Milky Disease Development in Field-Infected Japanese Beetle Larvae

GRANT ST. JULIAN, LEE A. BULLA, JR., AND GORDON L. ADAMS

Northern Regional Research Laboratory, Peoria, Illinois 61604

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By correlated visual and microscopical examination, milky disease of field-infected, third-instar Japanese beetle larvae is categorized into four phases. The phases are described as sequential disease symptoms I through IV. All four phases persist simultaneously throughout experimental incubation. Larvae die during all phases of the disease; however, the largest percentage of death is at phase II and III of the infectious process. At phase II, 90% of the total population of cell types in the infected hemolymph are vegetative cells; in phase III 65-76% are vegetative cells with 17-28% spores. The massive spore population (95% of population) that characterizes milky disease is designated phase IV; less than 30% of larvae reach this phase of the disease.

INTRODUCTION

The name "milky disease" refers to the milky appearance of the hemolymph of the Japanese beetle (Popillia japonica) larva after it becomes heavily infested with spores of either Bacillus popilliae or Bacillus lenti-norbus (Dutky, 1940). Spores of either bacterium are effective insecticidal agents against the Japanese beetle and some other susceptible hosts (Beard, 1945; Dutky, 1963; St. Julian and Hall, 1968; Tashiro, 1957).

Previously we determined the number of spores needed to infect larvae in soil and also outlined the pattern of development of B. popilliae within the larvae (St. Julian et al., 1970). The infectious process in these laboratory-infected larvae occurred in four phases, as determined by microscopical examination of the diseased hemolymph. We have now established (using the previously described phases of the infectious process as a reference) a correlation between visual gross symptoms and microscopic appearance of diseased hemolymph of field-infected larvae.

These data suggest important differences between laboratory- and field-infected larvae. Awareness of the differences could have a direct influence on the method and extent of spore application to field plots.

MATERIALS AND METHODS

Larvae. Third-instar larvae of the Japanese beetle, P. japonica, were collected by the Plant Protection Division, Agricultural Research Service, U.S. Department of Agriculture, from diseased areas in midwestern states. Their care and handling have been described by St. Julian et al. (1963). Data presented here summarize four separate experiments that involved a total of 12,000 larvae. The larvae were collected in the fall of 1969 and spring of 1970.

Experimental procedure. Larvae were maintained in wooden boxes (25 × 18 × 8 inches i.d.) containing finely sifted moistened soil to the depth of 6 inches. The soil came from field plots inhabited by the diseased larvae. Healthy larvae as controls were maintained in noninfected soil. Three thousand larvae each were placed into four boxes and incubated at 25-28°C for a total of 30 days. From each of three storage boxes, 150 live larvae were removed on days 1, 7, 14,
21, and 30. The live larvae then were examined for gross symptoms of milky disease. All dead larvae found at these time intervals were also examined for disease symptoms. The larvae were separated into four groups based on hemolymph turbidity. Each group of larvae was washed and bled as previously described (St. Julian et al., 1970). Differential microscopical count of cell types were made from the pooled hemolymph of each group with a Petroff-Hausser2 bacteria counter with phase-contrast optics at 1250X. The fourth storage box was allowed to incubate undisturbed for 30 days at which time all live and dead larvae were removed and examined as mentioned above.

Results

Recovery of spores from field soil. At the termination of the experiments, soil from the four storage boxes was combined and mixed thoroughly. Samples of 1, 10, and 100 g of combined soil were placed separately in Erlenmeyer flasks containing 3X (w/v) of sterile distilled water. The flasks containing soil and water were shaken at 300 rpm for about 3 hr. Flasks then were allowed to stand about 1 min and the supernatant water decanted away from settled soil; the supernatant then was centrifuged for 10 min at 1,800 g. The centrifugate was examined microscopically for B. popilliae spores. Control soil containing $1 \times 10^9$ spores/g of soil was examined in the same manner. Soil ($1 \times 10^9$ spores/g) previously has been shown to infect 50% of larvae (St. Julian et al., 1970).

