possible.
- Send the return sample with the transport document to NPPO-NL using the return envelope as soon as possible.

4 Troubleshooting

Feel free to contact Bart van de Vossenberg (b.t.l.h.van.de.vossenberg@minlnv.nl, +316 4635 2244) or Marcel Westenberg (m.westenberg@minlnv.nl, +316 4620 0605) in case you have any questions. Please note that both Bart and Marcel do not work on Thursday.

5 Ring test material provided

The table below states the items provided for the ring test and training sessions.

Table 2. Items provided for the TPS

Item	Description	Storage
Instruction booklet	This booklet	RT*
TPS sample set	12 vials with freeze dried samples including a positive isolation control (PIC) and negative isolation control (NIC).	RT
TPS back-up sample set	12 vials with freeze dried samples including a positive isolation control (PIC) and negative isolation control (NIC) identical to the TPS sample set.	RT
Return sample	1 vial containing freeze dried sample to be returned to NPPO-NL.	RT
LoA or import document	Letter of Authority (for EU members) or import document (for non-EU members) for the return sample.	RT
Return envelope	Addressed return envelope for return sample with LoA or import document	RT
Primers (conventional PCR, appendix 1)	2 vials containing 100 µl primer solution with a concentration of 10 µM. Primer names are stated in the protocol.	-20 °C
Primers and probes (real-time PCR, appendix 2)	4 vials containing 60 µl primer, 2 vials containing 30 µl probe solution with a concentration of 10 µM. Primer names are stated in the protocol.	-20 °C
Primers and probes (real-time PCR, appendix 3)	4 vials containing 50 µl primer, 3 vials containing 30 µl probe solution with a concentration of 10 µM. Primer names are stated in the protocol.	-20 °C

* Room Temperature

Table 2 (continued). Items provided for the TPS

Item	Description	Storage
Positive amplification controls (PACs)	4 vials containing 30 µl DNA of different concentration and different pathotypes of *S. endobioticum*	-20 °C
DNeasy® plant mini kit (Qiagen) (12 extractions)	• 2 ml tubes with 2 steel beads (12) • 1.5 ml microcentrifuge tubes (24) • 2 ml collection tubes (36) • AP1 buffer (6 ml) • RNase A (100 mg/ml) (60 µl) • P3 buffer (2 ml) • AW1 buffer (10 ml) • AW2 buffer (15 ml) • AE buffer (1 ml) • QIAshredder Mini spin column (lilac) (12) • DNeasy Mini spin column (white) (12)	RT
Molecular Grade Water	1 tube containing app. 15 ml molecular grade water used for reaction mix preparation.	RT

6 Ring test material to be provided by participant

TPS participants have to provide the following equipment, consumables and chemicals.

Equipment
- Pipettes (e.g. Eppendorf P10, P20, P100, P200, P1000)
- Beadbeater (e.g. BioSpec), TissueLyser (e.g. Qiagen) or Micro pestle (e.g. Eppendorf)
- Microcentrifuge (e.g. Eppendorf 5415D)
- Heat block or thermomixer (e.g. Eppendorf)
- Peltier-type thermal cycler with heated lid (e.g. Bio-Rad C1000)
- Real-time PCR machine (e.g. Bio-Rad CFX96)
- Agarose gel electrophoresis equipment (e.g. Biorad wide mini/midi sub™ cell)
- Agarose gel imaging-system (e.g. Syngene InGenius)

Consumables
- DNase and RNase free tubes/plates for (real-time) PCR
- Filter tips (e.g. Eppendorf for P10, P20, P100, P200, P1000)

Chemicals
- Premix Ex Taq™ DNA Polymerase (Perfect Real Time), 200 Rxns (Takara) (order number RR039A)
- GoTaq® Flexi DNA Polymerase (Promega) (order number M8301)
- dNTP PCR mix 10 mM each (e.g. Promega, order number U1511)

7 TPS experiments

In the TPS we prepared a total of twelve *unknown* samples numbered "sample 01" to "sample 12". You will receive a subset of 10 out of 12 unknown samples so it is possible that the samples you received do not have successive numbers (e.g. sample 01, 02, 04, 06, 07, …). Apart from the 10 *unknown* samples you will receive one positive and one negative isolation control (PIC and NIC). PIC, NIC and the unknown sample are defined as "sample set".

Figure 1 gives an overview of the experiments that have to be performed in the TPS. Below, a short description is given per step.

