A Rapid, Sensitive, and Cost-Efficient Assay to Estimate Viability of Potato Cyst Nematodes

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Accepted for publication 20 September 2011.

ABSTRACT

van den Elsen, S., Ave, M., Schoenmakers, N., Landeweert, R., Bakker, J., and Helder, J. 2012. A rapid, sensitive, and cost-efficient assay to estimate viability of potato cyst nematodes. *Phytopathology* 102:140-146.

Potato cyst nematodes (PCNs) are quarantine organisms, and they belong to the economically most relevant pathogens of potato worldwide. Methodologies to assess the viability of their cysts, which can contain 200 to 500 eggs protected by the hardened cuticle of a dead female, are either time and labor intensive or lack robustness. We present a robust and cost-efficient viability assay based on loss of membrane integrity upon death. This assay uses trehalose, a disaccharide present at a high concen-

tration in the perivitelline fluid of PCN eggs, as a viability marker. Although this assay can detect a single viable egg, the limit of detection for regular field samples was higher, ≈ 10 viable eggs, due to background signals produced by other soil components. On the basis of 30 nonviable PCN samples from The Netherlands, a threshold level was defined ($\Delta A_{\text{trehalose}} = 0.0094$) below which the presence of >10 viable eggs is highly unlikely (true for $\approx 99.7\%$ of the observations). This assay can easily be combined with a subsequent DNA-based species determination. The presence of trehalose is a general phenomenon among cyst nematodes; therefore, this method can probably be used for (for example) soybean, sugar beet, and cereal cyst nematodes as well.

Routine detection of specific plant pathogens in plant and soil samples is currently shifting from morphology-based inspection toward identification by means of specific DNA sequence signatures. The use of DNA for identification has several advantages; it is life stage independent, large and numerous plant or soil tissue samples can be handled in a short period of time (within days instead of weeks or months; lower chance of false negatives, especially in the case of low infestation levels), and these routine analyses can be done by people without a thorough classical phytopathological background. Downsides of this relatively novel approach are the costs of consumables and equipment.

A potential pitfall of DNA sequence-signature-based pathogen identification is related to the stability of this polymer. Contrary to RNA, for instance, DNA is a relatively stable molecule. Keeping in mind that both phyllosphere and soil are biodiverse, densely inhabited, and competitive habitats, any unprotected food source such as dead bacteria, fungi, or nematodes will be degraded in a relatively short time span. In one of the very few papers on this topic, MacMillan et al. (19) determined the half life of DNA from a nematode (infective Dauer larvae of *Phasmahabditis hermaphrodita*) in a sandy loam soil at ≈ 2.5 days. This could be different in the case of protected structures such as encysted nematodes. Here, we focus on the viability of cysts from the potato cyst nematodes (PCNs) *Globodera pallida* and *G. rostochiensis*. Being oligophagous obligate parasites of seasonal Solanaceous plant species such as tuber-bearing *Solanum* spp., they have to survive for a period of at least a few months in the absence of living host plants. PCNs have solved this problem by cyst formation. A cyst is a spherical or lemon-shaped, semipermeable holder that contains up to a few hundred nematode eggs. These eggs are surrounded by a chitinous cell wall. Within the

eggs, first- or second-stage juveniles (J1 or J2) will survive in a partly dehydrated condition because they are surrounded by a perivitelline fluid containing ≈ 0.34 M trehalose (24). Trehalose (α -D-glucopyranosyl- α -D-glucopyranoside) is a nonreducing disaccharide that, at high concentrations, allows for the desiccation of the nematode while preserving its membrane integrity. Trehalose is a very stable disaccharide; no degradation was observed upon heating a 4% (wt/vol) solution to 100°C for 24 h at pH 3.5 to 10 (11).

Trehalose-mediated cryptobiotic life stages are not unique among cyst nematodes; they have been documented for numerous other nematode species of genera *Tardigrades*, *Rotifers*, and *Crustacea* (23). In the case of PCNs, hatching occurs (once diapause is broken) upon exposure to host plant-released eclepins (20), and is preceded by a loss of solutes (including trehalose) from the perivitelline fluid (7).

Although unhatched J2s encapsulated in cysts can survive in soil for a prolonged period, each year a part will die because of spontaneous hatching (hatching in absence of a host plant) and another part will die by starvation or other causes such as fungal attack (in-egg mortality). The natural decline of PCN populations was estimated to be ≈ 50 to 60% for the first year (8,9); in the second year (keeping the field site fallow for another year), the viability of the remaining eggs decreased by $\approx 40\%$ (9). In a field experiment in Ireland with *G. rostochiensis*, spontaneous hatch accounted for $\approx 80\%$ of the mortality, whereas $\approx 20\%$ could be attributed to in-egg mortality (9).

Around the world, both species of PCN have quarantine status. In many countries, seed potato may be grown only if the foreseen growing area does not contain detectable levels of viable *G. pallida* or *G. rostochiensis* cysts, or, more accurately, cysts containing at least one viable, unhatched juvenile that is—upon hatching—able to infect a susceptible host. Increasingly, DNA-based detection tests are applied in cyst sample analyses for statutory preplant surveys. PCN-derived DNA signals show that living nematodes are present, or were present in the recent past.

