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Research

Effects of Cold Storage on Nondiapausing Eggs of the Western Corn Rootworm (Coleoptera: Chrysomelidae)

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Abstract

Western corn rootworm, *Diabrotica virgifera virgifera* LeConte, became much easier to research with the development of a nondiapausing rootworm strain. In the event that the eggs cannot be used immediately researchers have been known to delay egg hatch by storing the eggs at low temperatures. It is not well known how this technique could affect egg hatch or larval development, which could alter the results of an experiment. To test for this nondiapausing eggs of the western corn rootworm were stored at low temperatures to test for potential negative effects on hatch and larval development. Eggs were stored in either soil or agar and placed in refrigerators set to 4 or 8.5°C. Nondiapausing eggs were exposed to the cold for 1, 2, or 4 wk and then placed in a chamber set to 25°C. Eggs were then tested for average hatch percentage in Petri dishes and average larval recovery from containers with seedling corn. Results showed a significant reduction in percent hatch for eggs stored at 4°C for 4 wk. Larval recovery was significantly reduced in eggs stored for 4 wk at both 4 and 8.5°C. Within the treatments tested, egg storage for less than 4 wk in soil at 8.5°C provided the best hatch and larval recovery. Researchers wishing to store eggs may use these results to improve their rearing or testing of western corn rootworm.

Key words: western corn rootworm, rearing, cold temperature

Since it was first described in 1868, the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, has become one of the most economically important insect pests in the world (Chaing 1973, Meinke et al. 2009). The first recorded instance of western corn rootworm attacking corn (*Zea mays* L. (Poales: Poaceae)) was in 1909 by Gillette (1912). Currently, the western corn rootworm is the most damaging pest of corn in the world due to the high cost of pest control and crop yield losses, which are estimated to be in the billions of dollars for the United States alone (Metcalf 1986, Sappington et al. 2006, Gray et al. 2009). These extreme costs are due in part to the western corn rootworm's ability to adapt to control measures (Ball and Weekman 1962, Meinke et al. 1998, Levine et al. 2002, Gassmann et al. 2011).

Western corn rootworm became much easier to research with the development of a nondiapausing rootworm strain (Branson 1976). Normally, the western corn rootworm is univoltine, with oviposition primarily occurring in the soil of corn fields from July to early September (Levine and Oloumi-Sadeghi 1991). Eggs overwinter in the soil during which they are in a state of diapause. Diapause is broken around midwinter when the soil temperature is still below 11°C (Levine et al. 1992, Meinke et al. 2009). During this time, the eggs enter a state of chill-quiescence where they wait to hatch until the soil temperature is above 11°C and approximately 400 degree days have accumulated above that temperature (Meinke et al. 2009). In the U.S. Corn Belt, egg hatch typically occurs between late May and early June producing subterranean neonate larvae (Levine and Oloumi-Sadeghi 1991). The lengthy diapause process makes it advantageous for researchers to develop a nondiapausing strain of western corn rootworm. With the development of the nondiapausing strain, the generation time was reduced from ≈260 to ≈60 d in the laboratory (Hibbard et al. 1999).

The nondiapausing strain simplified research efforts and allowed researchers to observe resistance formation at an accelerated rate. Resistance formation to the toxin Cry3Bb1 was observed in nondiapausing western corn rootworm populations after three generations of continuous exposure in greenhouse experiments (Meihls et al. 2008). A few years later, field resistance to the Cry3Bb1 toxin was reported in Iowa corn fields (Gassmann et al. 2011). According

to farmers' accounts, the problem fields had been planted with the Cry3Bb1 producing corn for three consecutive years (Gassmann et al. 2011). The comparison of these two reports serves as a convincing example of the benefits of using nondiapausing western corn rootworm eggs to gain foresight into how natural populations may react to the measures used against them.

Although the use of nondiapausing eggs is more convenient for researchers, it must be noted that this trait is not naturally observed in the field. Many research projects using nondiapausing western corn rootworm eggs order the eggs from a nonlocal source. Normally, it would be preferred to use the eggs soon after they arrive. However, there are many factors that can delay the start of an experiment, especially those conducted in the field. In such situations, it may be necessary to store the eggs in a refrigerator to delay hatching in either soil or an agar water mixture (Palmer et al. 1977). For this study, we determined how storage temperature, media, and duration affect the hatch rate and larval development of nondiapausing western corn rootworm eggs.