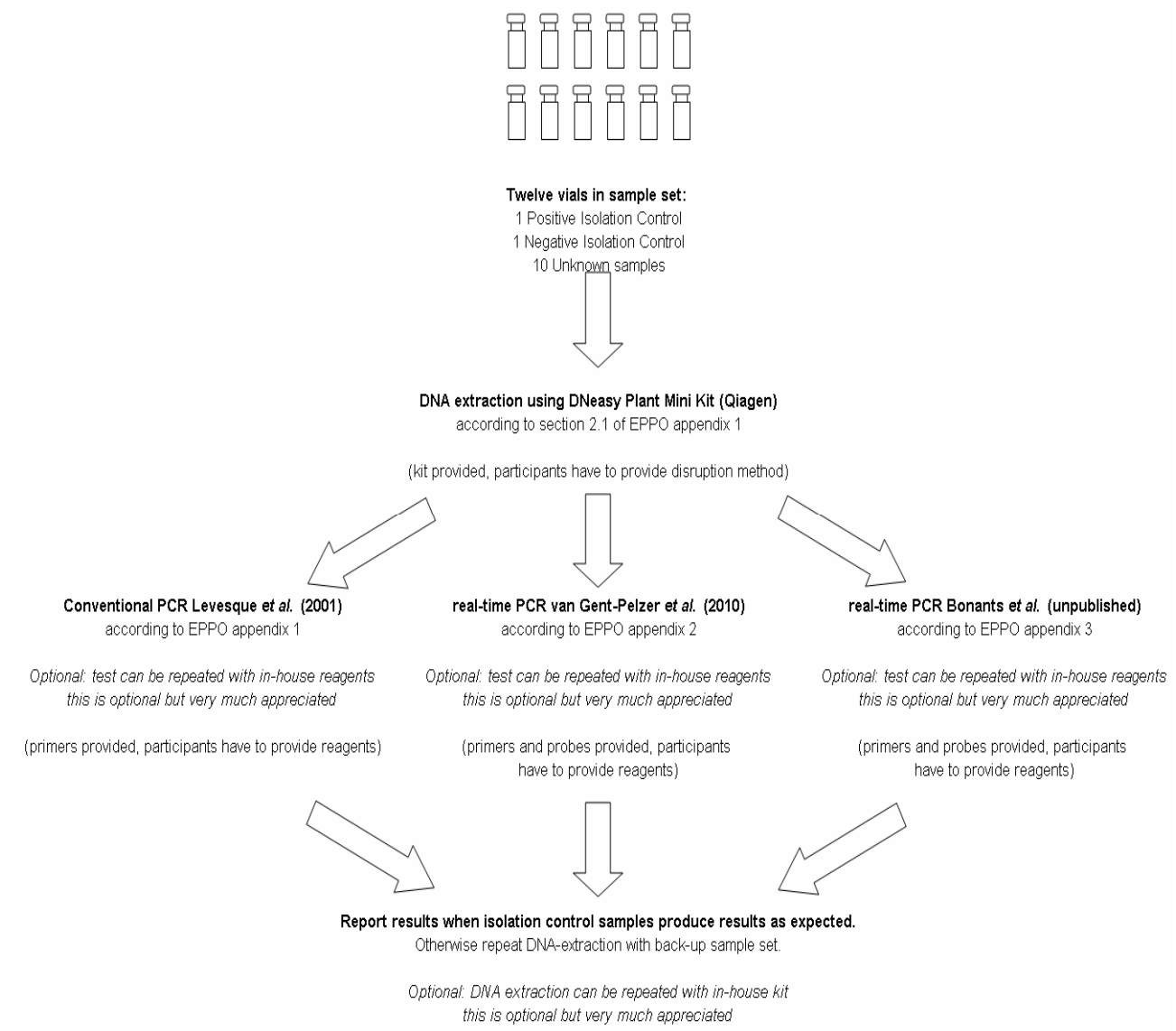

Figure 1. Overview of tests performed in the TPS

DNA-extraction

Two identical sample sets are provided; one is labelled "TPS Sample Set" the other one is labelled "TPS Back-up Sample Set". The back-up sample set is only used when DNA extraction of the "Sample Set" has failed (unexpected results for PIC and internal control Plant COX gene). Should this be the case, make sure to ask for a new set of DNeasy Plant Mini kit since we have provided aliquots for exactly 12 reactions. A new set of the DNeasy Plant Mini kit can be obtained from Bart or Marcel.

