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Effect of Storage Environment on Hatching of the Cyst Nematode *Globodera ellingtonae*

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Abstract: Globodera spp. eggs go through a diapause, which remains dormant until favorable hatching conditions are reached. Because of the regulatory concerns with cyst nematodes, it is often only possible to rear eggs for research in the greenhouse. However, hatch is often lower for greenhouse-produced eggs than for eggs obtained from the field. The goal of this research was to determine storage conditions for *Globodera ellingtonae* eggs produced in the greenhouse that would increase percentage hatch. Over 3 yr, *G. ellingtonae* greenhouse-produced eggs were stored in different environments (−20°C, 4°C, room temperature, and the field) in either dry or moist soil. Percentage hatch after exposure to the different environments was determined in potato root diffusate. Across two experiments, field-produced eggs had higher hatch rates (65.2%) than greenhouse-produced eggs (10.4%). Temperature did not have an appreciable influence on hatch of eggs stored dry in two experiments (2.8% to 8.4% and 3.8% to 8.6%), but hatch of eggs stored in moist soil was significantly higher than in dry soil at all temperatures except −20°C (26.8% and 28.7%). However, the ability of *G. ellingtonae* greenhouse-, microplot-, and field-produced eggs to reproduce on potato in field microplots was not different. Although it may not be possible to produce *G. ellingtonae* eggs in the greenhouse that have the magnitude of hatch as those produced in the field, hatching can be greatly increased by storing eggs in moist soil at either 4°C or room temperature.

Key words: diapause, dormancy, hatching, potato root diffusate, quiescence, rearing, senescence, storage.

Cyst nematodes (*Globodera* spp., *Heterodera* spp., and others) develop and undergo their first molt to a second stage juvenile (J2) while still within the eggshell. Depending on the species and environmental conditions, the unhatched J2 can remain in a dormant stage for many years (Evans, 1987; Turner and Rowe, 2006). Cyst nematodes may exhibit two types of dormancy, diapause and quiescence (Jones et al., 1998). Diapause is considered to be a state of arrested development in which hatching does not occur until certain environmental requirements are met. The extent of the diapause varies but some species, such as potato cyst nematodes *Globodera rostochiensis* (Wollenweber, 1925) Behrens, 1975 and *G. pallida* (Stone, 1973) Behrens, 1975 experience an obligate diapause during their first season of development (Turner and Rowe, 2006). Diapause is terminated in the spring when rising soil temperatures and adequate soil moisture is conducive for infection of a new potato (*Solanum tuberosum* L.) crop. The duration of obligate diapause is influenced by the photoperiod experienced by the plant on which the nematodes developed (Franco and Evans, 1979; Hominick et al., 1985; Hominick, 1986; Salazar and Ritter, 1993). Once diapause is completed, J2 may enter a quiescent state, which requires certain environmental cues for development to continue. These may include specific hatching stimuli produced by the host root system in root exudates that are often collected and referred to as potato root diffusates (PRD) (Widdowson, 1958). Dependence on these root-produced factors is a survival mechanism that is greater in species with a narrow host range. Quiescent nematodes hatch in high numbers when exposed to PRD, but vary by species in their ability to hatch in water (Turner and Rowe, 2006). Nematodes in diapause hatch poorly in both water and in root diffusate.

In 2008, a *Globodera* spp. which could not be placed within the species *G. rostochiensis* or *G. pallida* was found in Powell Butte, OR (Skantar et al., 2011). This nematode was elevated to a new species in 2012, *Globodera ellingtonae* Handoo, Carter, Skantar, and Chitwood, 2012 (Handoo et al., 2012). When *G. ellingtonae* was first detected in Oregon, very few cysts were available for research. Initially, host range studies were conducted using cysts collected from "hot spots" in the field at Powell Butte. However, ultimately, an abundant egg source was needed to conduct host range, pathogenicity, developmental biology, and hatching research. It was found that *G. ellingtonae* reproduced well on potato and tomato (*Solanum lycopersicon* L.) (Skantar et al., 2011) enabling the production of large number of cysts in the greenhouse. However, when greenhouse-produced eggs of *G. ellingtonae* were exposed to PRD they demonstrated low hatch (5%) indicating that the eggs were in diapause (Kroese et al., 2011). The ability to break diapause in greenhouse-produced eggs was of great importance to the *G. ellingtonae* research program to enable subsequent experimentation.

