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Comparison of Meldola's Blue Staining and Hatching Assay with Potato Root Diffusate for Assessment of *Globodera* sp. Egg Viability

Duncan Kroese,[1] Inga A. Zasada,[2] Russell E. Ingham[1]

Abstract: Laboratory-based methods to test egg viability include staining with Meldola's Blue and/or juvenile (J2) hatching assays using potato root diffusate (PRD). These two methods have not been tested under identical conditions to directly compare their assessments of *Globodera* egg viability. Using two bioassay strategies, cysts from a *Globodera* sp. population found in Oregon were subjected to both viability assessment methods. In strategy one, intact cysts were first stained with Meldola's Blue (primary staining) and eggs were then transferred to PRD (secondary hatching). In the second strategy, intact cysts were exposed to PRD (primary hatching) and then unhatched eggs were transferred to Meldola's Blue (secondary staining). Two different cohorts of cysts were evaluated using these experimental strategies: cohort 1 was comprised of cysts produced on potato in the greenhouse that exhibited low hatch when exposed to PRD and cohort 2 consisted of field-collected cysts whose eggs yielded significant hatch when exposed to PRD. Percentage viability was calculated and is expressed as the number of hatched J2 or unstained eggs/total number of eggs within a cyst. With field-produced cysts, primary staining with Meldola's Blue and hatching with PRD produced similar viability estimates, with averages of 74.9% and 76.3%, respectively. In contrast, with greenhouse-produced cysts the two methods yielded much lower and unequal estimates 32.4% to 2.2%, respectively for primary hatching and staining methods. In addition, J2 hatch from unstained (viable) greenhouse-produced eggs was 13.7% after secondary exposure to PRD compared to 61.5% for field-produced eggs. The majority of eggs remaining unhatched after primary exposure to PRD (> 87%) stained with Meldola's Blue regardless of cyst cohort. Staining with Meldola's Blue provided a conservative assessment of egg viability compared to hatch assay with PRD regardless of diapause.
Key words: cyst nematode, egg hatch, *Globodera*, juvenile, Meldola's Blue, method, potato root diffusate.

Determination of egg viability is important when considering the potential threat following *Globodera* cyst detection in the field. Proper determination of egg viability is also a key step in estimating potential crop losses due to the nematode and can guide the implementation of management practices (Back et al., 2004). Without knowing the viability of a population, the potential exists to either underestimate the threat of damage caused by a *Globodera* population or overestimate the viability of a population leading to unnecessary regulatory action or management.

Globodera eggs detected during processing of soil samples are generally assumed to be living unless showing visible signs of decay. This assessment is subject to interpretation by an individual, and therefore will vary between individuals and may not provide an accurate assessment of egg viability. Alternatively, laboratory-based methods that have been developed to test viability include egg staining and/or juvenile (J2) hatching assays using potato root diffusate (PRD) as a hatching stimulus. To our knowledge, side-by-side comparison of these two methods to assess *Globodera* egg viability has not been reported.

The measure of viability in a hatching assay using PRD is the emergence of a J2 from a PRD-treated egg. Though this method seems to provide a very accurate indication of viability at any one moment in time, it can be time consuming due to length of the assay and the need to grow plants to generate PRD. In addition, variability in the chemical composition of PRD has the potential to make comparisons of egg viability over time difficult. Potatoes grown under different environmental conditions certainly have the potential to produce diffusates comprised of different hatching factors at different concentrations, as was shown for aseptically-grown potato plants compared to plants grown in the greenhouse (Devine et al., 1996).

An alternative to using a hatching assay to determine egg viability is to use a staining method. Staining methods are less time and resource intensive as there is no need to produce hatching factors such as PRD. A number of different stains have been used to try to estimate nematode viability including Nile Blue A, phloxine B, and New Blue R (Southey, 1962; Moriarty, 1964; Ogiga and Estey, 1974). One of the most popular and consistent staining methods for assessing *Globodera* egg viability uses Meldola's Blue as the staining agent (Ogiga and Estey, 1974). The Meldola's Blue staining method has been used to assess *Globodera* egg viability when evaluating management practices (Twomey et al., 2000) and when conducting *Globodera* surveys of regulator significance (Rott et al., 2010). In this assay, eggs that do not stain are considered to be viable while stained eggs are not. Since varying degrees of egg staining occurs, the observer must make a judgment call to determine whether an egg is viable or not.

