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Evaluation of Fluorescent Stains for Viability Assessment of the Potato Cyst Nematodes *Globodera pallida* and *G. ellingtonae*

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Abstract

Potato cyst nematodes (PCNs), *Globodera pallida* and *Globodera rostochiensis*, are quarantine pests of potato which cause significant damage to production and farm gate revenue worldwide. Accurately assessing viability of PCN eggs is important for eradication and management programs. The goal of this study was to develop a quick and reliable fluorescent staining method to evaluate viability of *G. pallida* and *Globodera ellingtonae* eggs. The staining efficiency of eight fluorescent stains was evaluated using *G. pallida* eggs compared with the conventional Meldola's Blue (MB) staining method. The staining efficiency of the fluorescent stains ranged from 80.33 ± 2.99 (Sytox Green) to 100% (Acridine Orange) for non-viable eggs. Two stains were further evaluated for their efficiency in assessing viability of encysted eggs from five different greenhouse-reared *G. pallida* cyst sources which contained both viable and non-viable eggs. For the *G. pallida* cyst sources, viability ofencysted eggs were estimated to be 41.02 ± 3.81 to 62.66% ± 3.12% when stained with Acridine Orange (AO) and 79.52% ± 1.54% viability for *G. ellingtonae*. Both staining time and stain concentration were significant for staining efficiency of released and encysted eggs. Staining time and concentration were optimized for released eggs at 4 h at 10 µg/ml and for encysted eggs at 16 h at 25 µg/ml respectively for AO. Fluorescent stains accurately and rapidly assessed percent egg viability and were determined to be as sensitive as a seven-day incubation with the Meldola's Blue staining method.

Keywords

Globodera pallida, *Globodera ellingtonae*, Nematode Viability Assessment, Fluorescent Stains, Meldola Blue

1. Introduction

Biotrophic cyst nematodes are amongst the most economically damaging pests to cropping systems worldwide [1]. The potato cyst nematodes (PCNs) *Globodera pallida* (Stone) Behrens and *Globodera rostochiensis* (Wollenweber) Behrens have been estimated to cause 9% potato yield loss in the UK [2] [3]. Both *G. pallida and G. rostochiensis* originated in South America and spreaded to nearly all major potato growing regions of the world. Because of the ease of spread, and economic importance, potato cyst nematodes are listed as quarantine pests and subjected to strict regulation in many countries [4] [5].

In the United States, *G. pallida* was discovered in Idaho in 2006 [6] and is a regulated pest by the United States Department of Agriculture-Animal and Plant Health Inspection Services (USDA-APHIS) and by the Idaho State Department of Agriculture (ISDA) [7] [8]. A current map of the infestation in Idaho is available through the USDA-APHIS website: https://www.aphis.usda.gov/aphis/ourfocus/planthealth/plant-pest-and-disease-programs/pests-and-diseases/nematode/pcn). Phytosanitary regulations restrict potato cultivation or any other host crop in an infested field [7]. It takes only two cysts from two different soil samples with one of the cysts containing viable PCN eggs for the field to be considered infested. Infested fields are eligible to have some restrictions lifted when no viable eggs are no longer detected by the Meldola's Blue viability staining method and potato bioassays detect no reproduction of PCN [7]. One challenge for *G. pallida* control is the ability of encysted eggs to remain viable in soil for long periods (over 20 years) of time, and their capacity to infect potato after a long quiescent stage [9]-[11] thus making control by crop rotation difficult.

Quantifying viability of *G. pallida* eggs is important for both effective management, as well as eradication programs. Unfortunately, no efficient methods except time consuming and labor-intensive staining and microscopic evaluation of nematodes are available for this purpose. The conventional method of viability assessment of PCN is performed by using vital stains, such as Meldola's Blue and Nile Blue A [12]-[14]. The Meldola's Blue method is recommended by the Canada and United States guidelines on surveillance and phytosanitary actions for the *G. pallida* and *G. rosctochiensis* and also by the European Plant Protection Organization (EPPO) for routine assays of population densities from soil samples [4] [7]. But this method requires 7 days for adequately staining the eggs. Besides the use of vital stains, *in vitro* egg hatching assays [15] are also used for assessing egg viability, but this method does not account for unhatched eggs that may be viable but in diapause [16].