The objective of this research was to identify the storage conditions that would best break diapause in greenhouse-produced eggs. Over a 3-yr period, field- and/or greenhouse-produced cysts of *G. ellingtonae* were exposed to different overwintering environments and subsequent egg hatch in PRD was used to evaluate hatching response in the spring. Moisture and temperature were the two environmental variables that were considered within the context of diapause. The ability of *G. ellingtonae* eggs produced under different

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environments to reproduce on potato was also determined in a microplot experiment.

Materials and Methods

Hatching experiments were conducted over a 3-yr period, with modifications in experimental design occurring in each year. For all experiments, *G. ellingtonae* eggs were either collected directly from or reared from cysts collected from the Oregon State University Central Oregon Agriculture Research Center, Powell Butte, OR.

Experiment 1: Two different cohorts of cysts were used: (i) greenhouse-produced cysts reared on potato 'Modoc' during 2010 that were considered to be in diapause and which exhibited little hatch in PRD, and (ii) field-produced cysts from a 2008 potato crop, considered to have broken diapause and which hatched readily when exposed to PRD. The initial hatching dynamics of eggs from these two cohorts was previously reported (Kroese et al., 2011). Dry soil, 250 g, containing either greenhouse- or field-produced cysts was placed in 8- × 28-cm nylon bags that were tied closely. For each cohort, 21 bags were placed in each of three different environments. One set of bags was placed in a refrigerator at 4°C. Another set of bags was stored at room temperature. The remaining bags were placed in 3.7-liter pots, which were filled with soil. These pots were then transported to Powell Butte, OR, and buried in the soil to grade in three different locations on 15 October 2010. Bags (three from each cohort in each environment) were collected at monthly intervals from November 2010 to May 2011. Soil was removed from bags, spread in a 20- × 28-cm aluminum tray and allowed to air-dry for 1 wk. Cysts were extracted from the entirety of each sample using a USDA cyst extractor (Ayoub, 1981). Cysts were handpicked into water from the washed soil sample, counted, and stored at 4°C until used in assays, usually within 24 hr.

Cysts to be used in hatching assays were placed in a watch glass and cysts that were round and without large indentations were preferentially selected for inclusion in assays. Once selected, individual cysts were placed in a single well of a 96-well plate with 288 µl of water and 12 µl of PRD for a final concentration of 1:25 v/v solution. From each cohort/environment/replication five cysts were evaluated in PRD assays. The PRD was collected from 3-wk-old potato 'Modoc' plants grown and leached at USDA-ARS, Corvallis, OR, according to Widdowson (1958). Specifics of the 96-well PRD hatching assay have been previously described in detail in Kroese et al. (2011). Cysts remained in PRD for 1 wk, a period of time determined to result in the majority of hatch for *G. ellingtonae* (Zasada et al., 2013), at which point, the number of hatched J2 was counted in each well. After counting, cysts were individually removed from the well and cut open using a scalpel and forceps under a dissecting microscope. Unhatched eggs were then collected and counted on an inverted microscope at ×40. Percentage hatch was calculated as number of hatched J2/(number of hatched J2 + number of unhatched eggs)*100.

Experiment 2: Cysts for this experiment were produced in the greenhouse during Summer 2011 on potato 'Désirée' and considered to be in diapause. Approximately 250 g of dry, cyst-infested soil was placed into 50 nylon bags as described above. In November 2011, sets of bags (N = 10) were distributed into four different environments: a refrigerator at 4°C, a freezer at −20°C, room temperature, or the field at Powell Butte. The bags placed in the field were separated into wet and dry treatments where those in the wet treatment were buried in the field as they were and those in the dry treatment were placed in zip-closing plastic bags which were closed to prevent the sample from getting wet.

In April 2012, all of the bags were removed from their respective environments. Contents of the bags were treated as described in experiment 1. Extracted cysts from each sample were placed under a dissecting microscope and cut open using a scalpel and forceps to liberate eggs. Eggs were then collected and concentrated to approximately 100 eggs per 270-µl water before addition to a 96-well plate; for each sample eight wells were evaluated. After the eggs were added to wells, 30 µl of PRD was added to each well for a final concentration of 1:10 v/v PRD solution. The number of unhatched eggs was counted in each well using an inverted microscope at ×40. The plate was then allowed to sit for 7 d at room temperature, at which point the number of unhatched eggs in each well was counted again using an inverted microscope. Percentage hatch was calculated as number of hatched eggs/total number of eggs*100.