Materials and Methods

Insects

Nondiapausing western corn rootworm eggs were supplied by the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) North Central Agricultural Research Laboratory in Brookings, SD. Eggs were delivered in two ovipositional soil plates containing approximately 30,000 eggs each. Eggs were removed from the soil by washing them with water through a 60 mesh sieve (U.S. Standard Sieve Series Sieve 250 µm, Fisher Scientific, Hampton, NH). Eggs from both plates were mixed together and suspended in a 0.15% agar (CAS 9002-18-0, USB Corporation, Cleveland, OH) solution. The eggs in solution were then counted and calibrated to approximately 100 eggs/ml agar. The 100 eggs/ml agar solution was then used to inoculate the experiment. All eggs were used in the experiment the same day they arrived in lab.

Temperature, Media, and Duration Treatments

Eggs were divided among experimental treatments: two temperatures (4 and 8.5°C), three exposure lengths (1, 2, and 4 wk), and two types of storage medium (soil plates and 0.15% agar solution). For the agar storage medium, two 125-ml Erlenmeyer flasks were filled with ≈7,125 eggs each suspended in 0.15% agar and calibrated to ≈100 eggs/ml agar. The tops of the flasks were covered with Parafilm that had small holes poked in it. One flask was placed in a 2.7 cu ft mini fridge (Haier Group Corporation, Qingdao, China) maintained at 4°C, whereas the other was placed in a model LIFLY-VIEW incubator (Sheldon Manufacturing, Inc., Cornelius, OR) maintained at 8.5°C.

Soil plates were made by adding 40 ml of autoclaved soil passed through an 80 mesh sieve (180 µm) into a Petri plate (100 × 15 mm, Fisherbrand, Pittsburg, PA). The soil was mixed with 19 ml of water and spread to make a solid layer of moist soil. The soil surface was inoculated with ≈2,375 eggs, which were covered with a light layer of autoclaved sieved soil. The plates were closed and sealed with Parafilm. In total, six plates were made with three placed into the 4°C fridge and the remaining three placed into the 8.5°C chamber. At the end of each exposure period, the agar flasks and one plate were taken from each chamber, and the eggs were washed from the soil using a 60 mesh (250 µm) sieve and water. The eggs from each soil plate were suspended separately in a 0.15% agar solution and calibrated to ≈100 eggs/ml agar. Then, the eggs were used to inoculate 10 hatch plates and 10 seedling assays each. After being used to inoculate the hatch plates and seedling assays, the flasks containing the agar egg mixture were verified to be 100 egg/ml agar and adjusted if needed. The flasks were then returned to their respective chambers to be used for the next time variable.

Egg Hatch/Hatch Plates

Hatch plates were created by placing a moist filter paper into a Petri dish and inoculating ≈50 eggs onto the paper. The plates were sealed with Parafilm (5 cm width, Pechiney Plastic Packaging, Menasha, WI) and placed into a growth chamber (I-36LL, Percival Scientific, Perry, IA) maintained at 25°C to promote hatch (Branson et al. 1988). The initial total number of eggs was counted for each hatch plate upon creation to correctly determine percent hatch throughout the experiment. Plates were monitored daily for egg hatch. Larvae found in plates were counted as a hatched egg and removed. Plates were declared done after 2 wk of not finding any larvae. Plates were used to determine percent hatch and peak emergence time for each treatment.

Seedling Assay/Larval Recovery

For seedling assays, eggs were inoculated in 15 cm × 10 cm containers (708 ml, The Glad Products Company, Oakland, CA) at a density of ≈100 eggs per container. The eggs were then covered with ≈150 ml of growth medium consisting of 2:1 Missouri soil:LC1 mix (Sun Gro Horticulture Canada Ltd, Seba Beach, Canada), planted with ≈50 DK166 (Monsanto Company, St. Louis, MO) corn seeds, covered by an additional ≈300 ml of growth medium and then moistened with 100 ml of water. The containers were covered with plastic lids and placed in a growth chamber maintained at 25°C. After 4 d, the lids were removed to allow the corn seedlings to grow. Seedling assays for each treatment were analyzed 10 d after peak emergence had been observed in their corresponding hatch plates. Larval recovery data were taken by removing the soil and root material from the seedling assays and placing them in modified Tullgren funnels. The funnels were covered with lights fitted with 60-W light bulb and had half-pint mason jars filled with ≈150 ml of water attached to the bottom to collect escaping larvae. Jars were removed after 2 d and replaced with fresh jars, which remained on for an additional 2 d. Larvae recovered in jars were counted under a microscope and transferred to vials containing 95% ethanol.

Untreated Hatch/Larval Recovery

At the start of the experiment, 30 hatch plates and 30 seedling assays were created on the same day the eggs were delivered. The plates and assays were created similarly to those previously described and were placed in the 25°C chamber. Hatch data from the plates and larval recovery data from the assays were collected similarly to those previously described. This was performed to test the viability of the eggs before their use in the experiment.