Fluorescent dyes are seldom used for assessing nematode egg viability, but they are extensively used for precisely diagnosing cell viability and cell deaths [17]-[20]. Both cell permeant and non-permeant fluorescent dyes are available for assessing cell viability and differential staining of live and dead cells. The cell permeant dyes can freely diffuse through the membranes of live cells and the

non-permeant fluorescent dyes can only diffuse through compromised cell membranes [21]. The fluorescent dyes offer higher photo stability and brightness compared to non-fluorescent stains and do not require prolonged incubation time [22]. For nematodes, the success of staining methods depends on the stain permeability through its cuticle and lipoprotein membrane [12] [23]. The permeability is compromised by age, environmental factors such as temperature, chemical nematicides, nematode pathogens etc. [24]. In an earlier attempt, Perry and Feil [25] used the fluorescent dye, acridine orange (AO) to determine the viability of *G. rostochiensis*, and Twomey *et al.*, [26] used AO as an indicator of egg permeability in *G. rostochiensis* caused by contact with potato root diffusate. Similarly, AO has been used for distinguishing live and dead eggs of *Heterodera glycines* [24] [27] and to study the relationship between juvenile hatching and fluorescence of *H. glycines* eggs [28]. More recently Hajihassani and Dandurand [29] assessed the fluorescent dyes Sytox Green and Sytox Live/Dead green kit staining for viability staining of *G. pallida* for large particle flow cytometry.

In the present study, cell impermeant fluorescent stains were assessed for PCN egg viability. The goal of this study was to develop a fast and reliable fluorescent staining method for the evaluation of PCN egg viability. The effectiveness of the fluorescent stains was compared with the classic Meldola's Blue staining method.

2. Materials and Methods

2.1. Potato Cyst Nematode Cysts

Two *Globodera* species, *G. pallida* and *G. ellingtonae* were used for the study. Five different greenhouse reared cyst sources (A-E) from Idaho population of *G. pallida* reared on two different susceptible potato cultivars Desirée or Russet Burbank were used. Cysts were extracted from the soil by using an elutriator 16 weeks after infestation and planting and the harvested cysts were stored at 4˚C until experimental use [30] [31]. Morphological and molecular species confirmation of the *G. pallida* cyst was conducted [32]. *Globodera ellingtonae* cysts were provided by Dr. Inga Zasada (USDA-ARS, Corvallis, Oregon).

2.2. Fluorescent Stains and Microscopy

Eight cell impermeant fluorescent stains, Acridine Orange (AO), Laser Dye Styryl 751 (LDS 751), SYTO Orange 81, SYTO Orange 82, SYTO Orange 83, SYTO Orange 84, SYTO Orange 85 and Sytox Green were used to assess the PCN egg viability. All fluorescent stains were purchased from Thermo Fisher Scientific, Eugene, Oregon, USA. The vital stain Meldola's Blue (8-dimethylamino-2,3-benzophenoxazine hemi (zinc chloride) salt) used for the evaluation of fluorescent staining method was procured from Spectrum Chemical, New Brunswick, NJ, USA.

Examination of stained eggs was conducted using a Leica DMi8 fluorescent microscope (Leica microsystems CMS GmbH, Wetzlar, Germany) equipped with a metal halide light (Lumen 200 Fluorescence Illumination Systems, Prior

Scientific Inc., Rockland, MA, USA) source. A manual filter turret with filter cubes, 4',6-Diamidino-2-Phenylindole, dihydrochloride (DAPI) (Ex:390/70-DC:455-EM:470), fluorescein isothiocyanate (FITC) (Ex:470/40-DC:495-EM:525/50), Rhodamine (Ex:546/10-DC:560-EM:585/40) and Y5 (Ex:620/60-DC:660-EM:700/76) were used for visualizing different stains with varying fluorescent emission optima. Meldola's Blue stained eggs were analyzed under bright field. Fluorescence data for each stain were calculated and the fluorescent microscope is calibrated with focal check fluorescence microscope test slide #3 (Thermo Fisher Scientific). Photographs were taken in a darkened room at either 5×, 10× or 20× magnification with a Leica DFC 450 digital camera and processed with Leica Application Suite (LAS) software.