Experiment 3: Cysts for this experiment were produced in the greenhouse on potato 'Désirée' during the summer prior to the initiation of this experiment in November 2012. Approximately 64 g of cyst-infested dry soil was placed in a black nylon mesh bag as described above. A total of 40 bags were filled and placed into four main environments (N = 10): a refrigerator at 4°C, a freezer at −20°C, room temperature, or the field at Powell Butte. Prior to placement in each environment, a subtreatment was implemented in each environment with half of the bags (N = 5) remaining either dry or having 5.6 ml of water (approx. 73% of water-holding capacity determined to be 12%) mixed into the soil to evenly distribute the moisture. In all environments, except the field, initial moisture content was maintained by placing each bag in a partially closed zip-closing plastic bag.

The experiment was terminated in April 2013 by removing bags from the respective environments. At this time, cyst-infested field soil from the 2008 potato field

was collected for comparison in hatching assays. Drying of soil, extraction of cysts from soil, collection of cysts from extracted samples, PRD hatching assays, and calculation of percentage hatch were conducted as described for experiment 2.

Field microplot experiment: On 21 May 2012, 22-liter microplots were buried in a field at Powell Butte and filled with cyst-infested soil from: (i) greenhouse pots planted to potato 'Russet Burbank' in 2011 and stored over the winter at 4°C, (ii) microplots planted to potato 'Russet Burbank' at Powell Butte during 2011 that remained buried in the field through the winter, and (iii) the resident field site where potatoes had been grown in 2008. Each microplot received sufficient infested soil so that when mixed with uninfested soil from the planting site the resulting soil contained approximately 10 *G. ellingtonae* eggs/g soil. Microplots were planted with potato 'Russet Burbank' and irrigated and fertilized according to standard practices at the farm. Microplots were harvested on 9 September 2012 by removing the microplot from the ground, placing the soil in a wheelbarrow, mixing the soil, and collecting a 1-liter soil sample. Soil was dried and processed as described above. Eggs/cyst, cysts/gram soil, eggs/gram soil, and reproductive factors (Rf = final egg population/initial egg population) were determined to assess the level of reproduction for each source of inoculum.

Statistical analyses: Data are presented as means ± SE. Data were examined for significant differences by the Kruskal–Wallis test. Duncan's multiple range test was used to separate means only when Kruskal–Wallis was significant at $P \leq 0.05$ (SAS Institute, Cary, NC).

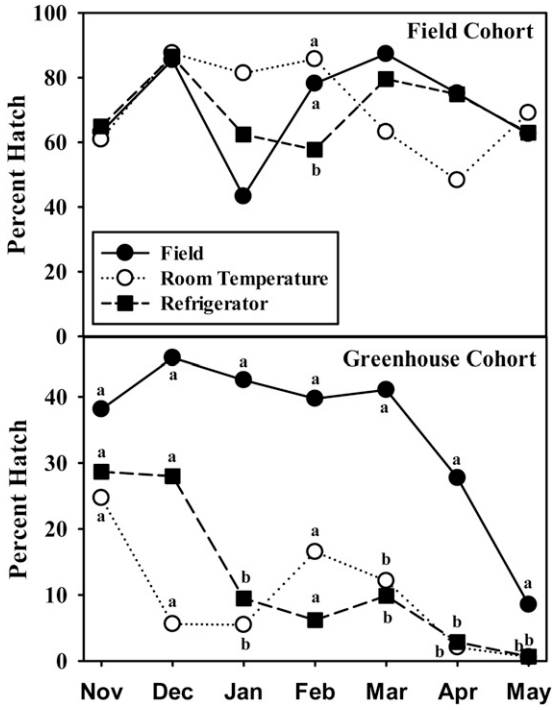

FIG. 1. Percentage hatch of greenhouse- and field-produced *Globodera ellingtonae* eggs after storage in the field, at room temperature, or in a refrigerator at 4°C from October 2010 until May 2011. Data points are means ($N = 3$). Values on any date that have the same letter are not significantly different according to Kruskal–Wallis test ($P \leq 0.05$). Means within the field-produced cohort were only different in February.

