Use of Coatings to Protect Lyophilized *Bacillus popilliae* from Moisture

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Received for publication 7 May 1973

Lyophilized cells of *Bacillus popilliae* were protected from moisture when suspended in pellets of tung oil polymer which were then coated with paraffin wax. The survival of the protected cells at various levels of relative humidity (RH) and under various storage conditions was determined. During 6 months of storage, moisture appeared to have little effect on survival of the cells when the RH level was 22% or less; but, at higher RH levels, survival declined upon storage. Viable cells were recovered when pellets were stored for 3 months at 35% RH, 2 months at 42% RH, 1 month at 50% RH, and 4 days in distilled water. Under field conditions, some cells survived at least 1 week of storage.

Spores of *Bacillus popilliae* Dutky have been used successfully in biological control of the Japanese beetle, *Popillia japonica* Newman (2). However, attempts to develop a method for the economical production of infective spores in vitro have had only limited success, and interest has developed in the possibility of using stabilized vegetative cells for control of the Japanese beetle.

Vegetative cells of *B. popilliae* have been preserved by lyophilization (1, 3, 5). The lyophilized cells maintain their morphological and cultural characteristics and ability to initiate disease in susceptible hosts (3). In a previous report (4), it was shown that lyophilized cells of *B. popilliae* survived for at least 1 year in dry soil, but viability was lost in less than 1 month when the relative humidity above the soil was 42% or higher. For lyophilized cells to be useful for control of the Japanese beetle, they must be protected from the destructive effects of moisture.

In this study (portion of a thesis presented by the senior author in partial fulfillment of the requirements for the M.S. degree in bacteriology at North Dakota State University), lyophilized cells of *B. popilliae* were incorporated into pellets of tung oil polymer and coated with paraffin wax to protect the cells from moisture.

**MATERIALS AND METHODS**

**Organism and medium.** The strain of *B. popilliae* used and the medium for growth have been described

1 Published with the approval of the Director of the North Dakota Agricultural Experiment Station as Journal Article no. 413.
maintained at 55 to 60 °C in a water bath, removed immediately by means of a small wire loop, and placed in a sterile petri dish for several minutes to allow the coating to harden.

Storage conditions. The various relative humidity (RH) levels were maintained with saturated salt solutions as described by Robinson and Stokes (7). Dry air (0% RH) was maintained in a desiccator containing CaSO₄. For exposure to the various moisture levels, the wax-coated pellets were placed in 50 g of sterile soil contained in pint jars with loosened screw-cap lids and placed in desiccators containing the saturated salt solutions. Pellets stored in water were placed in screw-cap dilution bottles containing 100 ml of sterile, distilled water and stored at room temperature. For exposure to field conditions, the pellets were buried approximately 2 inches (5.08 cm) below the soil surface of an outdoor garden.

The climatological statistics for the period of storage under field conditions during the month of June were as follows. During the month, the average temperature was 67.8 °F, with a range of 38 to 91 °F; the average RH was 59.9% and ranged from 43 to 97%; the total precipitation amounted to 9.99 inches (2.51 cm).

Viable counts of protected cells. Control and experimental counts were obtained by grinding the pellets individually in 10 ml of 0.1% tryptone solution in a Sorvall Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.), speed setting five, for 1 min. Serial dilutions were prepared in 0.1% tryptone, and appropriate dilutions were plated in replicate on five standard medium plates. Pellets removed from garden soil were rinsed for 2 min in a 0.1% solution of Roccal (Winthrop Lab., N.Y.) to remove surface contaminants prior to determination of viable cell counts. Plates were incubated for 4 days at 25 to 28 °C. Three pellets were used to determine each viable count, and recorded counts are log averages of the replicate counts.

RESULTS

Incorporation of lyophilized cells of B. popilliae into tung oil polymer pellets coated with paraffin wax increased the survival of the cells upon storage in the presence of moisture. Fig. 1 shows the survival of lyophilized cells contained in wax-coated pellets that were stored at various moisture levels. Immediately after application of the wax coating, three pellets gave an average count of 1.2 x 10⁶ viable cells per pellet. After 6 months of storage at 0, 11, and 22% RH, survival ranged between 0.3 and 1.3% of the original cells per pellet. At the higher RH values, viability of the protected cells decreased more rapidly. Some cells survived for 3 months at 33% RH, 2 months at 42% RH, and 1 month at 50% RH.