2.3. Optimization of Stains for PCN Egg and Cyst Staining

To optimize the staining protocol 20 surface sterilized and presoaked (24 h in water) *G. pallida* cysts were used for each stain. The cysts were transferred to a 2 ml eppendorf tube with 50 µl Barnstead GenPure water (Barnstead GenPure UV-TOC xCAD plus, Thermo electron LRD GmbH, Stockland 3, Germany), crushed using a Tenbroeck all glass tissue homogenizer (Fisher Scientific, USA). The egg suspension was allowed to settle for 5 minutes at room temperature and then centrifuged at 4800 g for 3 minutes at 4˚C using a refrigerated tabletop centrifuge (Eppendorf centrifuge 5810R, Eppendorf AG, 22331, Hamburg, Germany). The supernatant was removed and eggs resuspended in 1.5 ml GenPure water. Four different concentrations of stains, 5, 10, 25 and 50 µg/ml, for AO and LDS 751; and 5, 10, 25 and 50 µM/ml concentration of SYTO Orange 81 - 85 and Sytox Green stains were used. All stain dilutions were made using sterile GenPure water. For the optimization of stains, *G. pallida* eggs were heat killed using a modified method of Christoforo *et al.*, [33]. The freed eggs were heat treated for 2 h at 70˚C using a heating block and subsequently cooled on an ice before staining. For each concentration, 100 µl egg suspensions with an equal volume of staining solution were pipetted into 1.5 ml eppendorf tube and incubated in the dark at room temperature. Evaluation of staining efficiency was conducted at 1, 2, 4 and 8 h incubation times. To remove the residual stain, 50 µl of the egg suspension was centrifuged at 4800 g for 3 minutes and washed with 100 µl of GenPure water. The pelleted eggs were re-suspended in 10 µl of GenPure water and dispensed to a glass slidefor microscopic examination. An average of 65 eggs was counted for each replicate with three replicates per treatment, and the experiment was repeated. Fluorescent egg percentage was calculated by using the formula: % fluorescent eggs = (stained eggs/(stained + non-stained eggs)) × 100.

Two stains, AO and LDS 751 from the above experiment were further evaluated for application in staining eggs from whole *G. pallida* and *G. ellingtonae* cysts. Whole heat killed (cysts were incubated in boiling water for 7 minutes and cooled on an ice bath) cysts from source A were incubated in dark at room tem-

perature for 4, 8, 16 and 24 h in 10, 25 and 50 µg/ml concentration of AO and 25, 50 and 100 µg/ml final concentration of LDS 751. Thirty-six cysts for each treatment were stained and eggs from nine cysts were evaluated for each time interval at each concentration. The cysts were crushed, washed to remove excess stain and released eggs were re-suspended in 50 µl of GenPure water. Three-10 µl aliquots containing about 140 eggs per aliquot for each treatment were dispensed into a glass slide, visualized and scored under the microscope. The experiment was repeated.

2.4. PCN Viability Assessment Using Fluorescent Stains

Five different *G. pallida* and one *G. ellingtonae* cyst source was tested using AO and LDS 751 for viability assessment using the optimized staining parameters. Eggs that remained encysted from cyst sources were fluorescently stained and compared with Meldola's Blue staining assay [13]. Surface sterilized, presoaked cysts from each source were replicated 5 times for each treatment, and the experiment was repeated. After staining, cysts from each replicate were crushed, washed to remove excess stain, re-suspended in 50 ul of GenPure water, and a 10 µl aliquot containing an average of 158 eggs were dispensed and counted. Heat killed cysts from each cyst source were used as the positive control. Viability by Meldola's Blue stain was assessed after 7-day incubation time. Percent egg viability was calculated using the formula: % viability of eggs = (Non-stained Eggs/(Stained eggs + Non-stained eggs)) × 100.

2.5. Statistical Analysis

To evaluate the staining efficiency of the vital fluorescent stains a randomized complete design (RCD) of 9 stains, 4 different optimization times at 4 different concentrations of the stains were analyzed with the generalized linear mixed model (Proc GLIMMIX) procedure of SAS statistical package [34] assuming a binomial distribution for the number of stained eggs and a logit link function. The different stains, stain concentrations and staining times were treated as fixed effects and the significance of main (percent staining) and interaction effect (interaction of stain, concentration, stain* concentration) over time were tested. The level of significance was set for $p \leq 0.05$.