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<http://dx.doi.org/10.1094/PHYTO-02-11-0051>
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Especially in the case of in-egg mortality, it is conceivable that PCN DNA will still be detectable even though the nematodes inside are nonviable. To reduce the risk that samples on the basis of their DNA content test positive when they are, in fact, nonviable, DNA-based analysis of cyst samples of PCNs should be combined with a viability test.

Exposure to potato (or tomato) root leachate is an accepted method to check for egg viability (2). However, this test is too labor intensive and takes too much time for routine analyses (see Materials and Methods). Therefore, service laboratories assess the viability of crushed cysts on the basis of the morphology of nonhatched J2s (occasionally, viability stains such as MTT-formazan are used; 14). Standardization of these microscopic determinations has been proven to be difficult. High sample throughput in service laboratories (10 to 100 × 10³ samples each year) requires a more robust, objective, and preferably automatable test for rapid analysis of soil samples on PCN cyst viability. Here, we present an assay that uses trehalose as a viability marker. It is sensitive, robust, cost-efficient, and fast, and can easily be combined with a subsequent polymerase chain reaction (PCR) for species determination.

MATERIALS AND METHODS

PCN populations. Populations of *G. rostochiensis* (R11-7A) and *G. pallida* (D383 and Rookmaker [Rook 1-2b-Pa3]) were reared on the susceptible potato “Eigenheimer” in a greenhouse at 18°C and 16-h day length. Cysts were collected ≈3 months post inoculation, air dried, and kept at 4°C for at least 6 months (to lift diapause). Thereafter, hatchability was determined upon incubation in potato root diffusate (PRD; 15). Batches of fully viable cysts were stored at –80°C (10). A fully viable cyst is defined here by the hatchability of that cyst immediately after lifting diapause, given that it was grown under optimal conditions on a fully susceptible host.

In addition, cysts from *G. pallida* populations E400 1042, P 35 L1040, P 58 L1040, and P 59 L1040 were kindly supplied by Dr. T. van der Lee (Plant Research International, Wageningen, The Netherlands). Viability of these *G. pallida* populations was quantified as described above.

Nonviable PCN batches were kindly supplied by NAK Emmeloord (The Netherlands). These cysts were selected from soil samples collected from potato-growing areas in The Netherlands in the framework of statutory preplant surveys. On the basis of microscopic inspection as described in the European and Mediterranean Plant Protection Organization (EPPO) guideline PM 7/40 (2), three categories of nonviable cyst material were distinguished by the supplier: empty cysts, cysts with unidentifiable content, and cysts with dead larvae.

Essentially trehalose-free, nonviable cysts required as biological background for trehalose detection experiments were prepared by heating (99°C) ≈600 cysts from *G. pallida* population Rookmaker (Rook 1-2b-Pa3) in 50 ml of MilliQ water for 30 min. Subsequently, the supernatant was removed and replaced by another 50 ml of MilliQ water. After 2 days (at room temperature), the supernatant was removed again, and the batch of nonviable cysts was rinsed another two times with 50 ml of MilliQ. Subsequently, hand-picked dead PCN cysts were (i) used in an experiment to test the amplifiability of DNA extracted from dead cysts upon exposure to soil and (ii) mixed with viable cysts or eggs in the ratios required for testing.

Trehalose quantification. Single or multiple cysts, eggs, or both were transferred to a 0.2-ml PCR tube containing 10 µl of water (MilliQ quality). Trehalose was extracted from the nematode eggs by incubating the cysts for 30 min at 99°C using a thermocycler (MJ Biorad) or a Thermomixer (Eppendorf, Hamburg, Germany) (or similar heating device). For the crushing of cysts, a pestle was prepared by flaming the end of a 200-µl pipette

tip. After trehalose extraction from cysts by heating or crushing, samples were cooled down to room temperature and immediately processed further or stored at –20°C.

A detection kit (K-TREH 01/09 from Megazyme International Ireland Ltd., Wicklow, Ireland) was used to quantify the trehalose content of PCN cysts. In essence, trehalose is hydrolyzed to two units of D-glucose by trehalase. At the recommended pH 5.5, this trehalase of prokaryotic origin is active up to 40°C. D-glucose is phosphorylated by hexokinase in the presence of ATP. The resulting glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase and NADP⁺ to gluconate-6-phosphate, NADPH, and H⁺. Subsequently, the NADPH concentration is measured spectrophotometrically at λ = 340 nm. A NanoDrop 1000 (Wilmington, DE) was used to quantify the increase in absorbance (it can be replaced by any other spectrophotometer).

To each of the sample extracts (volume ≈10 µl), 12 µl of MilliQ water, 2 µl of imidazole buffer (2 M, pH 7.0) with 100 mM MgCl₂, 1 µl of a NADP⁺/ATP solution (12.5 + 36.7 µg/µl), and 0.2 µl of hexokinase and glucose-6-phosphate dehydrogenase (0.425 and 0.212 U/µl, respectively) were added. Samples were vortexed and cleared by centrifugation at 12,000 rpm for a few seconds. After ≈5 min, the absorbance at λ = 340 nm was measured (A₁). Subsequently, 1 µl of trehalase solution was added (0.490 U/µl) and the solution was vortexed again, followed by centrifugation for a few seconds at 12,000 rpm. After incubation for at least 5 min at room temperature, the absorbance of the supernatant was measured at 340 nm using a NanoDrop (A₂).