Data Analysis

Raw data are presented in all figures, while all statistical analyses were done with data that were square root ($x \pm 0.5$) transformed to meet all assumptions of normality. Hatch and seedling assays were analyzed separately as a randomized complete block in a three-way factorial arrangement (three exposure lengths, two temperatures, and two storage media types). Data were analyzed using PROC GLIMMIX of the SAS statistical package (SAS ver. 9.4) along with a Tukey's test (SAS Institute 2009). The model contained the main

effects of exposure length (1, 2, and 4 wk), temperature (4 and 8.5°C), and egg storage medium (soil and agar), and all two- and three-way interactions.

Results

Hatch

Eggs placed on hatch plates at 25°C at the start of the experiment hatched at an average rate of 55.27%. Analysis showed significant three-way interactions between exposure time, temperature, and egg storage medium (Table 1). The highest percent hatch occurred in eggs stored in soil for 2 wk at 8.5°C (81.86%) and eggs stored in agar for 1 wk at 8.5°C (75.72%), both of which were significantly greater than all other treatments with the exception of eggs stored in agar for 2 wk at 8.5°C (74.17%; Fig. 1). The lowest percent hatch occurred in eggs stored in agar at 4°C for 4 wk (45.55%), which was significantly lower than all other treatments (Fig. 1). The second lowest hatch occurred in eggs stored in soil for 4 wk at 4°C (60.85%) and was not significantly different from eggs stored in soil for 4 wk at 8.5°C (64.40%; Fig. 1). Percent hatch for eggs stored in soil for 4 wk at 8.5°C was also not significantly different from those stored in agar at 4°C for 1 and 2 wk (Fig. 1).

Table 1. Analysis of variance for percentage hatch and percentage larval recovery assays.

Analysis	Effect	df	*F* value	*P*
% Hatch	Exposure by medium × temperature	2, 99	5.63	0.0021
	Exposure by medium	2, 99	1.43	0.2087
	Exposure by temperature	2, 99	4.15	0.0063
	Temperature by medium	1, 99	7.67	0.0029
	Exposure	2, 99	42.89	<0.0001
	Medium	1, 99	8.53	0.0036
	Temperature	1, 99	32.65	<0.0001
% Larval recovery	Exposure by medium × temperature	2, 99	0.75	0.7303
	Exposure by medium	2, 99	6.12	0.0019
	Exposure by temperature	2, 99	1.05	0.4816
	Temperature by medium	1, 99	1.72	0.2027
	Exposure	2, 99	31.11	<0.0001
	Medium	1, 99	0.37	0.7703
	Temperature	1, 99	15.68	0.0001

Larval Recovery

Eggs placed in seedling assays produced an average of 33.4 larvae per assay. Analysis within treatment types showed no significant three-way interactions between exposure time, temperature, and egg storage medium in relation to larval recovery (Table 1). However, a significant two-way interaction between exposure time and storage medium was observed (Table 1). Analysis within the 4°C temperature, agar storage medium, soil storage medium, 1-wk exposure, 2-wk exposure, and 4-wk exposure showed no significant interaction. Within the 8.5°C temperature treatment, eggs stored in agar and soil for 4 wk had the lowest and second lowest average larval recovery, respectively, and were both significantly lower than eggs stored in agar for both 1- and 2-wk periods (Fig. 2).

Discussion

Research groups have used various methods to delay hatch of non-diapausing western corn rootworm eggs, using different media, temperatures, and storage times. These differences can affect the performance of western corn rootworm when taken out of storage. Consequently, it is important to understand the effect egg storage has on western corn rootworm and to relate that effect to the subsequent use of western corn rootworm. A thorough analysis of the effect these variables have on egg hatch and larval recovery provides valuable insight to these effects.

Significant differences in egg hatch and larval recovery as a result of applying different storage methods were observed after 4 wk of storage. Eggs stored in agar for 4 wk at 4°C had significantly lower percent hatch than any of the other treatments. This indicates egg storage in agar at colder temperatures for longer periods of time (i.e., >2 wk) is inferior to soil storage and results in reduced viability. We infer cold storage for short periods of time may enhance egg hatch. Although the percent hatch of eggs exposed to 25°C at the beginning of the experiment was lower than many of the other treatments, it still falls within an average that is often observed in western corn rootworm eggs of 53–60 percent hatch (Chaing 1973, Branson et al.