RESULTS

Experiment 1: Percentage hatch over time in the three environments within the greenhouse- and field-produced cohorts is shown in Fig. 1. In the greenhouse cohort, percentage hatch of eggs held in the refrigerator or at room temperature was initially near 30% but declined to low levels by January and hatch was never different between these two environments. Initial percentage hatch of the greenhouse cohort buried in the field environments was near 40% and it remained at this level until a decline in April and May. Percentage hatch of the greenhouse cohort stored in the field environment was significantly higher than hatch in the refrigerator and room temperature environments in January, March, April, and May. When averaged over all dates, percentage hatch of the greenhouse cohort in the refrigerator (12.2% ± 3.2%) and at room temperature (9.6% ± 2.4%) was not different, but both were significantly lower ($P \leq 0.0001$) than in the field environment (34.8% ± 3.9%). Percentage hatch of the field cohort was similar in all environments across all dates; the significant difference noted in February is likely due to happenstance. When averaged over all dates, mean percentage hatch of field cohort eggs in the refrigerator (69.8% ± 3.9%), room temperature (70.8% ± 4.2%), and the field (70.7% ± 3.8%) was not different ($P = 0.9609$). A comparison of percentage hatch over time between eggs from greenhouse and field cohorts within each of the three environments is illustrated in Fig. 2. In all three environments, percentage hatch in the field cohort was significantly higher than in the greenhouse cohort on every retrieval date except for the January evaluation.

Experiment 2: Table 1 summarizes the effect of storage under different environments on percentage hatch of *G. ellingtonae* eggs produced in the greenhouse. The lowest percentage hatch was observed in eggs stored in the freezer; this hatch was significantly less than in all other environments except when eggs were stored in a refrigerator. Percentage hatch of eggs stored at room temperature was not different from that of eggs stored in the refrigerator or from eggs kept dry in the field. Eggs from bags stored in the field which were exposed to moisture had substantially higher percentage hatch than eggs stored in all other environments and was similar to average hatch across time of greenhouse-produced eggs stored under similar conditions in experiment 1 (34.8% ± 3.9%).

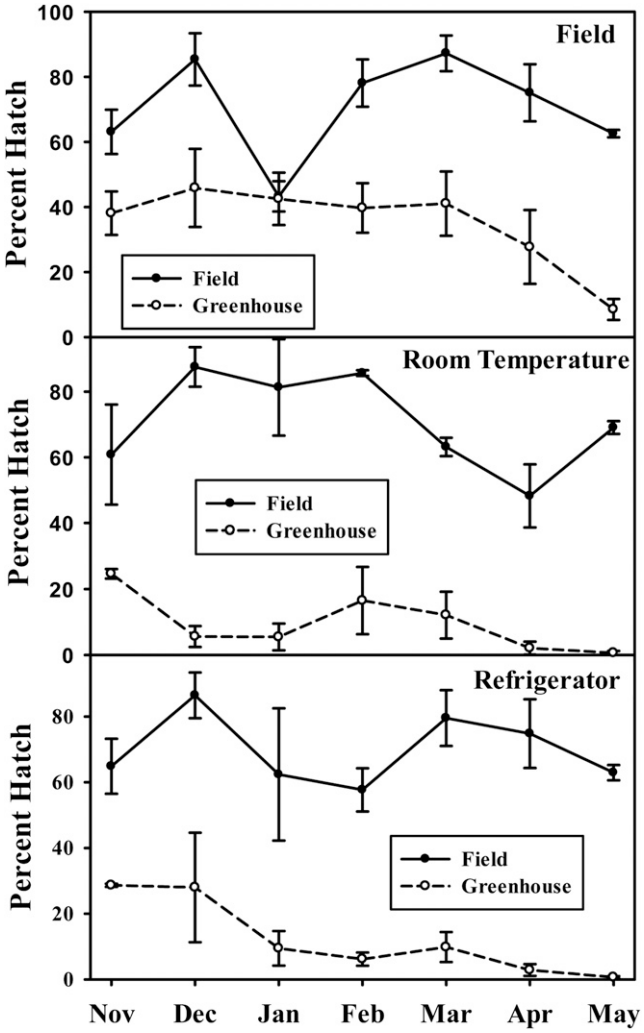

FIG. 2. Percentage hatch of greenhouse- and field-produced *Globodera ellingtonae* eggs after storage in the field, at room temperature, or in a refrigerator at 4°C from October, 2010 until May, 2011. Data points are means ($N = 3$) and SE. Percentage hatch was significantly greater for eggs from field-produced cysts compared to greenhouse-produced cysts for all dates in all environments except for cysts kept in the field on the January evaluation.