DNA extraction and ribosomal DNA amplification. In order to test whether the proposed trehalose-based viability assay is compatible with DNA-based species identification and, if so, whether a viability assay should precede or follow a species determination, cysts were crushed by use of homemade pestles that were prepared by flaming the end of a 200-µl pipette tip. Lysis was performed by adding an equal volume (mostly 10 µl) of lysis buffer containing β-mercaptoethanol (1% [vol/vol]), and proteinase-K (800 µg/ml) as described by Holterman et al. (12). Lysis took place in a Thermomixer (Eppendorf) at 65°C and 750 rpm for 2 h, followed by 5 min of incubation at 100°C. Lysate was used immediately or stored at –20°C. Before PCR, cyst lysate was diluted 100 times with MilliQ water. PCR was performed on 3 µl of (diluted) template in a total reaction volume of 25 µl. Species-specific PCR was essentially performed as described in EPPO guideline PM 7/40 (2), protocol D (2). This protocol exploits species-specific motives in the large-subunit ribosomal DNA. Amplification products (2 µl) were run on a 1.5% agarose gel stained with GelStar (2 µl per 100 ml) (Lonza, Rockland). To assess the size of the PCR products, 1 µl of a 1-kb Plus DNA Ladder (Invitrogen, Carlsbad, CA) was included.

Amplifiability of DNA extracted from dead cysts. To test for changes in the amplifiability of DNA extracted from dead PCN cysts (killed by heating in water, see section “PCN populations”) over time upon exposure to sandy soil from an agricultural field nearby Wageningen, individual cysts were buried in petri dish (9 cm, eight cysts per dish). To facilitate recollection of cysts, they were individually glued to the tip of a hair. At several time points (between 0 and 51 days after burying of the cysts in sandy soil), cysts were recollected and tested for amplifiable DNA as described above. Petri dishes with soil and cysts were kept at 18°C in the dark. Dishes were weighed every week, and loss in weight due to water evaporation was compensated for by the addition of an equivalent volume of tap water.

RESULTS

Extraction of trehalose from PCN cysts. Unhatched PCN juveniles are surrounded by a physically strong, chitinous egg wall, and several approaches were tested to quantitatively extract trehalose from cysts. We tested single treatments or combinations

of two of the following treatments: crushing, microwave, heating, and combination of heating and shaking (Table 1). Combinations 1 and 3 resulted in the highest trehalose concentrations in combination with limited variation between cysts. Combination 2 gave high trehalose concentrations per cyst but the consistency among individual cysts was low. This may have been due to crushing, a manual treatment that is difficult to standardize. When combinations 3 and 4 were compared, the same phenomenon was observed. Treatment 5 (microwave) resulted in barely any signal. In combination 6, the detected trehalose could be attributed fully to the crushing of the PCN cysts. Combination 7 showed the effect of incubation of cysts in water at room temperature; the trehalose values did not significantly differ from zero. Combination 1 (30 min of shaking at 99°C in water) resulted in the most

efficient and consistent extraction of trehalose from PCN cysts; therefore, it was selected as the preferred method for further experimentation.

Trehalose levels in PCN populations with various viabilities.

For five *G. pallida* populations, a comparison was made between hatchability values determined in a regular hatching assay and the viabilities as suggested by the trehalose quantities extracted from individual cysts (Table 2A and B). *G. pallida* population D383 was arbitrarily selected as a fully viable reference population. Apart from the viability, the quantity of trehalose released from a single cyst depends on the number of eggs it contains. Because the number of eggs per cyst may vary considerably (3), the quantity of trehalose extracted from a fully viable cyst may vary as well. This natural variation explains the >100% viability value

TABLE 1. Comparison of methods for the extraction of trehalose from single potato cyst nematode cysts (*Globodera pallida*)^a

Treatment	Extraction method						
	1	2	3	4	5	6	7
Crushing	–	+	–	+	–	+	...
Microwave (700W, 1 min)	...	...	...	...	+	+	...
Heating (30 min at 99°C)	...	...	+	+	...	...	...
Heating and shaking (30 min at 99°C, 900 rpm)	+	+	...	...	...	...	...
Average trehalose content (µg) per cyst (<i>n</i> = 3)	2.01	1.46	1.95	1.96	0.03	1.86	0.03
	$\sigma_{n-1} = 0.025$	$\sigma_{n-1} = 0.900$	$\sigma_{n-1} = 0.012$	$\sigma_{n-1} = 0.112$	$\sigma_{n-1} = 0.044$	$\sigma_{n-1} = 0.030$	$\sigma_{n-1} = 0.017$

^a Methods 1, 3, and 5 are single treatments, whereas methods 2, 4, and 6 consist of a combination of two treatments (method 7 is the untreated control).