3. Results

3.1. Staining Efficiency of Heat Killed Eggs

All fluorescent stains tested indicated a non-viable egg population of 80% or more, whereas Meldola's Blue indicated an average 51.5% non-viable population. Non-viable eggs intensely fluoresced green when stained with Sytox green or AO (**Figure 1**); red when stained with LDS 751; or orange when stained with the SYTO Orange series. Empty eggs were easily distinguished from intact eggs as the eggshell membrane of an empty egg was stained leaving a hollow transparent eggshell which was easily distinguished under the microscope (**Figure 1**). In our study, non-viable eggs were clearly distinguished from live as the dead eggs

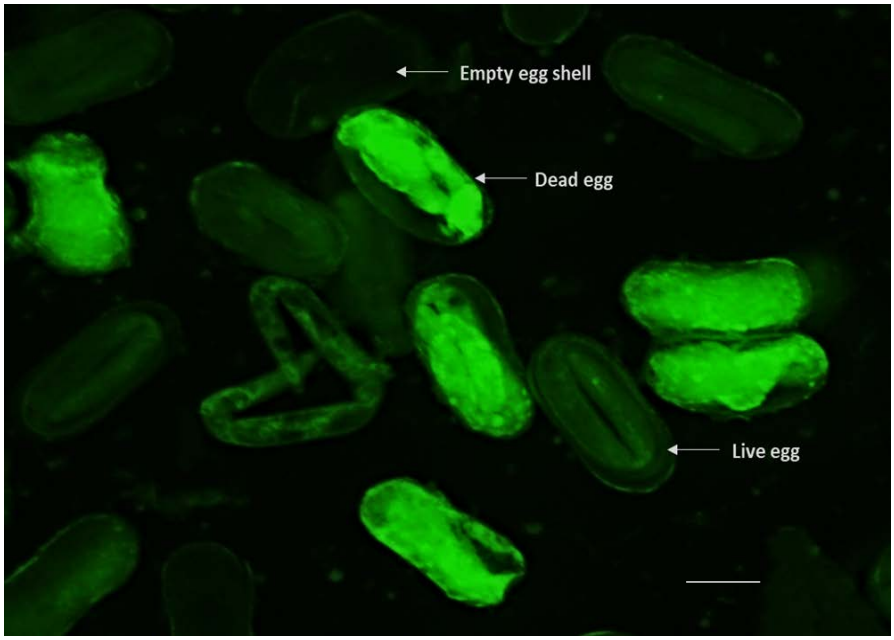

Figure 1. Acridine orange stained *G. pallida* eggs. The dead eggs are characterized by the deeply stained green fluorescent color whereas the live eggs with no intense fluorescence. The live eggs and empty eggs shells were distinguished by the granularity (absence of internal structures for the empty eggshell). Scale bar = 50 μM.

consistently gave a maximum fluorescent detection value of 255 whereas the live eggs have a mean value < 80 using LAS software.

All fluorescent stains required 4 h of staining and no increase in efficiency after 8 h of incubation (Table 1). Among the various stains tested, AO was the most efficient stain, as 87.61% ± 1.12% non-viable eggs were stained after 4 h at the lowest concentration tested (5 μg/ml); and 100% of the non-viable eggs were stained after 4 h at 25 μg/ml concentration (Table 1). When AO was used at higher concentrations (25 & 50 μg/ml), additional washing was required to remove high fluorescence from the background. An average of 98. 45% ± 0.86% of non-viable eggs were stained by LDS 751 (25 μg/ml) in 4 h (Table 1). SYTO Orange series 81 through 85, and Sytox Green stains (50 μM/ml) were less efficient as only 83% - 93% of the non-viable, heat-killed eggs were stained after incubation for 4 h (Table 1). Increased concentration of SYTO stains and incubation time did not increase staining efficiency (Table 1). Figure 2 shows the fluorescence of *G. pallida* eggs and juveniles stained with the fluorescent dyes AO, LDS 751 and SYTO Orange 85 with the control Meldola's Blue stain. The interaction between staining time, concentration and stain*concentration *time was significant ($p \leq 0.0001$). The interaction of concentration of stains and staining time on percent staining of released non-viable *G. pallida* egg is provided as supplementary Table S1 and Table S2.

3.2. Staining Efficiency of Heat Killed Cysts

Efficiency of two stains, AO and LDS 751 was further evaluated to estimate the

Table 1. Percent staining efficiency of eight fluorescent stains and Meldola's Blue on *G. pallida* eggs*.