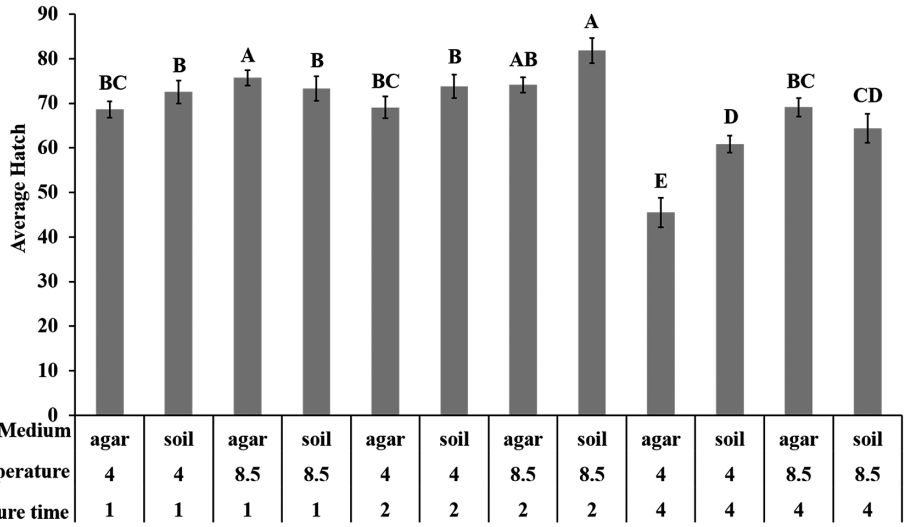

Fig. 1. Average (±SE) percent hatch of *Diabrotica virgifera virgifera* eggs stored at 4 or 8.5°C in agar or soil for 1, 2, or 4 wk. Differing uppercase letters indicate significant difference among treatments ($P \geq 0.05$).

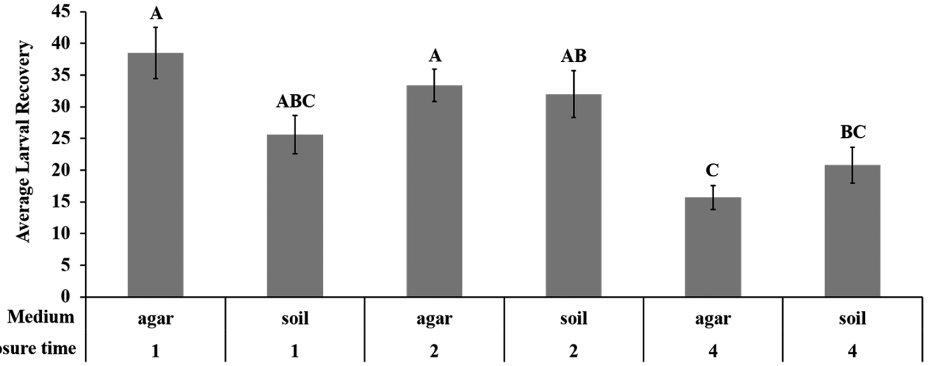

Fig. 2. Average (±SE) larval recovery of *Diabrotica virgifera virgifera* eggs stored at 8.5°C in agar or soil for 1, 2, or 4 wk. Differing uppercase letters indicate significant difference among treatments ($P \geq 0.05$).

1988). We use this information to infer that there were no negative effects on egg hatch at the start of the experiment and all effects seen within the treatments are due in part to the experimental treatments.

Eggs kept in storage for 4 wk showed a significant drop in larval recovery for both storage media at 8.5°C. These results do not directly correlate with the hatch data as previously described; however, they indicate that although long-term storage may not affect hatch significantly, it can affect larval development. Selection for nondiapausing eggs, as opposed to their wild state that undergoes an egg diapause, appears to influence the ability of these insects to tolerate colder conditions for extended periods of time, resulting in decreased larval survival.

Collectively, the results of this study suggest that storage in soil at 8.5°C for as brief a time as possible provides the best performance of western corn rootworm. In addition, these results alert western corn rootworm researchers to the influence of storage media on western corn rootworm performance. Many experiments involving the use of western corn rootworm eggs utilize hatch plates to account for effects of egg hatch on the outcome of the experiment. This normally involves storing a subsample of eggs on moist filter paper in a Petri dish. The dish is stored in the same area as the experiment and the eggs are observed daily to account for total egg hatch and egg emergence timing. Results from these hatch plates are used to document the effects seen in the experiment are not due to low or irregular egg hatch. However, our results show that storing eggs at low temperature for an extended period can have an influence on the larval viability while not affecting hatch. We propose recording larval recovery assays to account for the potential effects of egg storage on larval development and survival.

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