TABLE 2. Comparison between a conventional and trehalose-based viability test of cysts from the potato cyst nematode (PCN) *Globodera pallida*^a

A. PCN population	Viability (hatching assay) (%)	<i>n</i>	Cyst size		$(A_2 - A_1) - \Delta A_{\text{blank}}^b$ = $\Delta A_{\text{trehalose}}$	[Trehalose] (μg/μl) ^c	Trehalose/ cyst (μg) ^d	Average viability (%)	
D383	100	10	Small		0.3476 ^{Avg} (0.062)	0.0945 ^{Avg} (0.017)	2.47 ^{Avg} (0.44)	–	
	100	10	Medium		0.3781 ^{Avg} (0.016)	0.1027 ^{Avg} (0.004)	2.67 ^{Avg} (0.11)	–	
	100	10	Large		0.3760 ^{Avg} (0.009)	0.1036 ^{Avg} (0.003)	2.72 ^{Avg} (0.07)	–	
Total	–	30	–		–	–	2.62 ^{Avg} (0.28)	100	
Blank ^b	–	4	–		0.0013 ^{Avg} (0.0015)	–	–	–	
Inactivated trehalase ^c	–	7	–		–0.0014 ^{Avg} (0.0022)	–	–	–	
B. PCN population	Viability (hatching assay) (%)	<i>n</i>	Cyst	A ₁	A ₂	$(A_2 - A_1) - \Delta A_{\text{blank}}^b$ = $\Delta A_{\text{trehalose}}$	[Trehalose] (μg/μl) ^c	Trehalose/ cyst (μg) ^d	Average viability (%)
E400 1042	100	1	1	0.052	0.482	0.432	0.117	3.07	116
	...	...	2	0.048	0.473	0.427	0.116	3.04	
	...	...	3	0.050	0.475	0.427	0.116	3.04	
	...	...	4	0.042	0.464	0.424	0.115	3.02	
P 58 L1040	50	1	1	0.035	0.456	0.423	0.115	3.01	46
	...	...	2	0.031	0.030	0.001	0.000	0.01	
	...	...	3	0.025	0.259	0.236	0.064	1.68	
	...	...	4	0.024	0.025	0.003	0.001	0.02	
P 35 L1040	30	1	1	0.023	0.026	0.005	0.001	0.04	27
	...	...	2	0.022	0.020	0.000	0.000	0.00	
	...	...	3	0.037	0.420	0.385	0.105	2.74	
	...	...	4	0.024	0.023	0.001	0.000	0.01	
P 59 L1040	0	1	1	0.025	0.022	–0.001	0.000	–0.01	0
	...	...	2	0.023	0.021	0.000	0.000	0.00	
	...	...	3	0.024	0.020	–0.002	–0.001	–0.01	
	...	...	4	0.031	0.024	–0.005	–0.001	–0.04	
Blank ^b	–	–	–	0.018	0.016	–0.002	–	–	–

^a For one population (D383), trehalose content was determined for 30 individual cysts (three size classes). E400 1042, another fully viable population, was included to show possible population effects. P58 L1040, P35 L1040, and P59 L1040 show various degrees of reduced viability using single cysts for trehalose determinations).

^b $\Delta A_{\text{blank}} = A_2 - A_1$ (complete test without cyst). Avg. = average and numbers in parentheses = standard deviation.

^c [Trehalose] = $\Delta A_{\text{trehalose}} \times (\text{MW}_{\text{trehalose}} / \epsilon \times D \times 2)$ (in µg/µl). Avg. = average and numbers in parentheses = standard deviation.

^d Trehalose per cyst = [trehalose] µg/µl $\times$ 26.2 (in µg). Avg. = average and numbers in parentheses = standard deviation.

^e Trehalase inactivated by 15 min of incubation at 95°C.

for cysts from population E400 1042. For moderately viable populations such as P58 L1040 and P35 L1040, striking differences in the trehalose contents of individual cysts were observed (Table 2B). It suggests that such a population is a mixture of nonviable

TABLE 3. Trehalose contents of cysts and cyst-like material scored as nonviable^a

Sample ID ^b	Sample volume (μl)	$\Delta A_{\text{trehalose}}^c$
A1	72	0.003
A2	72	0.000
A3	65	0.001
A4	62	0.001
A5	45	0.000
A6	42	0.000
A7	66	-0.001
A8	74.5	0.002
A9	65	-0.001
A10	71.5	-0.003
B1	86.5	0.005
B2	116	0.003
B3	79.5	0.000
B4	118	0.003
B5	100	0.005
B6	22.5	0.005
B7	140	0.001
B8	64	0.004
B9	60	0.003
B10	97	-0.005
C1	110	-0.001
C2	57	0.000
C3	70.5	0.001
C4	54	0.000
C5	54.5	-0.001
C6	85	0.002
C7	72.5	0.005
C8	24.5	-0.003
C9	67.5	0.001
C10	115	0.007

^a Samples were randomly taken in early spring 2010 from statutory preplant surveys throughout The Netherlands. Upon crushing of cysts and cyst-like material, three categories were distinguished: empty cysts (A), cysts with unidentifiable content (B), and cysts with dead larvae (C). The condition of this degraded cyst material did not allow for a species determination anymore. Samples were kindly supplied by NAK Emmeloord (The Netherlands).

^b $\bar{x}$ (category A) 0.000, σ_{n-1} = 0.002, $\bar{x}$ (category B) 0.002, σ_{n-1} = 0.003, and $\bar{x}$ (category C) 0.001, σ_{n-1} = 0.003.

^c $\Delta A_{\text{trehalose}} = (A_2 - A_1) - \Delta A_{\text{blank}}$.

and viable cysts (and not a homogeneous population of cysts with intermediately viabilities). However, the number of observations was too low to draw any firm conclusion. It is noted that the viability values for PCN populations as determined by trehalose measurements do not deviate substantially from the viabilities established on the basis of hatchability.