Stain	Conc.	Staining percent			
		1 h	2 h	4 h	8 h
Acridine Orange	5 µg/ml	80.98 ± 2.02	83.20 ± 1.38	87.61 ± 1.12	87.93 ± 1.10
	10 µg/ml	90.82 ± 0.56	98.24 ± 0.16	99.45 ± 0.39	99.56 ± 0.31
	25 µg/ml	92.84 ± 0.73	98.79 ± 0.45	100.00 ± 00	100.00 ± 00
	50 µg/ml	93.73 ± 0.48	99.05 ± 0.67	100.00 ± 00	100.00 ± 00
LDS 751	5 µg/ml	44.30 ± 2.29	52.19 ± 2.71	61.47 ± 3.51	59.01 ± 2.60
	10 µg/ml	54.62 ± 3.40	80.85 ± 1.63	89.94 ± 2.23	83.63 ± 1.06
	25 µg/ml	64.45 ± 2.31	90.29 ± 0.49	98.93 ± 0.76	97.84 ± 0.94
	50 µg/ml	71.56 ± 2.01	91.45 ± 1.27	98.45 ± 0.86	98.10 ± 1.13
SYTO Orange 81	5 µM	20.94 ± 2.28	24.03 ± 2.38	25.63 ± 4.23	22.06 ± 2.35
	10 µM	33.35 ± 1.92	51.90 ± 2.03	59.09 ± 3.56	54.62 ± 1.71
	25 µM	48.23 ± 2.32	72.69 ± 1.54	81.15 ± 1.75	78.72 ± 1.69
	50 µM	54.80 ± 2.69	76.26 ± 2.56	88.83 ± 1.00	85.90 ± 1.21
SYTO Orange 82	5 µM	21.28 ± 2.08	21.26 ± 2.89	19.76 ± 1.95	20.38 ± 2.54
	10 µM	31.20 ± 2.00	46.65 ± 1.89	56.16 ± 4.09	64.68 ± 1.86
	25 µM	47.02 ± 1.06	70.67 ± 1.64	81.42 ± 1.34	73.73 ± 1.39
	50 µM	55.22 ± 1.22	81.24 ± 1.20	89.92 ± 0.85	85.77 ± 1.54
SYTO Orange 83	5 µM	35.24 ± 2.94	40.64 ± 2.29	39.91 ± 1.60	40.41 ± 1.65
	10 µM	60.08 ± 1.50	72.85 ± 1.60	77.16 ± 1.19	76.79 ± 1.70
	25 µM	65.41 ± 1.97	80.99 ± 0.48	93.34 ± 1.82	92.95 ± 0.92
	50 µM	71.85 ± 3.56	85.33 ± 2.26	93.43 ± 2.00	92.89 ± 1.60
SYTO Orange 84	5 µM	27.76 ± 2.17	27.60 ± 2.39	28.57 ± 2.86	27.57 ± 2.27
	10 µM	41.69 ± 0.52	60.73 ± 1.52	60.47 ± 3.07	63.75 ± 1.69
	25 µM	51.00 ± 3.67	79.69 ± 0.73	85.76 ± 1.81	82.85 ± 1.68
	50 µM	68.08 ± 3.20	82.28 ± 1.07	88.93 ± 1.34	88.92 ± 2.49
SYTO Orange 85	5 µM	55.89 ± 2.02	58.38 ± 3.45	57.43 ± 2.09	59.86 ± 0.79
	10 µM	73.66 ± 1.92	79.22 ± 1.95	82.17 ± 1.58	82.15 ± 0.62
	25 µM	85.36 ± 1.23	88.29 ± 2.61	90.09 ± 2.01	89.20 ± 1.99
	50 µM	86.28 ± 1.72	86.18 ± 0.87	92.28 ± 1.69	90.14 ± 1.72
SYTOX Green	5 µM	34.41 ± 1.62	36.17 ± 2.50	37.65 ± 0.85	39.62 ± 2.87
	10 µM	58.67 ± 0.82	63.99 ± 3.24	66.33 ± 3.10	68.83 ± 3.08
	25 µM	68.48 ± 1.46	76.40 ± 1.84	83.33 ± 1.43	77.80 ± 1.65
	50 µM	73.43 ± 2.25	79.37 ± 1.52	83.23 ± 1.14	80.33 ± 2.99
Meldola's Blue	0.05% w/v	26.04 ± 1.60	33.88 ± 1.77	42.08 ± 1.56	51.62 ± 1.43

*Values are means of two independent experiments with three replicates each followed by standard errors.

Table 2. Staining efficiency of AO and LDS 751 on *G. pallida* cysts*.