Disease symptoms. The extent of infection in an intact larva can be determined visually by gently pressing the larva posterior toward its anterior and observing the opacity and color of its hemolymph at the posterior.

Milky Disease Development

Visual observation enables the disease process to be categorized as four distinct phases. Phase I, the beginning of bacterial invasion into the hemolymph, is characterized by transparent hemolymph. At phase II of the disease the hemolymph is slightly gray; it is off-white at phase III, and milk white at phase IV of the infection. Microscopical appearance of larval hemolymph throughout the infectious process of milky disease is shown in Fig. 1. In phase I, the hemolymph contains only a few invading vegetative cells. Predominant vegetative proliferation in the hemolymph with limited spore formation indicates phase II of the infection. Intermediate phase III is characterized by concomitant vegetative growth and sporulation. In the terminal phase IV of the disease, there occurs massive accumulation of spores and typical milky white hemolymph.

The percent of larvae in each of the four phases of the infectious process during a 30-day observation period is shown in Table 1. As incubation time increased, the percent of larvae in the later phases of the disease increased. However, it is evident from these data that all phases occur throughout the incubation period. Death occurs at all phases.

\[2\] Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.
of the disease; the largest percent die during phases II and III of the infectious process. Significantly, most larvae (dead and live) exhibit the intermediate phase of infection (III), at which time spores do not predominate in the cell population.

Tables 2 and 3 show the quantitative pattern of \textit{B. popilliae} development in moribund and dead larvae, respectively. Vegetative growth and sporulation occur concomitantly so that spores form continuously and, ultimately, accumulate in large numbers. Total vegetative populations never exceed $3-4 \times 10^8$/ml of hemolymph. However, vegetative cell numbers seem to be higher in dead than in moribund larvae (Table 3, phase III); the contrary is true for spore numbers (Table 2, phase IV). Vegetative cells in phase II constitute essentially 100\% of the total population. In phase III 65-76\% are vegetative cells with 17-28\% spores. Less than 10\% of cells are prespores. Hemolymph contains about 99\% spores at phase IV.

**Recovery of Spores from Field Soil**

Of the $10^8$ spores per gram of control soil, the average number of spores recovered per gram was about $1 \times 10^8$. Spores could be isolated only from the 100-g samples of the test soil, and then the average number of spores recovered per gram of test soil was less than 1.

**DISCUSSION**

Until now it was presumed that most field-infected larvae died at phase IV and contained about $5 \times 10^6$ spores. Our data show that less than 30\% of larvae examined survive to phase IV although the microscopical pattern of growth and sporulation of \textit{B. popilliae} in the field-infected larvae is similar to larvae infected in the laboratory (St. Julian et al., 1970). Therefore, we believe that the sudden death of field-infected larvae may be due to rapid proliferation of $$

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Time of incubation (days)} & \textbf{I} & \textbf{II} & \textbf{III} & \textbf{IV} & \textbf{I} & \textbf{II} & \textbf{III} & \textbf{IV} \\
\hline
1 & 84 & 16 & 0.7 & 0 & 0 & 0 & 0 & 0 \\
7 & 18 & 76 & 3 & 2 & 39 & 50 & 11 & 0 \\
14 & 4 & 60 & 27 & 9 & 10 & 37 & 47 & 5 \\
21 & 3 & 9 & 68 & 16 & 3 & 11 & 80 & 7 \\
30 & 6 & 1 & 67 & 26 & 2 & 2 & 85 & 11 \\
30* & 18 & 36 & 39 & 7 & 0 & 28 & 71 & 1 \\
\hline
\end{tabular}
\caption{Visual Observation of the Infectious Process of Milky Disease in Japanese Beetle Larvae}
\end{table}

- Larvae incubated at 25-28°C in field soil from which they were collected; day 1 represents the first day larvae were placed in laboratory condition.
- Phase I = no visual evidence of infection, hemolymph is transparent. Phase II = hemolymph slight gray turbidity; vegetative cell proliferation. Phase III = off-white turbidity; concomitant vegetative growth, prespore formation and sporulation. Phase IV = milky white hemolymph containing massive spore population.
- On each designated day, 450 live larvae were examined.
- All dead larvae found each designated day were examined.
- Observed only after 30-day incubation at which time all larvae found dead or alive were examined.