Trehalose levels in nonviable natural PCN samples. For practical, often quarantine status-related purposes, PCN viability tests are used qualitatively (= presence or absence of infectious nematodes). To determine a threshold above which a trehalose signal is pointing at the presence of viable PCN cysts, three categories of nonviable cyst and cyst-like samples were screened. All samples were selected from statutory preplant surveys in potato-growing areas throughout The Netherlands. The condition of this (partially) degraded cyst material made it impossible to perform a species determination. The average $\Delta A_{\text{trehalose}}$ was 0.000 for empty cysts (n = 10), 0.003 for cysts with a nonidentifiable content (n = 10), and 0.003 for cysts with dead larvae (n = 10) (Table 3). Standard deviation within each of the categories was 0.002 to 0.003. The highest $\Delta A_{\text{trehalose}}$ value for nonviable PCN material was 0.007 (category C, sample 10). A Shapiro-Wilk test was performed to test whether these 30 samples represent a continuum of natural cyst degradation (cysts with dead larvae → cysts with a nonidentifiable content → empty cysts) that came from a normally distributed population. This test of normality gave a P value of 0.570 with an α level of 0.05. It was concluded that the data do not deviate from a normally distributed population. Given that $\approx 99.7\%$ of a normal distribution lies within three standard deviations of the mean (x = 0.0012, σ = 0.00273), we propose a threshold value for $\Delta A_{\text{trehalose}}$ of 0.0094.

Detection threshold of the trehalose-based viability assay. In order to verify whether a trehalose-based viability assay can be used for the detection of low quantities of viable (= infectious) PCN cysts or eggs as such or against a background of nonviable cysts, four series of samples were created. Series 1 (1, 5, and 25 viable cysts) gave a clear trehalose signal pointing at the presence of viable PCN eggs (Table 4). It is noted that the small extraction volume (10 μl) hinders a proper quantitative extraction of trehalose. The second series showed that a single viable cyst in a background of 24 nonviable cysts can be detected without any problem. In the third series, the trehalose signals from 1 to 20 viable PCN eggs were determined. It was shown that 10 or 20 viable egg produced a trehalose signal above the viability threshold defined at $\Delta A_{\text{trehalose}}$ = 0.0094. Single PCN eggs produced small

TABLE 4. Detection of trehalose in cysts or eggs of the potato cyst nematode (PCN) *Globodera pallida* (Rookmaker)^a

Series	Number of			A_1	A_2	ΔA	Viable PCN cysts or eggs ^b
	Viable cysts	Nonviable cysts	Viable eggs				
1	25			0.343	0.509	0.166	P
	5			0.074	0.400	0.326	P
	1			0.108	0.365	0.257	P
2	10	15		0.152	0.411	0.259	P
	5	20		0.149	0.418	0.269	P
	1	24		0.062	0.267	0.205	P
3			20	0.018	0.039	0.021	P
			10	0.017	0.037	0.020	P
			1	0.016	0.022	0.006	(P)
			1	0.017	0.019	0.002	(P)
			1	0.015	0.022	0.007	(P)
			1	0.016	0.021	0.005	(P)
			1	0.016	0.021	0.005	(P)
4		25	20	0.052	0.075	0.023	P
		25	10	0.054	0.071	0.017	P
Blank 1				0.017	0.017	0.000	
Blank 2				0.018	0.018	0.000	

^a Absorbance (A) refers to absorbance at a wavelength of 340 nm. Samples with a ΔA < 0.0094 were scored as not detected. P indicates presence of viable cyst or eggs as ΔA exceeds threshold value. $\Delta A_{\text{trehalose}} = (A_2 - A_1) - \Delta A_{\text{blank}}$.

^b (P) indicates that, although single eggs consistently gave small but consistent trehalose signals, they did not exceed the threshold. This is explained by the relatively clean nature of eggs samples as compared to the field samples analyzed in Table 3.

but consistent trehalose signals, but these signals did not reach the threshold level. It is noted that the threshold was defined on the basis of field samples with considerable background noise. Such noise was substantially reduced in the case of isolated cysts. In series 4, viable eggs were mixed with 25 nonviable cysts, and also here the resulting $\Delta A_{\text{trehalose}}$ were above the viability threshold (Table 4). Single eggs were not included in this series, because they were not expected to produce a signal above the threshold. In principle (i.e., when single isolated eggs are assayed), the newly developed trehalose-based viability assay is sufficiently sensitive to detect a single viable egg. However, in the case of field samples, the detection limit will be ≈ 10 viable PCN eggs in a background of 25 nonviable PCN cysts.

Compatibility of the trehalose-based viability assay with PCN species determination. If a viability assay points at the presence of viable PCN cysts, it could be relevant to perform a DNA-based species determination consecutively. To test the compatibility of both assays, an experiment was performed with fully viable cysts from a *G. rostochiensis* (R11-7A) and a *G. pallida* population (D383). Initial treatment of single cysts with or without crushing (CR) and with or without lysis buffer (LB) resulted in either the expected trehalose signal without any PCR product (treatments –CR, –LB and +CR, –LB) or with the expected PCR products in combination with a nonfunctioning trehalose assay (treatment +CR, +LB) (Table 5; Fig. 1A). It is con-

cluded that both crushing and lysis buffer are required for the release of PCR amplifiable DNA, whereas the presence of LB is noncompatible with trehalose determination. However, when LB is added after trehalose measurement, a proper PCR product is amplified (Fig. 1B). Crushing of the cyst is a necessary treatment for DNA extraction. However, this experiment shows that it does not matter whether the crushing takes place before (samples 9 to 12) or after (samples 1 to 4) trehalose determination (Table 5; Fig. 1). PCR primers were designed on species-specific motives in the LSU rDNA (2); the size of the main amplification products was ≈ 450 bp for *G. rostochiensis* and ≈ 600 bp for *G. pallida*.

