Infectivity of Spores of *Bacillus popilliae*
Produced on a Laboratory Medium

PAUL H. SCHWARTZ, JR., 1 AND EUGENE SHARPE

Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Moorestown, New Jersey 08057 and Northern Regional Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

Received August 8, 1969

No occurrence of milky disease resulted when third-instair larv of the Japanese beetle, *Popillia japonica*, were exposed to soil containing 2 billion spores/kg of strain NRRL B-2309M of the milky disease bacterium, *Bacillus popilliae*, produced on a laboratory medium, or when the larvae imbibed 200,000 of the spores of strain NRRL B-2309M in aqueous suspension; however, 24% of the larvae were infected when they were injected with 10,000 of the spores. In contrast, spores produced commercially2 in diseased larvae infected 60% of the larvae exposed to soil containing 2 billion spores/kg, 60% of the larvae that imbibed 280,000 spores in an aqueous suspension, and 40% of the larvae injected with 10,000 spores.

INTRODUCTION

Since Dutky (1940) isolated and identified the causal organism (*Bacillus popilliae*) of Type A milky disease, workers have attempted to grow the organism in laboratory media. At present, *B. popilliae* can be cultured in vitro with some degree of sporulation (Rhodes, 1965), and when the vegetative cells from these cultures are injected into larvae of the Japanese beetle, *Popillia japonica*, both infectivity (Pridham et al., 1964) and sporulation (Sharpe, 1966) occur.

The present study was made at the Japanese Beetle and European Chafer Investigations Laboratory at Moorestown, New Jersey, to determine whether spores produced on an artificial medium would cause an infection of milky disease when they were ingested by Japanese beetles.

MATERIAL AND METHODS

The third-instair beetle larv used in the study were collected in the field in Alleghany, Ashe, and Watauga Counties, North Carolina, or in Burlington County, New Jersey, and stored at 7°C until used. During the tests, the larv were held at 30°C and examined macroscopically on Monday, Wednesday, and Friday of each week until they were either milky or dead or had ceased to feed and had begun voiding the gut of its contents (prepupal stage). A blood sample from all dead or diseased larv was examined under phase contrast at 450 or 1,000 diameters to determine whether *B. popilliae* spores were present.

The in vitro spores used in the test were produced by *B. popilliae* NRRL B-2309M; in this strain, about 20% sporulation occurs in colonies placed on a specific agar medium. The propagation and maintenance

1 Present address: Entomology Research Division, Beltsville, Maryland 20705.

2 Fairfax Biological Laboratory, Clinton Corners, New York. Mention of a proprietary product does not necessarily imply its endorsement by the U.S. Department of Agriculture.
will be described by Sharpe et al. in a forthcoming manuscript. Sporulated cultures were dried and stored for as much as 3 months in screw-cap vials at room temperature (21° and 27°C).

Suspensions of the NRRL B-2309M spores and of dry blood films made from infected larvae were prepared in distilled water and stored at 4°C for as much as 2 years in vaccine bottles fitted with rubber stoppers. The aqueous suspensions (total volume of about 1 μl) were injected parenterally into each larva with a 1-ml tuberculin syringe, 27-gauge needle, and a Dutky-Fest microinjector (Dutky and Fest, 1942). Also, larvae were fed the aqueous suspension directly by placing 1 to 2 μl over the mouthparts with the microinjector and allowing the larva to imbibe the droplet. Larvae fed or injected with spore suspension were placed singly in tins containing uninfected moist soil and grass seed. Dust concentrates of strain NRRL B-2309M for inoculation of soil were prepared by mixing the spores with calcium carbonate [concentrations of spores were determined by the method of Dutky (1942)]; dust concentrates of B. popilliae spores from infected larvae were obtained commercially. The desired concentrations of dusts were mixed with soil, a 1 g mixture of redtop and white clover (1:1) seed/100 g of soil was added, and the soil was moistened with formaldehyde (40% USP solution) diluted 1:1,000 with water. The formaldehyde prevented damping-off of the clover seedlings. Then the soil was distributed into 3-oz tins (Ellisco style 22), and one larva was added to each tin (Dutky, 1942).