DNA is extracted according to section 2.1 of Appendix 1 of draft revision of EPPO standard PM7/28 (2) *Synchytrium endobioticum* (see appendix 1 of this booklet)

Make sure to extract DNA from the positive and negative isolation control (PIC and NIC) in parallel with the other samples.

When DNA extraction of the "sample set" was successful, the "TPS Back-up Sample Set" might be used for DNA extraction using a second DNA extraction kit you have available in-house. This part is optional, but your effort to do so will be very much appreciated and the results can be used to determine the performance criteria "robustness".

PCR tests

Three tests are performed for the detection of *S. endobioticum* in wart material. Each test has its own appendix in the draft revision of EPPO standard PM7/28 (2) *Synchytrium endobioticum*.

- Conventional PCR Levesque et al. (2001) is performed according to appendix 1 of the draft revision of EPPO standard PM7/28 (2) (see appendix 1 of this booklet)
- real-time PCR van Gent-Pelzer et al. (2010) is performed according to appendix 2 of the draft revision of EPPO standard PM7/28 (2) (see appendix 2 of this booklet)
- real-time PCR Bonants et al. (unpublished) is performed according to appendix 3 of the draft revision of EPPO standard PM7/28 (2) (see appendix 3 of this booklet)

Make sure to include the appropriate isolation (NIC and PIC) and amplification controls (PAC1 P1, PAC2 P1, PAC1 non-P1 and PAC2 non-P2) mentioned in the different appendices, see also table 3.

Table 3. Controls used in the different tests with corresponding appendices

Control	**Content**	**DNA extraction, appendix 1**	**Conventional PCR, appx. 1**	**real-time PCR, appx. 2**	**real-time PCR, appx. 3**
NIC	Healthy potato	x	x	x	x
PIC	*S. endobioticum* Pathotype 1 infected potato wart material	x	x	x	x
NAC	Molecular grade water used for reaction mix set-up		x	x	x
PAC1 P1	DNA from *S. endobioticum* Pathotype 1 infected potato wart material (undiluted)		x	x	x
PAC2 P1	DNA from *S. endobioticum* Pathotype 1 infected potato wart material (100x diluted)			x	x
PAC1 non-P1	DNA from *S. endobioticum* Pathotype 2 infected potato wart material (undiluted)				x
PAC2 non-P1	DNA from *S. endobioticum* Pathotype 2 infected potato wart material (100x diluted)				x

When the (real-time) PCR reactions were performed successful, the tests can be repeated with reagents you have available in-house. This part is optional, but your effort to do so will be very much appreciated and the results can be used to determine the performance criteria "robustness".

Interpretation of test results
The interpretation of the test results is described in EPPO appendices 1 to 3. Under routine conditions, laboratories should verify the cycle cut-off value when implementing tests for the first time. In this TPS, a cut-off value of Ct = 40,0 is used for the interpretation of results and additional verification of the cut-off value is not needed.

Report results
Use the provided Excel file to report the results. The file contains 5 worksheets; a general introduction, one on DNA extraction and one for each test. You are asked to provide qualitative results, Ct-values, a gel image (conventional PCR), an image of the amplification curves, threshold, baseline settings (real-time PCR) and additional information on thermal cyclers and real-time PCR equipment. Please follow the instructions in the Excel file.

8 Robustness

Robustness is defined as the degree of insensitivity of the results of a measurement to deviations in procedure, circumstances and nature of materials like these may occur in practice.

Within the TPS, the use of different thermal cyclers, real-time PCR equipment and software settings are used to define the robustness of the tests. Apart from these, participants are asked to perform additional experiments using in-house extraction kit and/or reagents. The additional work is *optional* but very much appreciated. The use of alternative DNA extraction protocols and/or reagents will also be used to define the robustness of the different tests.

For each test there is a section to report additional work performed in the results Excel file.

9 General advice

Make sure to prevent contamination or degradation of your samples. The following points are some general guidelines for working in molecular biological labs.