The goal of this study was to compare staining with Meldola's Blue to a hatching assay with PRD to assess *Globodera* egg viability. Specific goals were to determine if: (i) The two methods predict *Globodera* egg viability similarly; (ii) Assessment of egg viability by these two methods is influenced by diapause; (iii) Eggs unstained

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[1]Oregon State University, Department of Botany and Plant Pathology, Cordley 2082, Corvallis, OR 97331, USA
[2]USDA-ARS Horticultural Crops Research Laboratory, 3420 NW Orchard Ave., Corvallis, OR 97330, USA
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E-mail: inga.zasada@ars.usda.gov
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by Meldola's Blue (predicted as viable) hatch when exposed to PRD, and; (iv) Eggs that remain unhatched after exposure to PRD stain when exposed to Meldola's Blue.

Materials and Methods

Two different cohorts of cysts were used: field-produced cysts from prior seasons (considered not to be in diapause and which hatched readily in the presence of PRD) and greenhouse-produced cysts (considered to be in diapause and which exhibited little hatch in the presence of PRD). The egg hatching parameters of each cyst cohort are presented in Table 1. Field-produced cysts were collected from a field in Powell Butte, OR while greenhouse-produced cysts were reared on potato (*Solanum tuberosum*) 'Modoc' in a greenhouse.

Dried soil, 250 g, containing either field or greenhouse produced cysts was placed in 8 by 28 cm nylon bags, nine total per cohort, with the bags being tied closed and placed in a refrigerator at 4 °C. At five, six, and seven months after placement at 4 °C, three bags for each cohort were removed from the refrigerator and cysts were extracted using a USDA cyst extractor (Ayoub, 1980). Cysts were handpicked from washed soil samples and placed in water until used in assays, usually within 24 hrs. All collected cysts within each cyst cohort were placed under a dissecting microscope and cysts that contained eggs and appeared to be undamaged (i.e. round without any large indentations) were preferentially selected for inclusion in the methods comparison. Egg viability was assessed by two methods: hatching assay with PRD (primary hatching) then staining with Meldola's Blue (secondary staining) and staining with Meldola's Blue (primary staining) then hatching assay with PRD (secondary hatching).

Primary hatching assay with PRD and then secondary staining with Meldola's Blue: A 96-well plate assay system modified from Byrne et al. (2001) and Twomey et al. (1995) was used. Individual cysts (N = 13 cysts/cohort/sampling time) were placed into wells containing a 100 µl aliquot of 1:25 v/v PRD : water. PRD was either obtained from Roy Navarre (USDA-ARS, Prosser, WA) or collected from three-week-old potato 'Modoc' plants grown and leached at USDA-ARS, Corvallis, OR according to Widdowson (1958). Assay plates were sealed with parafilm, covered with aluminum foil to protect from light, and incubated at room temperature (~22°C). After seven days, the number of second-stage juveniles (J2) emerging from eggs in each well was enumerated at 40x using an inverted compound microscope (Leica, Wetzlar, Germany). In previous studies with the Oregon *Globodera* population, greater than 80% of J2 hatched from PRD-exposed eggs in a seven-day period (Zasada and Ingham, unpublished data). After counting, each cyst was removed from the well containing PRD using forceps and was placed in a BPI dish under a dissecting microscope. Using forceps and a surgical scalpel, the cyst was then cut open to liberate the remaining eggs. Once opened, the cyst was agitated slightly to remove any large masses and liberate eggs within the cyst. The freed eggs were then backwashed into a 50 ml tube using water and the final volume within the tube was brought up to 10 ml. A 1 ml subsample of this suspension was used to determine the number of eggs/ml at 40x using an inverted compound microscope.

Once the unhatched eggs from the primary hatching assay with PRD were counted, they were allowed to settle overnight in the refrigerator. From the bottom of the settled sample, 950 µl of liquid was removed and moved to a well on a 24-well plate. After all samples had been transferred to the 24-well plate, 50 µl of 1.0% w/v Meldola's Blue stain (Spectrum Chemical Corp., Gardena, CA) was added to each well, bringing the final concentration of stain within the well to 0.05% w/v. The assay plates were sealed with parafilm, covered with aluminum foil, and incubated at room temperature. After seven days, each sample was removed from the 24-well plate using a Pasteur pipette and dispensed over a 500-mesh sieve. Each sample was then rinsed under tap water to remove excess stain and backwashed into a 50 ml tube. Water was added to the tube for a final volume of 10 ml. The number of secondarily stained (nonviable) and unstained (viable) eggs within each sample was determined from a 1 ml sample examined at 40x using an inverted compound microscope.

