

A Quantitative Infection Method for Corn Borer Larva

An Honors Thesis (ID 499)

By

Kristi H. Dygert

Thesis Director

Harold L. Zimmack

Ball State University

Muncie, Indiana

May 1979

5/20
100
10
2-45
10
100
100

INTRODUCTION

I began my research in insect pathology under the direction of Dr. Harold Zimmack during spring quarter of 1977. I started by familiarizing myself with the internal anatomy and life functions of European corn borer larvae (Ostrinia nubilalis). During this time I became interested in developing a rapid screening technique for potential insect pathogens. This centered around the bacterially induced disruption of the peritrophic membrane of the insect's midgut. I was able to dissect out the membrane using a dissecting microscope and then discern its state of disruption or intactness with a light microscope.

The bacteria that was used in our lab was Bacillus thuringiensis, a known insect pathogen. It has recently been utilized in integrated control programs against the Spruce budworm and cabbage pests. The bacteria are considered harmless to man.

My biggest problem was in quantitatively infecting the larvae with the bacterial suspension. I tried various methods and finally found one that seemed to warrant further study. The method and its effectiveness will be described in this paper.

If my screening technique were to be valid I needed to find lethal dose (LD) levels for the corn borers

when treated with Bacillus thuringiensis. As it turned out this problem resulted in a research project in itself, and my research concerning peritrophic membranes was permanently postponed. This paper is concerned with explaining my infection method and resulting preliminary LD data, thus preparing the way for continued research by other persons in the Insect Pathology lab.

FACILITIES

For the past three quarters my research has been centered upon developing a new infection method. Prior to and during this time I worked in and had immediate access to the Insect Pathology Research Lab (CL 225) at Ball State University. A dissecting microscope, light microscope, and various implements were available to me. An insect incubator, refrigerator, and necessary corn borer larvae food medium materials were also present in the lab. The corn borer egg masses normally arrive weekly from the Ankeny, Iowa, Corn Borer Investigation Station. Cheryl Wibbens, an assistant working for Dr. Zimmack, reared the larvae I utilized in my experimentation. The bacteria I worked with was kept refrigerated, as directed by the manufacturer, until it was prepared in broth form for infection of the fifth instar corn borer larvae.

METHOD

The data I obtained was for fifth instar European Corn Borers. The pathogen utilized was Bacillus thuringiensis.

The procedure was that upon arrival of the egg masses from Ankeny, Dr. Zimmack or his assistant would transfer the egg masses to newly prepared food media. Upon their emergence from the egg mass groups of two or three larvae would be transferred to vials of fresh media. They were incubated at $30^{\circ}\text{C.} \pm 1^{\circ}$ throughout their larval development.

The larvae shed their head capsules when going from one instar stage to the next. By monitoring the larval development I could determine when they were in their early fifth instar stage. I started the bacterial suspension five days prior to that stage using DIPEL brand Bacillus thuringiensis in broth form. The bacteria is almost entirely in the spore stage by the end of the five day period. The quantity of bacteria added to the broth was varied to obtain different infecting levels.

On the day of the test run the bacterial spores and cells are counted using a hemocytometer. The counting chamber I use has Improved Neubauer ruling. The small squares are $1/400 \text{ mm}^2$ and the depth of the fluid between the cover glass and the ruled surface is $1/10 \text{ mm}$. Therefore, the volume of fluid covering one of the

smallest squares is $1/400 \text{ mm}^2$ x $1/10 \text{ mm}$ which is equal to $1/4000 \text{ mm}^3$. Five groups of sixteen of these smallest squares are counted out of the twenty-five groups in the chamber. The group in the center and each group from the four corners are counted. The fluid volume in the hemocytometer is: $1/400 \text{ mm}^2$ (area of smallest square) x 16 (number of squares per group) x 5 (number of groups counted) x $1/10 \text{ mm}$ (depth of chamber) = 0.02 mm^3 .

To obtain the number of cells and spores in 1 mm^3 multiply the cells counted in 0.02 mm^3 by 50. I used 0.01 cc of the bacterial suspension which is ten times the amount per 1 mm^3 . This will have to be multiplied in and any dilution factors are also taken into account. (Sample computation on the Data sheet.) Before counting, the bacterial suspension is thoroughly mixed with a Vortex Genie test tube mixer to avoid cell clumping.

Approximately twelve hours before the larvae are infected they must be removed from the food medium and put into Petri dishes, which contain wet paper toweling to prevent dessication of the insects. This period of starvation did not in any noticeable way adversely affect my control larvae and it is very helpful in persuading the corn borers to eat the food medium and thus the bacteria.

A special food media that is dryer than the standard media used in the lab is prepared the day of the test run in the manner outlined on the following page.

Instructions for preparation of the dry food media:

300 ml H₂O
13.5 g Agar
26 g Wheat germ
6 g Ascorbic acid
4.5 g Vitamin supplement
19.5 g Dextrose
19.5 g Casein
1.5 g Cholesterol
2.5 g Salt
10.5 g Yeast

Cook the agar with one-half of the water at 70°C for five minutes. Add the Wheat Germ and cook for fifteen more minutes. In the blender dissolve the ascorbic acid and vitamin supplement in the remaining water and then add the rest of the dry ingredients to the blender and mix for two minutes. Add the cooked material to this mixture and blend for two more minutes. Pour this mixture into autoclaved glass petri dishes to a depth of approximately 1/8 inch. The media will solidify quickly. It should be used as soon as possible as it tends to collect moisture and becomes sticky.