- Keep different steps (DNA extraction, preparation of reaction mixes, amplification and detection) of the test protocol physically separated, preferably using separated labs. A one-way-system should be maintained between separated labs.
- Assign pipettes, lab coats and consumables to each laboratory.
- Always wear latex gloves while handling reagents and DNA or RNA samples to prevent contamination. Vinyl or nitrile gloves can be used for people allergic to latex. Preferably wear non-powdered latex gloves to prevent inhibition of the PCR reaction. Change gloves frequently and keep tubes closed whenever possible.
- Work on ice as much as possible in order to prevent enzyme activity loss.
- Make sure to store extracts, PCR products, buffers, enzymes et cetera at the correct temperature.
- DNA or RNA contaminated glassware and other re-usable objects (e.g. micro-pestles) are to be cleaned with 1% sodium hypochlorite solution (at least 10 min), rinsed with deionised water and sterilised at 121 ºC.
- Routinely clean laboratory tables and door handles using 1% sodium hypochlorite solution. Rinse with water afterwards.

Appendices

Appendix 1. detection of *S. endobioticum* in potato wart material using conventional PCR

Appendix 2. detection of *S. endobioticum* in potato wart material using real-time PCR

Appendix 3. identification of *S. endobioticum* pathotype 1 in potato wart material using real-time PCR

Appendix 4. TPS checklist

Appendix 1. Detection of *S. endobioticum* in potato wart material using conventional PCR

1. General Information

1.1. Detection of *S. endobioticum* in potato warts using conventional PCR developed by Levesque *et al.* 2001

1.2. The conventional PCR was first published by Levesque *et al.* in 2001, but described in detail by van den Boogert *et al.* in 2005. The PCR reaction mix was updated by NPPO-NL in 2013 and validated in an international test performance study as such.

1.3. Primers F49 (5'-CAACACCATGTGAACTG-3') and R502 (5'-ACATACACAATTCGAGTTT-3') amplify 472 bp of the internal transcribed spacer (ITS) region.

1.4. Amplification is performed in a thermal cycler with heated lid, e.g. T100 Thermal cycler (Bio-Rad).

2. Methods

2.1. Nucleic Acid Extraction and Purification

Potato wart material (max. 100 mg) is extracted using a modified Plant Tissue mini protocol from the DNeasy® Plant Mini Kit (Qiagen).

2.1.1. Transfer wart material (max. 100 mg) to a 2 mL microcentrifuge tube. Add two steel beads (e.g. 4 mm diameter) to the vial when mechanical disruption is used

2.1.2. Add 400 µL Buffer AP1 and 4 µL RNase A stock solution (100 mg/mL). Buffer AP1 may form precipitates upon storage. If necessary, warm to 65°C to redissolve

2.1.3. Disrupt the sample either manually (e.g. with a micro pestle) or mechanically (e.g. with a BeadBeater or TissueLyser)

2.1.4. Incubate the mixture for 10 min at 65°C. Mix 2 or 3 times during incubation by inverting tube

2.1.5. Centrifuge 1 min at 20,000 x g and transfer the supernatant (= lysate) to a new 1.5 mL microcentrifuge tube

2.1.6. Add 130 µL Buffer P3 to the lysate and mix.

2.1.7. Incubate for 5 min on ice

2.1.8. Centrifuge the lysate for 5 min at 20,000 x g

2.1.9. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 mL collection tube

2.1.10. Centrifuge for 2 min at 20,000 x g

2.1.11. Transfer 450 µL of the flow-through fraction from step 10 into a new 1.5 mL microcentrifuge tube

2.1.12. Add 675 µL (1.5 volumes) of Buffer AW1 to the cleared lysate, and mix by pipetting.

2.1.13. Pipet 650 µL of the mixture from step 12, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 mL collection tube.

2.1.14. Centrifuge for 1 min at 6000 x g

2.1.15. Place the DNeasy Mini spin column into a new 2 mL collection tube and repeat step 11 with remaining sample.

2.1.16. Place the DNeasy Mini spin column into a new 2 mL collection tube, add 500 µL Buffer AW2,

2.1.17. Centrifuge for 1 min at 6000 x g.

2.1.18. Place the DNeasy Mini spin column into a new 2 mL collection tube, add 500 µL Buffer AW2 to the DNeasy Mini spin column.

2.1.19. Centrifuge for 2 min at 20,000 x g to dry the membrane.

2.1.20. Transfer the DNeasy Mini spin column to a 1.5 mL microcentrifuge tube, and pipet 50 µL Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15 - 25°C).

2.1.21. Centrifuge for 1 min at 6000 x g. The flow through contains the extracted DNA.

2.1.22. After DNA extraction, no additional DNA clean-up is required. Either use extracted DNA immediately or store it at –20 °C until use.