Stain	Conc.	Staining percent			
		4 h	8 h	16 h	24 h
Acridine Orange	10 µg/ml	84.06 ± 1.63	91.18 ± 0.86	93.71 ± 1.54	92.88 ± 1.75
	25 µg/ml	90.90 ± 0.88	96.01 ± 1.16	100.00 ± 00	100.00 ± 00
	50 µg/ml	94.03 ± 0.63	99.63 ± 0.26	100.00 ± 00	100.00 ± 00
LDS 751	25 µg/ml	75.22 ± 2.75	83.40 ± 1.72	86.17 ± 2.34	85.95 ± 2.46
	50 µg/ml	79.70 ± 1.31	88.60 ± 1.57	95.94 ± 0.35	95.11 ± 0.85
	100 µg/ml	80.28 ± 1.75	89.30 ± 0.84	96.58 ± 0.99	95.15 ± 0.63
Meldola's Blue	0.05% w/v	41.96 ± 1.21	49.06 ± 2.84	70.04 ± 2.41	76.99 ± 1.95

*Values are means of two independent experiments with three replicates each followed by standard errors.

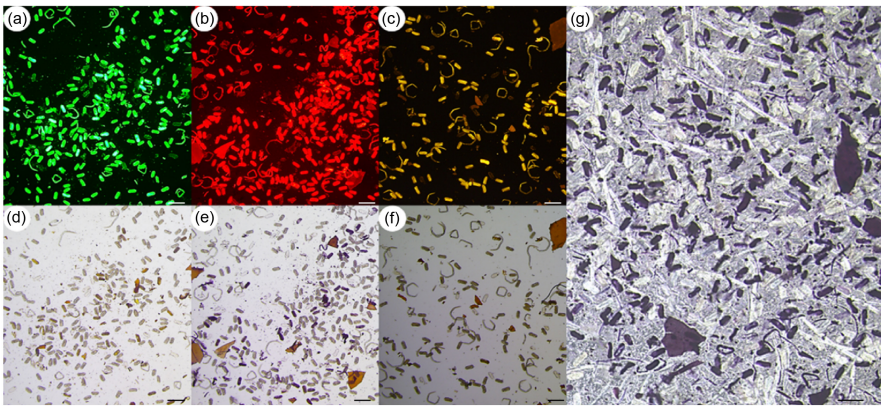

Figure 2. Comparison of fluorescent stains vs Meldola's Blue staining. (a)-(c) are heat killed *G. pallida* eggs stained with AO, LDS 751 and SYTO Orange 85 stains respectively, (d)-(f) are the bright field image of the corresponding eggs and G is the Meldola's Blue stained eggs. The AO gave deep green fluorescence for the dead *G. pallida* eggs while LDS 751 and SYTO Orange series stains gave red and orange fluorescence respectively for the dead eggs. Scale bar = 50 µM.

percentage of non-viable, heat killed *G. pallida* encysted eggs. No difference was found in the efficiency of these two stains whether eggs were released or remained encysted prior to staining. However, a significantly greater percentage of eggs were stained when AO was used compared to LDS 751 or Meldola's Blue. Unlike free eggs, whole cysts required higher concentrations of both AO and LDS 751 (25 µg/ml, and 50 µg/ml respectively). Only 93.7% of the non-viable, heat killed eggs were stained at 10 µg/ml concentration in 16 h but increased to 100% when a 25 µg/ml AO concentration was used. The percent non-viable eggs stained with LDS 751 was 86.1% when using a concentration of 25 µg/ml while increasing the concentration to 50 µg/ml resulted in a 95.9% staining efficiency (Table 2). A significant interaction between staining time, concentration and stain*concentration *time was found ($p \leq 0.0001$) (supplementary Table S3 and Table S4).

Table 3. Comparison of percent viability of 5 *G. pallida* cyst sources and the *G. ellingtonae* assayed using AO, LDS 751 and MB staining methods.

Stain	Conc.	Time	GP Source A	GP Source B	GP Source C	GP Source D	GP Source E	GE
Acridine Orange	25 µg/ml	16 h	41.02 ± 3.81	44.36 ± 3.59	51.46 ± 3.87	57.85 ± 5.14	62.66 ± 3.12	79.52 ± 1.54
LDS 751	50 µg/ml	16 h	47.34 ± 1.30	53.20 ± 1.82	55.53 ± 4.23	61.89 ± 3.85	65.20 ± 3.44	86.25 ± 1.78
Meldola's Blue	0.05% w/v	7 days	39.21 ± 6.24	41.00 ± 1.96	43.58 ± 4.78	51.95 ± 5.62	52.55 ± 4.03	90.10 ± 1.10

*Values are means of two independent experiments with three replicates each followed by standard errors. GP—*G. pallida*, GE—*G. ellingtonae*.

Table 4. Interaction of different *G. pallida* cyst sources and stains on assessing viability.