Amplifiability of DNA from dead PCN cysts. To see whether amplifiable DNA could be extracted from dead intact cysts from the PCN *G. pallida* upon exposure to a natural agricultural field, dead cysts buried in a petri dish filled with sandy soil were recollected at several time points. This qualitative experiment showed clearly that, even after 51 days direct exposure to soil, some cysts allowed for the amplification of DNA fragment of ≈ 670 bp (Fig. 2). It is concluded that, in this case of artificial in-egg mortality (i.e., killing of unhatched, encysted juveniles by incubation in water at 99°C), the speed of DNA degradation is retarded substantially compared with the degradation of DNA from dead nematodes directly exposed to soil (e.g., Dauer larvae of *P. hermaphrodita*, as mentioned above).

TABLE 5. Compatibility of trehalose viability test with DNA-based species determination for the potato cyst nematode (PCN) species *Globodera rostochiensis* and *G. pallida*^a

Number	PCN populations ^b	CR or LB	$(A_2 - A_1) - \Delta A_{\text{blank}} = \Delta A_{\text{trehalose}}^c$	[Trehalose] ($\mu\text{g}/\mu\text{l}$) ^d	Trehalose/cyst (μg) ^e	PCR product ^f	Compatibility of trehalose assay and DNA amplification
1	R11-7A	– CR – LB (30 min at 99°C)	0.335	0.091	2.38	A 1–2	Trehalose: + DNA: –
2			0.364	0.099	2.59		
3	D383		0.249	0.068	1.77	A 3–4	
4			0.109	0.030	0.78		
	Follow up treatment	CR and LB after trehalose measurement				B 1–2 B 3–4	DNA: + Compatible
5	R11-7A	– CR + LB (30 min at 99°C)	–0.015	–0.004	–0.11	A 5–6	Trehalose: – DNA: –
6			–0.001	0.000	–0.01		
7	D383		–0.057	–0.015	–0.41	A 7–8	
8			–0.050	–0.014	–0.36		
	Follow up treatment	CR and LB (first LB was heat inactivated) after trehalose measurement				B 5–6 B 7–8	DNA: + Not Compatible
9	R11-7A	+ CR – LB (30 min at 99°C)	0.378	0.103	2.69	A 9–10	Trehalose: + DNA: –
10			0.343	0.093	2.44		
11	D383		0.263	0.071	1.87	A 11–12	
12			0.361	0.098	2.57		
	Follow up treatment	LB after trehalose measurement				B 9–10 B 11–12	DNA: + Compatible
13	R11-7A	+ CR + LB (30 min at 99°C)	–0.003	–0.001	–0.02	A 13–14	Trehalose: – DNA: +
14			–0.017	–0.005	–0.12		
15	D383		–0.027	–0.007	–0.19	A 15–16	
16			–0.010	–0.003	–0.07		
	Follow up treatment	None				B 13–14 B 15–16	DNA: + Not Compatible
	Blank ^d		$\Delta A_{\text{blank}} = 0.002$				

^a A combination of crushing (CR) and exposure to nematode lysis buffer (LB; 12) is required for DNA extraction. Various treatments and treatment orders were tested (in duplicate).

^b Fully viable PCN populations: *G. rostochiensis* R11-7A and *G. pallida* D383.

^c $\Delta A_{\text{blank}} = A_2 - A_1$ (complete test without cyst).

^d [Trehalose] = $\Delta A_{\text{trehalose}} \times (\text{MW}_{\text{trehalose}} / \epsilon \times D \times 2)$ (in $\mu\text{g}/\mu\text{l}$).

^e Trehalose per cyst = [trehalose] $\mu\text{g}/\mu\text{l} \times 26.2$ (in μg).

^f PCR = polymerase chain reaction (Fig. 1).

DISCUSSION

In parallel with changes in veterinary and humane diagnostics, routine detection of plant pathogens is inevitably shifting toward DNA-based detection. A potential disadvantage of the use of DNA for plant pathogen detection is the stability of this polymer. Even a nonviable pathogen could, for some time, give rise to a positive signal in DNA-based detection assays. Because most pathogens live in highly competitive environments, DNA degradation after death will usually be a matter of hours or days. However, this might be different when pathogens are protected by a reinforced cell wall or the equivalent; physically strong and hard-to-degrade layers surrounding the pathogen in some specialized life stages (e.g., to survive temporary absence of host plants). In those cases, there could be a considerable time lag between viability of the pathogen and DNA detectability. Hence, on particular occasions, it could be useful to accompany a DNA-based detection assay with a viability test.