Primary staining with Meldola's Blue and then secondary hatching assay with PRD: A 96-well assay, with a single cyst per well (N = 13 cysts/cohort/sampling time), was used as described above. Cysts were first exposed to 0.05% w/v Meldola's Blue in 100 µl of water per well. Once all cysts had been selected and placed in stain, the assay plates were sealed with parafilm, covered with aluminum foil, and incubated at room temperature. After seven days, each cyst was removed from the well with forceps and rinsed with water to remove excess stain. The washed cyst was then placed in a BPI dish under a dissecting microscope and cut open using forceps and scalpel. Once opened, the cyst was agitated slightly to remove any large masses and liberate any eggs within the cyst. The freed eggs were then backwashed into a 50 ml tube with water

Table 1. Hatching characteristics of *Globodera* eggs retained in cysts collected from a field at Powell Butte, OR (Field) and from cysts from the Powell Butte population reared on potato 'Modoc' (*Solanum tuberosum*) in the greenhouse (Greenhouse).[a]

	Greenhouse	Field
Egg hatch (%)[b]	5 (0 – 95)	72 (0 – 100)
Maximum number of juveniles emerging from cysts	197	493
Cysts from which any hatch occurred when exposed to potato root diffusate (%)	20	96

[a] N = 45 cysts.
[b] Average egg hatch = # juveniles/(# juveniles + eggs remaining in cysts)*100 after exposure to potato root diffusate for 7 days. Values are means and ranges.

and the final volume brought up to 10 ml. Primary stained and unstained eggs within each sample were determined from a 1 ml subsample at 40x using an inverted compound microscope.

Once all samples from the staining assay were counted, samples were allowed to sit in the refrigerator overnight to settle. The next day, 960 μl of liquid was removed from the bottom of the settled sample and moved to a well on a 24-well plate. After all samples were transferred, 40 μl of full strength PRD was added to each well, bringing the final concentration of PRD in each well to 1:25 v/v. Plates were then sealed with parafilm and the number of unstained eggs and already hatched J2 were determined at 40x using an inverted compound microscope. Plates were then covered with foil and held at room temperature. After seven days, the total number of J2 within each well was determined at 40x using an inverted compound microscope.

Statistical analyses: At each sampling date 13 cysts of each cohort were selected (see above) for inclusion in each method (N = 39). Raw data was analyzed nonparametrically because the assumptions of analysis of variance (normality and homogeneity of variance) were not met. Differences in sampling date and methods were determined using the Kruskal-Wallis and Scheirer-Ray-Hare tests. Data are presented as means ± standard errors (SE). Data were analyzed with the statistical package JMP (SAS Institute, Cary, NC).

Results

Primary egg viability assay comparison and diapauses influence: There was no difference in percentage egg viability within each cyst cohort collected on different sampling dates ($P > 0.10$), therefore the data were combined for analyses (N = 39). There was a significant interaction between cyst cohort and viability assessment method ($P < 0.001$) (Fig. 1). Primary staining with Meldola's Blue and hatching assay with PRD both estimated egg viability similarly in the field cohort ($P = 0.831$). However, egg viability in the greenhouse cohort was estimated to be 30% greater by staining with Meldola's Blue than by the hatching assay with PRD ($P < 0.001$) where less than 3% of PRD-exposed eggs hatched (Fig. 1).

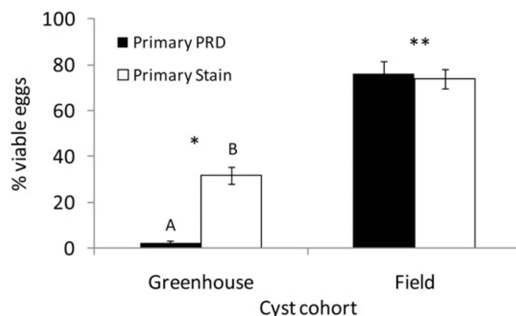

Fig. 1. Comparison of primary staining with Meldola's Blue and primary hatch assay with potato root diffusate (PRD) for assessment of *Globodera* egg viability. Differences between cyst cohorts (*, **) and within the greenhouse cohort (A and B) are significant according to the Scheirer-Ray-Hare test ($P < 0.001$). Each column represents the means ± standard error of N = 39.

Secondary staining with Meldola's Blue: For both cohorts of cysts the majority of eggs that did not hatch when exposed to PRD did stain when subsequently exposed to Meldola's Blue. Of the unhatched PRD-treated eggs from the field cohort 87.6 ± 3.1% were stained after exposure to Meldola's Blue. The percentage of PRD-treated eggs from the greenhouse cohort stained by Meldola's Blue was 97.6 ± 1.4%.