Glass tubes are cut to a length of 4 cm and their diameter is 3 mm. The glass tubes are autoclaved before their use in the experimentation. One of the ends of the glass tube is put into the dry food media and it picks up about a 1/8 inch section of this food. That end of the tube is then sealed with melted paraffin to prevent any leakage of the bacterial suspension that will be added. The paraffin should be at a low temperature just between the liquid and solid stage. This will avoid burning the food media in the tube.

The bacteria are maintained in a dispersed state in their fluid media by using the Vortex mixer throughout the testing. One one-hundredth of a cubic centimeter of the bacterial suspension was added to the dry food in the glass tube via a syringe and needle. This amount of fluid mixed well with the dry media.

The insect is held in one hand and the freshly prepared tube is in the other hand. The insect's head is directed into the tube and he is carefully prodded in from behind with a finger. After the insect has crawled into the tube, a cotton plug is inserted into the open end to prevent his escape. This is repeated with all of the test insects.

After the insects are all in their tubes, they are incubated and faithfully monitored. When an insect has consumed all the food in his tube, that insect is placed into a fresh vial with the standard food medium. Each insect is then observed to see if it dies or pupates healthily. Some insects pupate, but die in the process. These are also included in the mortality count.

The entire process usually requires about three hours. This will vary depending upon the number of insects being infected. Monitoring is done intermittantly throughout an additional five or six hour time block.

It was imperative to find out if the method itself would harm the insects. Prior to my testing with bacteria I used sixty-five control insects without any fatalities.

My controls underwent the exact same process that was described previously except instead of adding a bacterial suspension to the dry media I added 0.01 cc of nutrient broth to it. I noticed that not all of these insects were eating the food media and upon closer examination I found that light capsule color definitely appeared to be a distinguishing characteristic of the larva which consumed all of their food.

DATA SHEET

I. Efficiency of Infection Method - Control Insects

Number of controls = 65
 Number consuming food = 31
 Percent efficiency of method = 48%

II. Efficiency of Infection Method - Test Insects

Number of insects = 94
 Number consuming food = 59
 Percent efficiency of method = 63%

III. Bacterial Counts and Corresponding Mortality

<u># Tested</u>	<u># Consuming Food</u>	<u># Dead</u>	<u># Bacteria Ingested</u>	<u>% Fatality</u>
30	19	9	734,500	47.4%
29	19	13	2,430,000	68.4%
35	21	19	5,525,000	90.5%

IV. Hemocytometer Sample Calculation

1469 (Bacterial count from 5 square groups) x 50
 (Volume factor) x 10 (Infection factor) = 734,500

DISCUSSION

The primary goal of my research was to develop a method that could be used to conveniently and accurately infect the corn borer larvae. This has been a fundamental drawback in many of the research projects in the Insect Pathology Lab.

There are several other methods of infection presently used in the lab. They include: immersing larvae in a test tube of bacterial suspension, applying the bacteria in a nutrient broth to the egg masses pre-emergence, and infecting with a microapplicator. The first two methods are not quantitative, and therefore are unacceptable for my purposes. The microapplicator has proven very difficult to use and very little success has been reported with it in our lab. The technique requires that the insect be infected via a needle, which can easily damage the insect. I believe my method to be a more natural means of infection because the insects are not injured in the process.

The efficiency of my method is reported on the data sheet. The control and test group results are different for the following reason. When I began using my control larvae, I noticed that those with lighter head capsules were consuming the food media in their tubes much more readily than were the dark headed insects. The **head** capsules are light brown in the early part of an instar stage, but they darken to an almost black tone

at the end of the stage. I used insects with lighter head capsules for the remainder of my control group and as my test insects. This is probably why the test insects exhibited higher method efficiency than did the controls.

The data concerning the Lethal Dose levels is only preliminary, and should not be considered conclusive. The results will have to be replicated to demonstrate validity, and many levels will have to be obtained if an LD standard curve is desired.

The standard curve for bacterial broth concentrations could also be constructed using absorbance readings, (Spectronic 20), rather than the hemocytometer counts. Another possible method would be to use serial dilutions and plating to obtain bacterial colony counts. These and other methods of obtaining a bacterial count might be considered by persons using my infection method.

The bacterial counts I obtained should probably be considered to be the minimum number of bacteria present. It frequently takes several hours for the insects to consume their dry food medium and bacterial suspension mixture. In this time the bacterial cells could conceivably vegetate in the food media and the corn borer subsequently could ingest more bacteria than recorded. The counts should therefore be considered minimum until more study is conducted.

CONCLUSIONS

Solving unforeseen problems is an important aspect of any research. Although I had to abandon the study of the peritrophic membrane for the endeavor described in this paper, I feel that my time was well spent. However, the study of the peritrophic membrane as a screening technique is important, and should be fully studied once LD levels are conclusively determined.

The preliminary LD data indicates levels of approximately 50, 70, and 90. Lethal dose levels of 50 and 90 percent are very important for research, and if my values are replicated and verified the research projects in the Insect Pathology Lab will be greatly facilitated.

The hemocytometer method of obtaining bacterial counts is promising, but more testing is required before anything conclusive can be said about the reliability of this procedure. An accurate bacterial count must be obtained for my infection method to be utilized in the lab with other research projects.

My method is an effective means of giving each larva a known amount of a bacterial suspension in a nontraumatic fashion. With continued utilization, this technique may prove to be even more efficient than noted in this paper. Other researchers should study the LD problem, employing the technique that has now been worked out for their use.