Experiment 3: Hatch of greenhouse-produced eggs stored wet or dry under different temperature conditions is presented in Table 2. Percentage hatch was similar from all environments when eggs were stored dry. Although percentage hatch of eggs stored in the field was significantly different than hatch of eggs stored in the refrigerator and the freezer, the actual differences between the means were relatively small (4.4% to 4.8%) and probably not biologically meaningful. Among the different environments when eggs were stored wet, percentage hatch of eggs stored in the freezer was substantially less than in all other environments. This suggests that eggs are more susceptible to damage from freezing when the soil is moist. The highest percentage hatch was observed for eggs stored wet in the field and was significantly higher than hatch of eggs stored at room temperature. There was no difference in percentage hatch of eggs stored wet in the refrigerator and at room temperature. Percentage hatch of eggs stored wet in the freezer was significantly less than that of eggs stored dry in the freezer. However, under all other temperature conditions, percentage hatch was significantly higher when eggs were stored wet. Once again, percentage hatch of greenhouse-produced eggs stored in wet soils in the field during experiment 3 (33.7%) was similar to that observed in experiment 1 (34.8%) and experiment 2 (26.8%).

TABLE 1. Effect of storage under different environmental conditions on percentage hatch of *Globodera ellingtonae* eggs produced in the greenhouse.

Environment[a]	Percent hatch[b]
Freezer (−20°C)—dry	2.8 ± 0.6 d[c]
Refrigerator (4°C)—dry	5.2 ± 1.2 cd
Room temperature—dry	6.6 ± 1.3 bc
Field—dry	8.4 ± 1.4 b
Field—wet	26.8 ± 2.7 a

[a] Environmental conditions under which cysts with eggs were kept from November 2011 to April 2012.
[b] Percentage of hatched eggs after exposure to PRD for 7 d.
[c] Values followed by the same letter are not significantly different according to Kruskal–Wallis test ($P \leq 0.05$). Each value represents the mean ± SE ($N = 10$).

Experiment 4: There were no significant differences in eggs/cyst, cysts or eggs/g soil, or reproduction factors between inoculum produced in the greenhouse, in field microplots, or collected from the field site (Table 3).

DISCUSSION

Globodera spp. go through a diapause stage in which juvenile development within the egg is arrested and remains dormant until favorable hatching conditions are reached (Evans, 1987). For example, Gonzalez and

TABLE 2. Effect of storage under different environmental and moisture conditions on percentage hatch of *Globodera ellingtonae* eggs produced in the greenhouse.

	Percent hatch[b]	
Environment[a]	Dry[c]	Wet[d]
Freezer (−20°C)	7.9 ± 1.2 a	3.6 ± 0.6 c*[e]
Refrigerator (4°C)	8.6 ± 1.8 a	28.4 ± 2.9 ab*
Room temperature	5.6 ± 1.2 ab	23.9 ± 3.0 b*
Field	3.8 ± 1.1 b	33.7 ± 1.0 a*
Cysts from field[f]		60.5 ± 3.8

[a] Environmental conditions under which cysts with eggs were kept from November 2012 to April 2013.
[b] Percentage of hatched eggs after exposure to PRD for 7 d.
[c] Air-dried for 1 wk and then kept dry.
[d] Initial soil moisture 9%.
[e] Values followed by the same letter are not significantly different according to Kruskal–Wallis test ($P \leq 0.05$). *indicates significant difference between moisture regimes. Each value represents the mean ± SE ($N = 5$).
[f] Cysts were collected from field soil from site where potatoes were grown in 2008 and source of inoculum for greenhouse-produced cysts. Data not included in statistical analysis.

TABLE 3. Reproduction of *Globodera ellingtonae* from different inoculum sources in field microplots planted to potato 'Russet Burbank' during 2012.

Egg source	Eggs/cyst	Cysts/gram soil	Eggs/gram soil	Rf[a]
Greenhouse[b]	266 ± 23[c]	0.56 ± 0.10	140 ± 24	14.0 ± 2.4
Microplot[d]	270 ± 24	0.72 ± 0.13	191 ± 38	19.1 ± 3.8
Field[e]	255 ± 19	0.46 ± 0.06	116 ± 15	11.6 ± 1.5
Pr > F	0.8120	0.2808	0.4085	0.4084

[a] Reproductive factor = final egg density/initial egg density (10 eggs/g soil).
[b] Eggs recovered from greenhouse cultures produced during 2011 by inoculating potato 'Russet Burbank' with eggs obtained from the field site where potato had been grown in 2008. Eggs were stored at 4°C through the winter.
[c] Values are means ± SE ($N = 10$).
[d] Eggs recovered from field microplots produced during 2011 by inoculating potato 'Russet Burbank' with eggs obtained from the field site where potato had been grown in 2008. Eggs were maintained in the microplots through the winter.
[e] Eggs were recovered from the field site where potato had been grown in 2008.