The soil was moistened and the seed were replenished as necessary throughout the tests.

RESULTS AND DISCUSSION

No infections resulted when 50 larvae were exposed to soil containing 2 billion spores/kg of B. popilliae strain NRRL B-2309M for 28 days: 36% of the exposed larvae survived to the prepupal stage, compared with 40% of 25 larvae exposed to untreated soil. The other larvae died from unknown causes. In contrast, 60% of 25 larvae were infected when they were exposed to soil containing 2 billion spores/kg of B. popilliae prepared from a commercial dust, and only 12% survived to the prepupal stage. None of the larvae exposed to untreated soil developed milky disease.

According to Dutky (1963), a dose of 2 billion spores/kg should cause about 84% diseased larvae after 28 days at 30°C. However, in the present test, even the commercially prepared dust was 24% below this figure. Perhaps we would have had a higher percentage of diseased larvae if the natural rate of mortality of the native larvae had not been so high. We do not understand the failure of the NRRL B-2309M strain to induce infection, but survival of larvae exposed to untreated soil and to soil treated with strain NRRL B-2309M compared favorably, an indication that the exposed larvae were not dying from apparent infections of milky disease.

Since exposure in soil to B. popilliae strain NRRL B-2309M did not result in any infectivity, we compared the infectivity of B-2309M spores and of spores from dried blood films of infected larvae. Since both sources of B. popilliae were held at least 4 months in the dry state, no viable rods existed in the cultures.

Table 1 shows that when 10,000 spores of B-2309M were injected into each of 25 larvae, 24% of the larvae developed the disease compared with 40% of those injected with spores from the dried blood film. St. Julian and Hall (1968), using strain NRRL B-2309, obtained rates of infection of 7.4 to 22.2% when they injected strain NRRL B-2309 at a rate of 100,000 spores/larva. Strain NRRL B-2309M spores were therefore as viable or more viable...
than strain NRRL B-2309, but only 60% as viable as the spores from the dried blood film. By Dutky's (1963) Equation 4,

\[(\% \text{ infection}) = \frac{1}{2} \times 3.051 \log_{10} N - 5.2\]

the percentage of larvae infected by the dried blood film should have been 49% for 10,000 spores and 16% for 1,000 spores. Thus, these spores had not lost their infectivity (Table 1). Also, the percentage of larvae infected by injection of strain NRRL B-2309M spores proved that the strain had not lost infectivity by aging. However, we could not be sure that the failure of these spores to infect larvae in the feeding dust tests was not caused by such factors as the weak solution of formaldehyde or the soil pH. To eliminate these factors as a cause of loss of viability and to assure ourselves that each larva was getting a dose of spores that should initiate infection, we administered droplets of spore suspension containing 200,000 spores of strain NRRL B-2309M to each of 50 larvae, droplets containing 280,000 spores from a dried blood film in the same way to each of 50 larvae and droplets of water to each of 25 larvae.

The B-2309M spores did not infect any larvae, but spores from the blood film infected 60% of the larvae, and one larva fed a water droplet became infected (as a result of a natural infection present in the larvae). During the 28-day test, 52% of the larvae were fed water only, 55% the droplets containing spores of B-2309M, and 20% the droplet containing spores of a blood film survived to the prepupal stage. It appeared that the larvae fed 200,000 spores of B-2309M were not adversely affected. Since the dose of B-2309M spores administered to the larvae in this test was 20 times greater than the dose that caused 24% infection when it was injected, we felt that it had been sufficient to induce an infection. B. popilliae strain NRRL B-2309M was therefore not infectious to Japanese beetle when it was administered to larvae via the alimentary canal at the doses tested. The reasons for the lack of infectivity were not apparent.

### REFERENCES