Pellets stored in distilled water showed an average cell survival of 11.5% after 4 days but no survival after 2 weeks. Under field conditions, 25.7% of the cells survived 1 week, but no cells were recovered after 4 weeks.

![Fig. 1. Survival of lyophilized B. popilliae at various levels of RH. Cells were contained in tung oil polymer pellets coated with paraffin wax.](image)

DISCUSSION

The results indicate that the survival of lyophilized B. popilliae in the presence of moisture was increased when the cells were incorporated into pellets of tung oil and coated with paraffin wax. Lingg and McMahon (4) found that lyophilized cells of B. popilliae could not survive in soil for even 1 month when the RH above the soil was 42% or higher.

Although some protection was given to the cells, the moisture-proofing ability of the paraffin wax coating was limited. Paraffin wax is a moistureproof coating only when maintained as a continuous film. The thin layer of wax that was applied to the pellets was brittle and easily cracked. Once the coating had cracked, moisture was able to enter the pellets and destroy the cells. It may be possible to increase the durability of the wax coating by incorporating certain modifiers into the paraffin wax. Recent experiments indicate that the addition of a small percentage of rubber may cause a significant increase in the protective ability of the coating and thereby improve cell survival in the pellets. Cells incorporated into tung oil pellets coated with a 97:3 (wt/wt) mixture of paraffin and rubber cement have remained viable for at least 10 weeks when stored in distilled water.

The tung oil polymer proved to be a useful primary coating for the cells. Tung oil, or Chinawood oil, is a natural drying vegetable oil that absorbs oxygen from the air and forms a solid, water-resistant polymer. The drier, tri-n-octylaluminum, increased the polymerization rate of the oil and enabled the formation of uniform pellets from drops of the quickly polymerizing oil.

The results show that lyophilized cells can be coated to protect them from adverse conditions. If the wax coating can be improved, the use of
vegetative cells of B. popilliae for control of the Japanese beetle may be possible. The coated cells must be infective upon ingestion by beetle larvae. The coatings used, a natural plant product and a wax, have potential as substances that larvae will be able to ingest. If some of the lyophilized cells are released into the gut of the larvae, by mechanical disruption or digestion of the pellet, infection may occur. When injected, vegetative cells produce infection at much lower dosages than spores. According to St. Julian et al. (8), the injection of 300 to 1,000 viable vegetative cells into the hemolymph of larvae causes 50 to 80% of the larvae to become grossly infected. By comparison, 10⁶ to 10⁸ spores must be injected to gain a comparable percentage of infection. These workers believe that a relatively large number of spores, compared to vegetative cells, are needed for optimal infectivity because only a small number of spores germinate in the larvae hemolymph. Studies to determine infectivity of pelleted cells are planned and will be the subject of a further report.

Although the purpose of this study was to develop a method of preserving vegetative cells for use in biological control, results obtained suggest other uses for such stabilized cells. Cells lyophilized by the procedure used in this study and stored in vacuo at room temperature for 6 months showed a survival rate of 2.8% of the original cells (J. A. Cloran, M.S. thesis, North Dakota State Univ., Fargo, 1973). When cells were incorporated into tung oil pellets coated with paraffin wax and stored at 0% RH for 6 months, 1.3% of the cells per pellet retained viability. These results suggest that the technique may be useful for preserving stock cultures. A culture collection of pelleted, lyophilized cells would be compact and easily stored, handled, and maintained. One small vial could contain enough pellets to start over 100 cultures. If the surface of a pellet became contaminated during handling or storage, it could be disinfected by rinsing the pellet in a common disinfectant prior to crushing of the pellet for initiation of a new culture.

This method of preserving stock cultures is somewhat similar to the technique described by Nagel and Kunz (6). Their method involves the coating of glass beads with a mixture of broth culture and horse blood. Beads are then stored in a freezer at −70 C. Pelleted, lyophilized cells may be stored in the refrigerator or at room temperature.

ACKNOWLEDGMENTS
We thank E. Kohn, formerly of the Department of Polymers and Coatings, North Dakota State University, for suggesting the use of the tung oil polymer, and J. R. McDermott for his technical assistance in preparation of the polymer.

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