Phillips (1996) observed that the mean hatching rate of 3-yr-old populations from the field, which presumably were no longer in diapause, was more than double (44.5%) that of newly reared populations from the greenhouse (19.1%), which were assumed to be in diapause. Results from the current study are even more divergent, with percentage hatch of old eggs from the field over 60% and that of new eggs from the greenhouse less than 10%. Obtaining eggs that have undergone and completed diapause for use in research can be difficult. This is particularly challenging when a *Globodera* sp. is detected in a new region and scientists are interested in doing research on the nematode. Because *G. rostochiensis* and *G. pallida* are both regulated pests in the United States, planting a host crop on infested ground is not permitted. Therefore, the ability to obtain cysts that are produced and overwinter in the field is limited. This was also a complicating factor when the new species, *G. ellingtonae* was discovered since densities in the field were extremely low (<0.4 cysts/kg soil). Although many cysts can be produced in the greenhouse on host plants (i.e., potato or tomato; Zasada et al., 2013), physiologically they appear to possess developmental attributes that are not entirely identical to cysts produced in the field, with diapause being the most notable. Obtaining eggs with high hatching rates is critical for conducting extensive research objectives. For example, the EU protocol for screening potato varieties for resistance to *Globodera* spp. states that hatch rates need to be 60% (Anonymous, 2007), a level currently unobtainable with greenhouse-produced eggs.

Little research has been done on mechanisms that influence induction or breaking of diapause. One factor that may influence diapause is the photoperiod the plant host experiences during nematode development. Hominick et al. (1985) observed diapause in *G. rostochiensis* and recorded a low rate of hatch in the fall and winter after harvest and a faster rate of hatch the following spring and summer. Diapause was attributed to signals passed to the developing females and eggs by the plant and speculated that this signal may be photoperiod. This was supported by Hominick (1986) who found that eggs produced in complete darkness had a prolonged diapause period while those from plants grown under constant light expressed little of a diapause response. Eggs produced on plants grown under a 14/10 day/night hour light regime or outdoors had an intermediate diapause response. Hominick et al. (1985) and Hominick (1986) concluded that photoperiod, acting on the potato, affects developing females and influences the hatching mechanism of the developing juveniles. These observations are supported by those of Salazar and Ritter (1993) in which juveniles raised on potato grown under long-day conditions had higher rates of hatch than those grown under short-day regimes. They concluded that increasing day length during multiplication prevented the establishment of diapause. Similarly, Franco and Evans (1979) reported that more juveniles hatched from cysts obtained under 16-hr days than under 12-hr days. Therefore, *G. rostochiensis*, and to a lesser extent *G. pallida*, can develop a diapause that is determined during development and may be prevented by increasing long-day conditions.

Age of cysts and/or environmental conditions may also influence diapause which is frequently stated to be broken in the spring. Aspects of the current study are similar to that reported by Muhammad and Evans (1997). Cysts of *G. rostochiensis* were produced on potato plants grown in pots outdoors and either extracted and stored dry at 20°C (new cysts) or retained in the soil that was returned to the pots, which were buried outdoors for 1 yr (old cysts). In this case, their "new cysts" resembled our cysts stored at room temperature and their "old cysts" were similar to our cysts stored moist in the field. They evaluated percentage hatch over the next 9 months. On nearly all sample dates, percentage hatch was significantly higher for eggs from old cysts than from new cysts. They concluded that new cysts experienced some form of dormancy that was lost by old cysts that had been stored outdoors. Similar results were observed by Hominick et al. (1985) and Hominick (1986) who reported a slow hatch response of *G. rostochiensis* in the October, December, and February following harvest and a faster hatch the subsequent April, June, and October. However, in another experiment, hatch of juveniles from plants grown outdoors was low and did not increase in the spring. Although Hominick (1986) described this response as an absence of diapause it may have been due to diapause that was not broken over the period of time observed similar to that for eggs stored dry in the current study. In 3 yr of study, hatch frequency in the spring remained low in all storage environments when soil was dry. Therefore, during the course of these experiments, time alone was not responsible for breaking diapause.