Viability assessment method	Stain Conc.	Percent viability
Acridine Orange	25 µg/ml	51.47 ± 1.43 a*
LDS 751	50 µg/ml	56.63± 1.43 a
Meldola's Blue	0.05% w/v	45.66 ± 1.43 b

*The data is based on 5 different *G. pallida* cyst sources and means with different letters are significantly different as determined by Tukey's HSD comparison testat $p < 0.05$.

3.3. Comparison of Fluorescent Stains vs Meldola's Blue for Assessing PCN Egg Viability

Viability assays using either AO or LDS 751 were compared to the Meldola's Blue protocol. Egg viability of the five greenhouse reared *G. pallida* cysts sources ranged from 41.0% ± 3.8% (Source A) through 62.3% ± 3.1% (Source E) when stained with AO; 47.3% ± 1.3% through 65.2% ± 3.4% when stains with LDS 751; and 39.2% ± 6.2% to 52.6% ± 4.0% with Meldola's Blue staining (Table 3). The interaction between cyst sources and stain was not significant ($p \geq 0.15$) which indicates that the cyst sources responded similarly to each viability assessment method. However, the type of stain used had a significant effect on the percent viability assessment ($p \leq 0.001$) (Table 4).

4. Discussion

Accurate determination of PCN egg viability is important in estimating the potential crop loss due to a nematode infestation and for appropriate regulatory and/or management actions. Even though several staining methods are in use for the detection of PCN egg viability, use of a vital stain and its visual observation is considered to be the most accurate, reliable and widely accepted method for viability assessment [33]. The current vital stain used for viability assessment of PCN, Meldola's Blue, has an incubation of 7 days for adequate staining of eggs. This is time consuming and can skew in favor of a non-viable population [35]. This method is also unsuccessful for staining aged specimens, typically producing yellow-colored specimens, and in some cases, specimens may be partially stained in either the head area or at the tail end [12]. The eight fluorescent stains examined in this study indicate that the use of fluorescent stains is reliable for PCN egg viability assessment.

Acridine Orange is a powerful stain to measure cells undergoing apoptosis and useful in fixed tissues and cells [36]. Twomey *et al.* [26] used the fluorescent stain AO as an indicator of egg permeability triggered by potato root diffusate in a closely related PCN species *G. rostochiensis*. In the present study, almost 100% of the heat killed *G. pallida* and *G. ellingtonae* eggs were stained by AO which is in agreement with Twomey *et al.* [26]. Staining with AO accurately estimated non-viable eggs for both *G. pallida* and *G. ellingtonae*. Meyer *et al.* [24] reported that AO is useful for distinguishing between viable and non-viable *Heterodera glycines* eggs. LDS 751 stained over 98% of the heat killed eggs within 4 h. LDS 751 undergoes fluorescence enhancement upon binding to nucleic acids but with negligible fluorescent enhancement upon binding to proteins used to discriminate intact and damaged cells. The non-viable eggs with compromised membrane allowed the dye to effectively stain the eggs a deep read color with minimal egg membrane staining. In addition, LDS 751 is used for the differentiation of apoptotic from non-apoptotic cells [37]. This property of the LDS 751 stain can be further exploited for staining an aging population of viable *G. pallida* eggs. Since both AO and LDS 751 are fixable nucleic acid stains, these stains will be more stable with little or no photo bleaching over time.

The SYTO family of fluorescent stains exhibits 450 to 1000 fold fluorescence enhancement upon binding nucleic acids [22] and are extensively used to determine viability/apoptosis of various cell lines [37]-[39]. The comparatively high staining efficiency of the SYTO stains can be exploited for viability assessment of PCN under laboratory conditions. The low cytotoxic effect [39] of the SYTO stains may be an advantage for using in downstream processing, such as hatching assays. However, one disadvantages of the SYTO stains is their sensitivity to storage conditions compared to the other fluorescent stains. In a previous study, Hajihassani and Dandurand [29] used Sytox Green stain for fluorescent staining and sorting of *G. pallida* using flow cytometer observed an increased viability of eggs upon increasing the incubation time. The study also reported a 7% - 14.6% greater viability estimate of *G. pallida* eggs than Meldola's Blue staining. A loss of fluorescence for the SYTO stains subsequent to the initiation of apoptotic cascade is well documented [40] [41]. Self-quenching of SYTO molecules during cell death can result in loss of fluorescence and an overestimation of the viability. Apoptotic chromatic condensation, decrease in the number of SYTO binding sites as the chromatin condensation and/or RNA degradation during apoptosis, alterations in binding of SYTO to mitochondrial DNA and decrease in mitochondrial uptake of SYTO molecules, the loss of plasma membrane integrity during the celldeath can all be possible reasons for the fluorescence loss by SYTO stains [38]-[44].