2.2. Conventional PCR

2.2.1. Master Mix conventional PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	13.8	N.A.
Colorless GoTaq Flexi buffer (Promega)	5x	5.0	1x
$MgCl_2$ (Promega)	25 mM	1.5	1.5 mM
dNTP's (Promega)	10 mM each	0.5	0.2 mM
Primer F49	10 µM	1.5	600 nM
Primer R502	10 µM	1.5	600 nM
GoTaq DNA Polymerase (Promega)	5 U/µL	0.2	1 Unit
Subtotal		24.0	
Genomic DNA extract		1.0	
Total		25.0	

*Molecular grade water should be used preferably. Alternatively sterile (autoclaved or 0.45 µm filtered), purified (deionised or distilled) and nuclease-free water can be used.

2.2.2. PCR conditions: 2 min 95 °C, 35x (30 sec 95 °C, 30 sec 57 °C, 30 sec 72 °C), 5 min 72 °C, quick cooling to room temperature

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: DNA extraction from healthy potato material (max. 100 mg)
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: DNA extraction from *S. endobioticum* (e.g. pathotype 1) infected potato wart material (max. 100 mg).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of undiluted DNA extracted from *S. endobioticum* (e.g. pathotype 1) infected potato wart material (max. 100 mg).

3.2. Interpretation of results

In order to assign results from this test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of 472 bp

When these conditions are met:

- A test will be considered positive if amplicons of 472 bp are produced
- A test will be considered negative, if it produces no band or a band of a different size
- Tests should be repeated if any contradictory or unclear results are obtained

Appendix 2. Detection of *S. endobioticum* in potato wart material using real-time PCR

1. General Information

1.1. Detection of *S. endobioticum* in potato warts using real-time PCR developed by van Gent-Pelzer *et al*. (2010)

1.2. The test is designed to amplify 84 bp of the internal transcribed spacer 2 (ITS2) sequence of *S. endobioticum* and 79 bp of the cytochrome oxidase subunit 1 (COX) of plant DNA as internal control.

1.3. Forward primer SendoITS2F (5'-TTTTTACGCTCACTTTTTTTAGAATGTT-3'); reverse primer SendoITS2R (5'-CTGCCTCACACACCACATACA-3') Sendo probe2 (5'-AATTCGAGTTTGTCAAAAGGTGTTTGTTGTGG-3') FAM label and Eclipse Dark Quencher (EDQ); forward primer COX F (5'-CGTCGCATTCCAGATTATCCA-3'); reverse primer COX RW (5'- CAACTACGGATATATAAGRRCCRRAACTG-3'); probe COXSOL 1511T (5'-AGGGCATTCCATCCAGCGTAAGCA–3) Yakima Yellow label and Black Hole Quencher 1 (BHQ1).

1.4. Amplification is performed in a real-time PCR thermal cycler with heated lid, e.g. CFX96 (Bio-Rad).

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Potato wart material (max. 100 mg) is extracted using a modified Plant Tissue mini protocol from the DNeasy® Plant Mini Kit (Qiagen). See also section 2.1 of appendix 1.

2.1.2. After DNA extraction, no additional DNA clean-up is required. Either use extracted DNA immediately or store it at –20 °C until use.

2.2. Real-time PCR van Gent-Pelzer *et al*. (2010)

2.2.1. Two simplex reactions are prepared; one for *S. endobioticum* detection, and one for amplification of the Plant COX gene as internal control.

2.2.2. Master Mix real-time PCR *S. endobioticum* detection

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	10.25&	N.A.
2x Premix Ex Taq (TaKaRa)	2x	15.0	1x
ROX Reference Dye/Dye II (TaKaRa)		Use when needed #	
SendoITS2F	(10 µM)	0.75	250 nM
SendoITS2R	(10 µM)	0.75	250 nM
Sendo probe2	(10 µM)	0.25	83 nM
Subtotal		27.0	
Genomic DNA extract		3.0	
Total		30.0	

* Molecular grade water should be used. Alternatively sterile (autoclaved or 0.45 µm filtered), purified (deionised or distilled) and nuclease-free water can be used.

& The Molecular Grade Water volume is reduced to 9.65 µL when ROX Reference Dye/Dye II is used.