Secondary hatching assay with PRD: For both cyst cohorts, not all eggs remaining unstained after exposure to Meldola's Blue hatched when exposed to PRD (Table 2). This overestimation of viability by staining with Meldola's Blue was less pronounced for the field cohort ($P = 0.047$), in which 18% of unstained eggs did not hatch than for the greenhouse cohort ($P = 0.009$) where 60% of unstained eggs did not hatch. It is interesting to note that the viability of eggs produced by the greenhouse cohort as determined by secondary hatch assay with PRD (13.0%) was higher after primary staining with Meldola's Blue compared to when exposed to PRD alone (2.2%).

Discussion

Regardless of the state of diapause of the *Globodera,* egg staining with Meldola's Blue consistently provided a conservative estimate of egg viability. For older eggs, which were assumed to have passed through diapause, the assessment of egg viability was almost identical with the two methods. For newly produced eggs, the majority of which were assumed to be in diapause, staining with Meldola's Blue always estimated greater egg viability compared to hatch assay with PRD.

Globodera spp. go through a diapause stage in which juvenile development within the egg stops and the egg is left dormant until favorable hatching conditions are

Table 2. Comparison of *Globodera* egg viability by primary staining with Meldola's Blue and secondary hatch of Meldola's Blue-exposed eggs with PRD.

Cyst cohort	Method	
	Primary Staining[a] (% unstained viable eggs)	Secondary Hatching[a] (% J2 hatch)
Field[b]	74.0 ± 4.3 a[c]	61.0 ± 5.0 b
Greenhouse[b]	32.0 ± 5.4 A	13.0 ± 3.0 B

[a] Eggs were first stained with 0.05% w/v Meldola's Blue (primary stain) for one week and stained vs. unstained eggs determined. All eggs from the primary staining assay were then transferred to 1:25 v/v PRD : water for one week (secondary hatching) and the number of J2 hatching from unstained eggs determined.

[b] Field cysts were collected from Powell Butte, OR and greenhouse cysts were produced from the Powell Butte *Globodera* populations inoculated onto potato (*Solanum tuberosum*) 'Modoc'.

[c] Values within a cyst cohort followed by a different letter are significantly different according to Kruskal-Wallis test ($P < 0.001$). Lower case letters are used for the field cohort and upper case letters are used for the greenhouse cohort. Each value represents the means ± standard error of N = 39.

reached (Evans, 1987). Factors that may lead to a break in diapause include photoperiod and light intensity, exposure to a minimum environmental temperature for a certain period of time, or chemical hatching factors (Salazar and Ritter, 1993). Whether an egg is in diapause or not could have a significant influence on its characterization as viable or nonviable according to the tested viability methods. For the hatching assay with PRD, an egg in diapause would not hatch and would therefore be considered as nonviable even though it would have the potential to hatch later when diapause is broken. Staining methods seem to solve this problem to some extent. Eggs that are in diapause remain unstained whether or not they are viable, not whether or not they will hatch in their current state. However, since staining methods are unaffected by diapause, they give no indication of hatch densities that might occur at any one time, only over the entire lifespan of the cyst.

With similar viability results from the two methods for field-produced cysts, it is safe to say that hatching and staining assays predict egg viability similarly. However, since the two methods appear to be influenced by diapause differently, one method may be more useful than the other based on the desired course of action. From a management perspective, due to the tendency of staining with Meldola's Blue to overestimate ability to hatch at a given moment, this method might cause a producer to overestimate damage potential and apply unnecessary and possibly expensive control agents. On the other hand, from a regulatory standpoint, where the assessment of viability is more critical than ability to hatch at that moment, this overestimation from the staining method would be acceptable.

It is also important to note that while hatching assays with PRD appeared to provide a very accurate estimate of ability to hatch at a particular time, there is no standard for producing potato root diffusate. Depending on the potato species used to produce PRD as well as the method used to collect PRD the resulting diffusates may be extremely variable in their composition (Devine et al., 1996). Since egg hatch is stimulated only after exposure to hatching factors, the concentration and composition of these compounds within the PRD is extremely important to generate an accurate assessment of egg hatch. Because of variability in PRD chemistry between different batches another disadvantage of this method may be the inability to compare results across time or between laboratories.

Our hatching assay with PRD in this study was seven days based upon work with this *Globodera* population that consistently demonstrated that 80% of total hatch of field-produced eggs occurred within seven-days of exposure to PRD (Zasada and Ingham, unpublished data). Similarly, in the current study, 76% of eggs from the field cohort hatched in seven days. However, this amount of time may have been insufficient to fully realize egg hatch from greenhouse-produced cysts. A longer incubation period of greenhouse-produced eggs in PRD may have resulted in similar estimates of viability compared to staining with Meldola's Blue.