In the past, a range of viability stains has been tested on both pathogenic and non-plant-pathogenic nematodes. Boyd (4) used an aqueous solution of iodine and potassium iodide to stain dead second-stage larvae of *G. rostochiensis*. For free-living nematode genera *Diplogasteroides* and *Diplogasteritus*, eosin-Y dye was employed to identify dead individuals (6). Upon testing a number of stains on eight nematode genera (four plant-parasitic and four nonparasitic), Ogiga and Estey (21) selected two dyes, Meldola Blue and Nile Blue A, as being the more dependable stains for the identification of dead nematodes. However, apart from the labor-intensive nature of these assays, none of these stains are suitable for routine use because there clearly is an element of subjectivity in these stain-based viability assessments.

Various viability tests based on DNA stains or other dyes have been developed for bacterial and fungal pathogens. In the case of the chytrid fungus *Batrachochytrium dendrobatidis*—a fungus implicated in the global decline of amphibians—a combination of

two fluorescent DNA stains (namely, SYBR14 and propidium iodide) was tested on zoospores. Motile (viable) zoospores stained green, whereas nonviable zoospores turned red (22). Another dye used for viability assays is fluorescein diacetate (FDA). This apolar molecule diffuses across the plasma membrane, and intracellular esterase activity results in the release of optically active fluorescein (13). FDA-based vitality assays depend on the permeability of the cellular structures under investigation. Fungal spore coats are not necessarily permeable to FDA. In the case of teliospores from *Tilletia controversa*—the causal agent of dwarf bunt in wheat—Chastain and King (5) had to use extracts of teliospores (instead of intact spores).

In most instances, techniques involving viability stains require subsequent microscopic analysis. As an alternative, dyes such as ethidium bromide monoazide (EMA; 18) that block the PCR reaction could be used to detect dead propagules. However, the application of this dye for viability tests is not straightforward. Kobayashi et al. (17) tested this dye to discriminate between viable and dead bacterial cells of *Staphylococcus aureus* and *S. epidermidis*. For this particular case, EMA was shown to be an unreliable viability agent.

Dye-independent approaches, for example, make use of the intrinsic instability of mRNA. Kaucner and Stinear (16) were among the first to apply this principle to assess the viability of *Giardia* cysts and *Cryptosporidium parvum* oocysts (two protozoan parasites) in fresh-water samples. The same principle was exploited to estimate the viability of the opportunistic fungal pathogen *Cryptococcus neoformans*. Here, both the primers and the internal control were based in the capsular CAP10 gen mRNA. Although this methodology was shown to be specific and sensitive for clinical specimens (1), this application requires relatively expensive materials.

In the case of cysts from PCNs, current viability tests are time consuming and labor intensive (hatching assays; see Materials and Methods) or insufficiently robust (microscopic inspection of crushed cysts or eggs; see Materials and Methods). To avoid any accessibility problem, we set out to develop a viability assay without the use of a dye or molecular probe, yet making use of the loss of selective permeability of membranes upon death. This implies that any measurable component with a steep concentration gradient across the membrane (highest concentration at the inside) could potentially be used as a viability marker. In

Fig. 1. Compatibility of a trehalose-based viability test with potato cyst nematode species determination. **A and B.** Amplification products resulting from a polymerase chain reaction (PCR) using large subunit ribosomal DNA-based species-specific primers. **A.** Samples 1 to 16 as described in Table 5 as used in the trehalose assay. **B.** The same samples after a follow-up treatment as described in Table 5. On the extreme left and right, a 1-kb Plus DNA Ladder was included. Gr: *Globodera rostochiensis*, Gp: *Globodera pallida*. Size of the amplification products appears to differ slightly between A and B, caused by the type of DNA stain used. GelStar, a highly sensitive stain, affects migration of PCR products in a concentration-dependent manner.

Fig. 2. Amplification of a 674-bp fragment from the large subunit of the ribosomal DNA from *Globodera pallida* cysts upon heating in water for 30 min at 99°C followed by incubation in a sandy soil taken from a local agricultural field. Samples 1 to 6: six randomly taken fully viable cysts (control). Samples 7 to 12: six randomly taken cysts just after heat treatment. Samples 13 to 18: six individual cysts recollected after 22 days of direct exposure to soil. Samples 19 to 20: two individual cysts recollected after 51 days of direct exposure to soil.

the particular case of PCNs, we choose trehalose because of its very high concentration in the perivitelline fluid. Detection of the disaccharide involves only two steps: its hydrolysis into two glucose molecules and, subsequently, the detection of glucose.

Given the small, economical margins in arable farming, the cost-efficiency aspect of this new assay could be relevant (in our hands, US\$0.04 to 0.07 per sample). It is noted that the total reaction volume for trehalose detection in our experiments remained low ($\approx 25 \mu\text{l}$). This reaction volume can be upscaled according to number of cysts analyzed. Depending on the working volume, a single trehalose detection kit can be used for 1,000 to 10,000 viability tests. To the best of our knowledge, this is the most cost-efficient and straightforward viability assay for plant-parasitic nematodes reported so far. We wish to underline that the threshold value for PCN viability as given in this article ($\Delta A_{\text{trehalose}} = 0.0094$; below this value the presence of >10 viable eggs [1/20 to 1/50 of a single cyst] is highly unlikely) was established on the basis of Dutch PCN populations scored as nonviable. The confidence level of 99.7% was chosen by the authors of this article and does not necessarily reflect a degree of certainty that is required for statutory purposes. If desired, the detection threshold of this assay could be optimized further by reducing the background signal produced by non-target soil components (e.g., by sample purification). Implementation of this methodology should be preceded by the analysis of local PCN populations. In combination with the desired confidence level, this would result in a new threshold value that might deviate from the value presented here.