The ROX Reference Dye/Dye II is used for normalization of the fluorescent signal when working with Applied Biosystems (AB) real-time PCR instruments. For AB 7000/7700/7900HT and 7300 Real-Time PCR Systems, use 0.6 µL ROX Reference Dye (50x), final concentration 1x. For the AB 7500 Real-Time PCR System use 0.6 µL ROX Reference Dye II (50x), final concentration 1x. When the ROX Reference Dye or Dye II is used, reduce the volume of molecular grade water with 0.6 µL per reaction.

2.2.3. Master Mix real-time PCR for plant DNA amplification

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	10.5&	N.A.
2x Premix Ex Taq (TaKaRa)	2x	15.0	1x
ROX Reference Dye/Dye II (TaKaRa)		Use when needed #	
COX F	(10 µM)	0.6	200 nM
COX RW	(10 µM)	0.6	200 nM
COXSOL 1511T	(10 µM)	0.3	100 nM
Subtotal		27.0	
Genomic DNA extract		3.0	
Total		30.0	

* Molecular grade water should be used. Alternatively sterile (autoclaved or 0.45 µm filtered), purified (deionised or distilled) and nuclease-free water can be used.
& The Molecular Grade Water volume is reduced to 9.9 µL when ROX Reference Dye/Dye II is used.
# The ROX Reference Dye/Dye II is used for normalization of the fluorescent signal when working with Applied Biosystems (AB) real-time PCR instruments. For AB 7000/7700/7900HT and 7300 Real-Time PCR Systems, use 0.6 µL ROX Reference Dye (50x), final concentration 1x. For the AB 7500 Real-Time PCR System use 0.6 µL ROX Reference Dye II (50x), final concentration 1x. When the ROX Reference Dye or Dye II is used, reduce the volume of molecular grade water with 0.6 µL per reaction.

2.2.4. PCR conditions: 95 ºC for 10 min, 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included. These are used for each series of nucleic acid extraction and amplification of the target organism:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: DNA extraction from healthy potato material (max. 100 mg)
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: DNA extraction from *S. endobioticum* (e.g. pathotype 1) infected potato wart material (max. 100 mg).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Two positive amplification controls (PAC1 and PAC2) to monitor the efficiency of the amplification: amplification of undiluted and 100x diluted DNA extracted from *S. endobioticum* (e.g. pathotype 1) infected potato wart material (max. 100 mg).

In addition to the external positive controls (PIC, PAC1 and PAC2), an internal positive isolation control is used to monitor each individual sample separately (specific amplification of plant COX gene).

3.2. Interpretation of results

The cycle cut off value for both *S. endobioticum* and the Plant COX gene is set at 40, and was obtained using the equipment, materials and chemistry used as described in this appendix. When necessary the Ct cut off value should be determined for the required control.

The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time. To assign results from real-time PCR-based tests the following criteria should be followed:

Verification of the controls

- NAC should be negative (Ct > cut off) for both *S. endobioticum* and the Plant COX gene.
- NIC should be negative (Ct > cut off) for *S. endobioticum*, and for the Plant COX gene produce an exponential amplification curve with a Ct value below the cut off value.
- PIC, PAC1 and PAC2 should produce an exponential amplification curve, and a Ct value below the cut off value for both *S. endobioticum* and the Plant COX gene.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve, and a Ct value below the cut off value for *S. endobioticum*.
- A test will be considered negative, if it produces no exponential amplification curve and/or a Ct value equal or above the cut off value for *S. endobioticum*, and for the Plant COX gene an exponential amplification curve, and a Ct value below the cut off value.
- Tests should be repeated if any contradictory or unclear results are obtained.

Appendix 3. Identification of *S. endobioticum* pathotype 1 in potato wart material using real-time PCR

1. General Information

1.1. Detection of *S. endobioticum* in potato warts using real-time PCR developed by Bonants *et al*. (unpublished) and optimised by HLB, Wijster, the Netherlands.

1.2. The test is designed to amplify 122 bp of a region containing a pathotype 1(P1) versus non-pathotype 1(non-P1) specific single nucleotide polymorphism (SNP) identified by CroPs analysis. Both *S. endobioticum* P1 and non-P1 strains can be identified in a duplex reaction. In addition, 79 bp of the cytochrome oxidase subunit 1 (COX) of plant DNA can be amplified as internal control.