Among environmental factors, temperature is stated as the most important cue for the termination of obligate diapause, with a fixed period of exposure to low temperatures considered necessary for breaking the arrested development (Wright and Perry, 2006). Salazar and Ritter (1993) found a large amount of variability in the effect of storage temperature on hatching as it was dependent on day length and species of *Globodera*. After 12 weeks of storage, populations of *G. pallida* and *G. rostochiensis* produced under short day-length conditions hatched poorly when stored at 13°C and not at all when stored at 22°C. Under a moderate day-length regime, *G. pallida* had a high rate of hatch when stored at 13°C but a low rate of hatch when stored at 22°C. The opposite trend was true for *G. rostochiensis* although percentage hatch was much less. Percentage hatch was high for both species when stored under either temperature for eggs produced on plants grown under long day length. Franco (1979) reported that the optimum temperature for hatching was between 15°C and 20°C for *G. rostochiensis* and between 10°C and 20°C for *G. pallida*. Optimum hatching temperature for *G. ellingtonae* is not known. In the current study, when greenhouse-produced eggs of *G. ellingtonae* were kept in dry soil, exposure to different temperature regimes had no effect on the percentage of eggs that hatched after exposure to PRD. When averaged over all years, hatch of eggs stored at constant temperatures in a freezer (5.4%), in a refrigerator (8.7%), at room temperature (7.3%), or at variable temperatures in the field (6.1%) was nearly identical and not appreciably different than that observed before storage (9.8%). This would suggest that temperature alone may not be an important factor for breaking diapause in *G. ellingtonae*.

Moisture is another factor reported to influence diapause. Janssen et al. (1987) mentioned that storing moist or air-dried cysts at −80°C, −20°C, 4°C, or 21°C did not accelerate hatching but no data were presented. Percentage hatch of eggs in whole new cysts (12 weeks after inoculation) placed in PRD was 3% while that of eggs in cysts cut in half was 40%. This was compared to 75% hatch for eggs from 1-yr-old air-dried cysts. The authors concluded that the water regime in cysts and cutting the cyst wall were essential to bypassing diapause. However, while they stated that allowing new cysts to desiccate before cutting the cyst strongly reduced the number of hatched juveniles, no data were presented. Our studies also suggest that moisture is an important factor. When eggs were stored in moist soil in all these environments except the freezer, percentage hatch was significantly higher (average over all environments in experiment 3 = 28.7%) than in comparable dry environment (6.0%). This value was similar to the average percentage hatch (31.8%) of eggs from moist soil buried in the field in experiments 1, 2, and 3.

It is possible that by storing eggs in a moist environment that somehow the juveniles in the egg are primed to respond to chemical cues from PRD. Initially, when eggs are placed in PRD there is little or no hatch, presumably because this is the period of time when the permeability of the lipoprotein membrane of the eggshell changes in response to the presence of PRD (Perry and Beane, 1982). With this change in eggshell permeability, solutes (trehalose) leave the egg allowing for rehydration of the unhatched juveniles (Ellenby and Perry, 1976). Since hydration is part of this process, keeping eggs hydrated may expedite hatching.

Percentage hatch of greenhouse-reared eggs from all the environments examined during all of our experiments remained substantially less than that measured for eggs collected from the field site where potatoes had been grown in 2008 (70.4% in 2011, 60.5% in 2013). Therefore, some factor other than temperature or moisture must be regulating hatch. Furthermore, in spite of the difference in percentage hatch between greenhouse-produced eggs stored dry which were in diapause and field-produced eggs which had passed through diapause, there was no difference in the reproductive factor between these sources of eggs when used as inoculum in field microplots. This observation is supported by results from Muhammad (1994) who found that there was no difference in infectivity between juveniles of *G. pallida* obtained from eggs in diapause and those which had overcome diapause. This suggests that diapause may break more readily when eggs are inoculated to a host plant outdoors than is indicated by hatching assays conducted in the laboratory.

In summary, the current research demonstrated that moisture is a key component of breaking diapause during storage regardless of the environment in which eggs are stored. The percentage hatch of greenhouse-produced eggs could potentially be increased by increasing the photoperiod of culture host plants in the greenhouse and keeping soil moist from harvest until inoculum is needed for research.

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