\textit{B. popilliae} vegetative cells rather than to accumulation of spores.

A wide variety of strains develop from \textit{B. popilliae} spores (Sharpe et al., 1970). From the standpoint of larval infection, in vitro grown vegetative cells are of two types: (1) those that grow slowly in the hemolymph and cause disease phases I through IV and (2) cells that grow rapidly and kill larvae at phases II or III before massive spore production. The same substrains seem to develop in field-infected larvae. Injection of 100-5,000 in vitro grown vegetative cells per larva is required to obtain 50\% infectivity (Pridham et al., 1964). A challenge dose of
TABLE 2
MICROSCOPICAL OBSERVATIONS OF Bacillus popilliae IN HEMOLYMPH OF MORIBUND JAPANESE BEETLE LARVAE

<table>
<thead>
<tr>
<th>Time of incubation (days)</th>
<th>Cells/ml hemolymph from moribund larvae at phases I-IV of infection (count × 10⁶)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Veg</td>
</tr>
<tr>
<td>1</td>
<td>0.0001</td>
</tr>
<tr>
<td>7</td>
<td>0.003</td>
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<td>14</td>
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<td>21</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>30²</td>
<td>0.00001</td>
</tr>
</tbody>
</table>

² Same as footnote a, Table 1.
³ Average differential microscopical counts made from pooled larval hemolymph with Petroff-Hauser bacteria counter under phase-contrast optics at 1250X.

TABLE 3
MICROSCOPICAL OBSERVATIONS OF Bacillus popilliae IN HEMOLYMPH OF DEAD JAPANESE BEETLE LARVAE

<table>
<thead>
<tr>
<th>Time of incubation (days)</th>
<th>Cells/ml hemolymph from dead larvae at phases I-IV of infection (count × 10⁶)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Veg</td>
</tr>
<tr>
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</tr>
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<td>0.0006</td>
</tr>
<tr>
<td>30²</td>
<td>0</td>
</tr>
</tbody>
</table>

² Same as footnote a, Table 1.
³ Same as footnote b, Table 2.
⁴ Same as footnote e, Table 1.

approximately 5,000-10,000 viable vegetative cells of B. popilliae kills larvae within 3–5 days without the development of milky disease symptoms or microscopical evidence that spores were formed in the larvae. The dead larvae contain a massive vegetative population. By comparison, a high incidence of milky disease (90%) can be obtained by injecting 10⁶ B. popilliae spores per larva. In addition, challenge doses up to 4 × 10⁶ spores per larva never causes the nonspecific death associated with the injection of large numbers of vegetative cells (St. Julian and Hall, 1968). The number of spores needed for oral infection is also high. Concentrations of 1 × 10⁶ spores per gram of soil result in
50% infection (St. Julian et al., 1970). It is our contention that such large numbers of spores are needed to infect larvae because of the extremely low germination and outgrowth rate of the bacterial spores. Spores of _B. popilliae_ do not respond to common germinating agents, and the germination rate and subsequent outgrowth of these spores range from only 2 to 10% (St. Julian et al., 1967).

Our data indicate that the natural buildup of _B. popilliae_ spores in field plots from diseased larvae does not occur as rapidly as was previously assumed. Therefore, we believe that to effectively control Japanese beetle larvae, heavy concentrations of _B. popilliae_ spores (> $1 \times 10^9$ spores per square inch of land surface) must be applied in such manner as to become immediately available to the larvae. In addition, annual or semiannual application with available _B. popilliae_ strains is recommended.

**Acknowledgments**

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**References**