1.3. Forward primer FW_P1+2b (5'-xxx-3'); reverse primer RV_P1+1 (5'-xxx-3'); Probe_F1_P1VIC (5'-xxx-3') VIC label en xx quencher; Probe F18_SHFAM (5'-xxx-3') FAM label en xxx quencher; forward primer COX F (5'-CGTCGCATTCCAGATTA TCCA-3'); reverse primer COX RW (5'-CAACTACGGATATATAAGRRCCRRAA CTG-3'); probe COXSOL 1511T (5'-AGGGCATTCCATCCAGCGTAAGCA–3) Yakima Yellow label and Black Hole Quencher 1 (BHQ1)

1.4. Amplification is performed in a real-time PCR thermal cycler with heated lid, e.g. CFX96 (Bio-Rad).

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Potato wart material (max. 100 mg) is extracted using a modified Plant Tissue mini protocol from the DNeasy® Plant Mini Kit (Qiagen). See also section 2.1 of appendix 1.

2.1.2. After DNA extraction, no additional DNA clean-up is required. Either use extracted DNA immediately or store it at –20 °C until use.

2.2. Real-time PCR Bonants *et al*. (unpublished)

2.2.1. One duplex and one simplex reactions is prepared; one for *S. endobioticum* P1 and non-P1 identification, and one for amplification of the Plant COX gene as internal control.

2.2.2. Master Mix real-time PCR *S. endobioticum* P1 and non-P1 identification

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	9.75&	N.A.
2x Premix Ex Taq (TaKaRa)	2x	15.0	1x
ROX Reference Dye/Dye II (TaKaRa)		Use when needed #	
FW_P1+2b	(10 µM)	0.75	250 nM
RV_P1+1	(10 µM)	0.75	250 nM
Probe_F1_P1VIC	(10 µM)	0.25	83 nM
Probe F18_SHFAM	(10 µM)	0.5	166 nM
Subtotal		27.0	
Genomic DNA extract		3.0	
Total		30.0	

* Molecular grade water should be used. Alternatively sterile (autoclaved or 0.45 µm filtered), purified (deionised or distilled) and nuclease-free water can be used.

& The Molecular Grade Water volume is reduced to 9.15 µL when ROX Reference Dye/Dye II is used.

The ROX Reference Dye/Dye II is used for normalization of the fluorescent signal when working with Applied Biosystems (AB) real-time PCR instruments. For AB 7000/7700/7900HT and 7300 Real-Time PCR Systems, use 0.6 µL ROX Reference Dye (50x), final concentration 1x. For the AB 7500 Real-Time PCR System use 0.6 µL ROX Reference Dye II (50x), final concentration 1x. When the ROX Reference Dye or Dye II is used, reduce the volume of molecular grade water with 0.6 µL per reaction.

2.2.3. Master Mix real-time PCR for plant DNA amplification

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	10.5&	N.A.
2x Premix Ex Taq (TaKaRa)	2x	15.0	1x
ROX Reference Dye/Dye II (TaKaRa)		Use when needed #	
COX F	(10 µM)	0.6	200 nM
COX RW	(10 µM)	0.6	200 nM
COXSOL 1511T	(10 µM)	0.3	100 nM
Subtotal		27.0	
Genomic DNA extract		3.0	
Total		30.0	

* Molecular grade water should be used. Alternatively sterile (autoclaved or 0.45 µm filtered), purified (deionised or distilled) and nuclease-free water can be used.
& The Molecular Grade Water volume is reduced to 9.9 µL when ROX Reference Dye/Dye II is used.
# The ROX Reference Dye/Dye II is used for normalization of the fluorescent signal when working with Applied Biosystems (AB) real-time PCR instruments. For AB 7000/7700/7900HT and 7300 Real-Time PCR Systems, use 0.6 µL ROX Reference Dye (50x), final concentration 1x. For the AB 7500 Real-Time PCR System use 0.6 µL ROX Reference Dye II (50x), final concentration 1x. When the ROX Reference Dye or Dye II is used, reduce the volume of molecular grade water with 0.6 µL per reaction.

2.2.4. PCR conditions: 95 ºC for 10 min, 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included. These are used for each series of nucleic acid extraction and amplification of the target organism:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: DNA extraction from healthy potato material (max. 100 mg)
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: DNA extraction from *S. endobioticum* (e.g. pathotype 1) infected potato wart material (max. 100 mg).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Two positive amplification controls (PAC1 and PAC2) to monitor the efficiency of the amplification of *S. endobioticum* pathotype 1 (P1): amplification of undiluted and 100x diluted DNA extracted from *S. endobioticum* P1 infected potato wart material (max. 100 mg).
- Two positive amplification controls (PAC1 and PAC2) to monitor the efficiency of the amplification of non-pathotype 1 *S. endobioticum* strains (non-P1): amplification of undiluted and 100x diluted DNA extracted from non-P1 *S. endobioticum* (e.g. pathotype 2) infected potato wart material (max. 100 mg).