Presumably, this viability assay can be used as well for other high-impact cyst nematode species such as *Heterodera glycines*, *H. schachtii*, or *H. avenae*. It is conceivable that this principle—exploiting the loss of selective permeability of membranes upon death—can also be used to determine the viability of survival propagules of other plant pathogens and animal parasites.

ACKNOWLEDGMENTS

We thank the Dutch FES program “Strengthening Plant Health Infrastructure”, subprogram: “Plant pathogen viability” for financial support; the NAK Emmeloord and Plant Research International (Bio-interactions unit) for their kind supply of well-characterized batches of viable and nonviable PCNs; and A. Hakman and P. van Haperen—both M.Sc. student at Wageningen University—for their help in the experiment on DNA survival in soil.

LITERATURE CITED

- Amjad, M., Kfoury, N., Cha, R., and Mobarak, R. 2004. Quantification and assessment of viability of *Cryptococcus neoformans* by LightCycler amplification of capsule gene mRNA. J. Med. Microbiol. 53:1201-1206.
- Anonymous. 2009. *Globodera rostochiensis* and *Globodera pallida*. EPPO Bull. 39:354-368.
- Arntzen, F. K., Mulder, J. G., and Visser, J. H. M. 1994. Rapid and non-destructive assessment of the number of eggs in cysts of potato cyst nematodes by weighing. Fundam. Appl. Nematol. 17:299-302.
- Boyd, A. E. W. 1941. Determination of death in the larvae of the potato root eelworm. Nature 148:782.
- Chastain, T. G., and King, B. 1990. A biochemical method for estimating viability of teliospores of *Tilletia controversa*. Phytopathology 80:474-476.
- Chaudhuri, N., Dick, R. I., Engelbrecht, R. S., and Austin, J. H. 1966. Staining of free-living nematodes by eosin-Y dye. Nematologica 12:337-342.
- Clarke, A. J., Perry, R. N., and Hennessy, J. 1978. Osmotic stress and the hatching of *Globodera rostochiensis*. Nematologica 24:384-392.
- Den Ouden, H. 1960. Periodicity in spontaneous hatching of *Heterodera rostochiensis* in soil. Nematologica (Suppl.) II:101-105.
- Devine, K. J., Dunne, C., O’Gara, F., and Jones, P. W. 1999. The influence of in-egg mortality and spontaneous hatching on the decline of *Globodera rostochiensis* during crop rotation in the absence of the host potato crop in the field. Nematology 1:637-645.
- Folkertsma, R. T., Helder, J., Gommers, F. J., and Bakker, J. 1997. Storage of potato cyst nematodes at -80°C . Fundam. Appl. Nematol. 20:299-302.
- Higashiyama, T. 2002. Novel functions and applications of trehalose. Pure Appl. Chem. 74:1263-1269.
- Holterman, M., van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J., and Helder, J. 2006. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades. Mol. Biol. Evol. 23:1792-1800.
- Hoppe, R. W., and Bavister, B. D. 1984. Evaluation of the fluorescein diacetate (FDA) vital dye viability test with hamster and bovine embryos. Anim. Reprod. Sci. 6:323-335.
- James, C. E., and Davey, M. W. 2007. A rapid colorimetric assay for the quantitation of the viability of free-living larvae of nematodes in vitro. Parasitol. Res. 101:975-980.
- Janssen, R., Bakker, J., and Gommers, F. J. 1987. Circumventing the diapause of potato cyst nematodes. Neth. J. Plant Pathol. 93:107-113.
- Kaucner, C., and Stinear, T. 1998. Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. Appl. Environ. Microbiol. 64:1743-1749.
- Kobayashi, H., Oethinger, M., Tuohy, M. J., Hall, G. S., and Bauer, T. W. 2009. Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermidis* by ethidium bromide monoazide. Lett. Appl. Microbiol. 48:633-638.
- Lee, J. L., and Levin, R. E. 2006. Use of ethidium bromide monoazide for quantification of viable and dead mixed bacterial flora from fish fillets by polymerase chain reaction. J. Microbiol. Methods 67:456-462.
- MacMillan, K., Blok, V., Young, I., Crawford, J., and Wilson, M. J. 2006. Quantification of the slug parasitic nematode *Phasmarhabditis hermanni* from soil samples using real time qPCR. Int. J. Parasitol. 36:1453-1461.
- Mulder, J. G., Diepenhorst, P., Plieger, P., and Brueggemann-Rotgans, I. E. M. 1996. Hatching agent for the potato cyst nematode C. P. I. Luxan. The Netherlands. Patent US 5585505 (A).
- Ogiga, I. R., and Estey, R. H. 1974. The use of Meldola’s blue and Nile blue a, for distinguishing dead from live nematodes. Nematologica 20:271-276.
- Stockwell, M. P., Clulow, J., and Mahony, M. J. 2010. Efficacy of SYBR 14/propidium iodide viability stain for the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. Dis. Aquat. Org. 88:177-181.
- Watanabe, M. 2006. Anhydrobiosis in invertebrates. Appl. Entomol. Zool. 41:15-31.
- Womersley, C., and Smith, L. 1981. Anhydrobiosis in nematodes I. The role of glycerol, myo-inositol and trehalose during desiccation. Comp. Biochem. Physiol. 70B:579-586.