Results from this study clearly demonstrate that fluorescent stains can reliably assess viability of both *G. pallida* and *G. ellingtonae*. The fluorescent stains assessed required minimal staining time compared to the Meldola's Blue staining. Use of fluorescent stains can be further developed for fluorescence-based high throughput sorting of PCN life stages such as assessing viability of field derived

encysted eggs through use of automated cell imaging systems and/or large particle flow cytometers.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary

Table S1. Effect of concentration of stains on percent staining of released non-viable *G. pallida* eggs.

Stain	5 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml
AO	85.32 ± 1.02^{a}	97.63 ± 0.46^{a}	98.31 ± 0.39^{a}	98.25 ± 0.39^{a}
LDS751	53.83 ± 1.55^{b}	79.38 ± 1.42^{b}	93.93 ± 0.92^{b}	94.49 ± 0.80^{b}
SYTO81	22.82 ± 1.25^{e}	49.62 ± 1.85^{f}	71.48 ± 1.33^{e}	78.45 ± 1.22^{e}
SYTO82	20.67 ± 1.20^{e}	49.57 ± 1.86^{f}	69.25 ± 1.35^{e}	80.15 ± 1.20^{e}
SYTO83	38.98 ± 1.50^{c}	72.13 ± 1.58^{c}	85.87 ± 1.11^{c}	87.35 ± 0.98^{c}
SYTO84	27.86 ± 1.37^{d}	57.22 ± 1.82^{e}	76.72 ± 1.25^{d}	83.36 ± 1.11^{d}
SYTO85	57.78 ± 1.54^{b}	79.43 ± 1.34^{b}	88.08 ± 0.91^{c}	89.08 ± 0.86^{c}
Sytoxgreen	36.95 ± 1.50^{c}	64.64 ± 1.72^{d}	76.90 ± 1.12^{d}	79.35 ± 1.13^{e}
MB	-	-	-	37.87 ± 1.14^{f}

T grouping for stain least squares means (Alpha = 0.05). A single standard concentration (0.05% w/v) of the MB stain is used for the study. LS-means with the same letter are not significantly different.

Table S2. Effect of staining time on percent staining of released non-viable *G. pallida* eggs across the different concentrations.

Time	5 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml
1 h	39.50 ± 1.15^{b}	57.51 ± 1.37^{c}	68.24 ± 1.06^{d}	69.66 ± 0.97^{c}
2 h	42.61 ± 1.17^{ab}	74.15 ± 1.29^{b}	85.14 ± 0.86^{c}	83.69 ± 0.88^{b}
4 h	44.91 ± 1.21^{a}	79.93 ± 1.29^{a}	92.95 ± 0.75^{a}	91.36 ± 0.71^{a}
8 h	44.86 ± 1.22^{a}	79.86 ± 1.31^{a}	90.69 ± 0.79^{b}	90.69 ± 0.73^{a}

T grouping for time least squares means (Alpha = 0.05). LS-means with the same letter are not significantly different.

Table S3. Effect of concentration of stains on percent staining of encysted non-viable *G. pallida* eggs.

Stain	10 µg/ml	25 µg/ml	50 µg/ml
AO	91.11 ± 0.71^{a}	97.86 ± 0.33^{a}	98.66 ± 0.23^{a}
LDS751	83.35 ± 1.08^{b}	91.43 ± 0.63^{b}	92.08 ± 0.56^{b}
MB	-	-	60.81 ± 1.20^{c}

T grouping for stain least squares means (Alpha = 0.05). LS-means with the same letter are not significantly different. A single standard concentration (0.05% w/v) of the MB stain is used for the study.

Table S4. Effect of staining time on percent staining of encysted non-viable *G. pallida* eggs across the different concentrations.

Time	10 µg/ml	25 µg/ml	50 µg/ml
4 h	69.48 ± 1.67^{c}	86.21 ± 1.15^{c}	88.92 ± 0.90^{c}
8 h	78.74 ± 1.39^{b}	92.93 ± 0.76^{b}	96.74 ± 0.60^{b}
24 h	86.10 ± 1.06^{a}	98.30 ± 0.39^{a}	98.38 ± 0.37^{ab}
48 h	86.76 ± 0.99^{a}	98.05 ± 0.43^{a}	98.06 ± 0.43^{b}

T grouping for time least squares means (Alpha = 0.05). LS-means with the same letter are not significantly different.