In addition to the external positive controls (PIC, PAC1 and PAC2), an internal positive isolation control is used to monitor each individual sample separately (specific amplification of plant COX gene).

3.2. Interpretation of results

The cycle cut off value for both *S. endobioticum* P1, non-P1 and the Plant COX gene is set at 40, and was obtained using the equipment, materials and chemistry used as described in this appendix.

The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time. To assign results from real-time PCR-based tests the following criteria should be followed:

Adjusting threshold settings

- Use PAC1 and PAC2 *S. endobioticum* P1 and non-P1 to determine the threshold setting for probe Probe_F1_P1VIC for each real-time PCR run. Non-P1 strains give a false positive VIC signal without producing exponential amplification curves. Increase the VIC threshold setting so that the false positive non-P1 signal does not exceed the threshold.

Verification of the controls

- NAC should be negative (Ct > cut off) for both *S. endobioticum* P1, non-P1 and the Plant COX gene.
- NIC should be negative (Ct > cut off) for both *S. endobioticum* P1, non-P1 and produce an exponential amplification curve, and a Ct value below the cut off value for the Plant COX gene.
- PAC1 and PAC2 *S. endobioticum* P1 should produce an amplification curve, and a Ct value below the cut off value for both *S. endobioticum* P1 (VIC) and the Plant COX gene. Please note that P1 strains can give a false positive FAM signal (non-P1 probe).
- PAC1 and PAC2 *S. endobioticum* non-P1 should produce an exponential amplification curve, and a Ct value below the cut off value for both *S. endobioticum* non-P1 (FAM) and the Plant COX gene. The VIC signal should be negative (Ct > cut off and/or no exponential amplification curve)
- PIC should produce an exponential amplification curve, and a Ct value below the cut off value according to the pathotype used.

When these conditions are met:

- A test will be considered positive for *S. endobioticum* P1 if it produces an amplification curve, and a Ct value below the cut off value for *S. endobioticum* P1 (VIC) . Please note that P1 strains can give a false positive FAM signal.
- A test will be considered positive for *S. endobioticum* non-P1 if it produces an amplification curve, and a Ct value below the cut off value for *S. endobioticum* non-P1 (FAM).
- A test will be considered negative, if it produces no exponential amplification curve and/or a Ct value equal or above the cut off value for *S. endobioticum* P1 and non P1, and for the Plant COX gene an exponential amplification curve, and a Ct value below the cut off value.
- Tests should be repeated if any contradictory or unclear results are obtained.

Appendix 4. TPS checklist

What	When	Check
Order TPS material to be provided by participant	Before start of TPS	
Did you receive the package intact? If not, contact coördinators	Upon arrival TPS package	
Place items provided at the correct temperature	Upon arrival TPS package	
Provide endorsement stamp for the transport document (LoA) of the return sample	Upon arrival TPS package	
Send the return sample (with transport document) to NPPO-NL using the return envelope	Upon arrival TPS package – after providing LoA endorsement stamp	
DNA extraction of 10 unknown samples + 2 internal controls using DNeasy Plant mini kit (Qiagen), appendix 1	mid-June - end of August	
Performing conventional PCR described by Levesque *et al.* (2001) using GoTaq® Flexi DNA Polymerase (Promega), appendix 1	mid-June - end of August	
Performing real-time PCR described by van Gent-Pelzer *et al.* (2010) using Premix Ex Taq™ DNA Polymerase (TaKaRa), appendix 2	mid-June - end of August	
Performing real-time PCR described by Bonants *et al.* (unpublished) using Premix Ex Taq™ DNA Polymerase (TaKaRa), appendix 3	mid-June - end of August	
DNA extraction of back-up sample set	only when: 1. DNA extraction on original sample set failed 2. DNA extraction using an alternative DNA extraction kit (*optional when extraction of original set was successful but very much appreciated)*	
Performing conventional and real-time PCR assays with alternative reagents	*optional but very much appreciated*	
Report results using the provided Excel file	before 30 August 2013	