It was interesting to note that hatch of greenhouse-produced eggs was much greater after primary staining with Meldola's Blue and then secondary hatching with PRD compared to hatch when only exposed to PRD alone. Change in membrane permeability (Perry and Feil, 1986) may explain these phenomena. Many laboratories pre-soak cysts in water prior to exposure to PRD (Byrne et al., 2001; Twomey et al., 1995). Hydrated eggs may be more amenable to changes in membrane permeability; a period of 24 hours in PRD was sufficient for permeability changes and juvenile water uptake to occur in freed eggs (Ellenby and Perry, 1976).

In summary, the specific goals of this work determined that: (i) Estimates of egg viability by the two methods were similar for eggs that had over wintered in the field but were significantly higher with the staining method for young eggs produced in the greenhouse; (ii) Both methods appeared to be influenced by diapause since estimates of viability were lower for young greenhouse eggs than for eggs that had overwintered in the field. This difference was greater for the hatching assay; (iii) The majority (82%) of field eggs that did not stain hatched when exposed to PRD but only 40% of unstained greenhouse eggs hatched, and; (iv) For both cohorts of cysts the majority of eggs that did not hatch when exposed to PRD did stain when subsequently exposed to Meldola's Blue. Because of the complication from diapause, hatching assays with PRD to estimate viability should only be done on eggs from cysts that have overwintered. In contrast, Meldola's Blue appeared to be less influenced by diapause and may be more generally applicable.

Literature Cited

Ayoub, S. M. 1981. Plant Nematology. An Agricultural Training Aid. Sacramento, CA: NemaAid Publications.

Back, M. A., Heath, W., Edwards, S., Haydock, P. P. J., Grove, I. G. G., and Minnis, S. 2004. Development of a robust assay to assess the viability of potato cyst nematodes. Department for Environment, Food and Rural Affairs. CSG 15.

Byrne, J. T., Maher, N. J., and Jones, P. W. 2001. Comparative responses of *Globodera rostochiensis* and *G. pallida* to hatching chemicals. Journal of Nematology 33:195–202.

Devine, K. J., Byrne, J., Maher, N., and Jones, P. W. 1996. Resolution of natural hatching factors for golden potato cyst nematode, *Globodera rostochiensis*. Annals of Applied Biology 129:323–334.

Ellenby, C., and Perry, R. N. 1976. The influence of the hatching factor on the water uptake of the second stage larva of the potato cyst-nematode *Heterodera rostochiensis*. Journal of Experimental Biology 64:141–147.

Evans, A. A. F. 1987. Diapause in nematodes as a survival strategy. Pp. 180–187 in J. A. Veech and J. A. Dickson, eds. Vistas on Nematology, Hyattsville, MD: Society of Nematologists.

Moriarty, F. 1964. The efficacy of Chrysoidin, New Blue R and Phloxine B for determining the viability of beet eelworm, *Heterodera schachtii* Schm. Nematologica 10:644–646.

Ogiga, I. R., and Estey, R. H. 1974. The use of Meldola Blue and Nile Blue A, for distinguishing dead from living nematodes. Nematologica 20:271–276.

Perry, R. N., and Feil, J. J. 1986. Observations on a novel hatching bioassay for *Globodera rostochiensis* using fluorescence microscopy. Revue Nematology 9:280–282.

Rott, M., Lawrence, T., Belton, M., Sun, F., and Kyle, D. 2010. Occurrence and detection of *Globodera rostochiensis* on Vancouver Island, British Columbia: an update. Plant Disease 94:1367–1371.

Salazar, A., and Ritter, E. 1993. Effects of day length during cyst formation, storage temperature of cysts on the *in vitro* hatching of *Globodera rostochiensis* and *G. pallida*. Fundamental and Applied Nematology. 16:567–572.

Southey, J. F. 1962. New blue R, a stain that differentiates between living and dead nematodes. Nematologica 8:201–208.

Twomey, U., Raftery, T., Devine, K., and Jones, P. 1995. An improved procedure for assaying hatching activity of potato root diffusates towards *Globodera rostochiensis*. Nematologica 41:258–268.

Twomey, U., Warrior, P., Kerry, B. R., and Perry, R. N. 2000. Effects of the biological nematicide, DiTera, on hatching of *Globodera rostochiensis* and *G. pallida*. Nematology 2:355–362.

Widdowson, E. 1958. Observations on the collection and storage of potato root diffusate. Nematologica 3:173–178.