The Immune Response of the Tobacco Hornworm (*Manduca sexta*) and Its Effect on Bacterial Growth

Sawyer T. Duffey and Jeremy D. Blaschke
Union University Department of Biology, Jackson, TN

Abstract

The immune response of the tobacco hornworm (*Manduca sexta*) is comparable to the mammalian immune response. Antimicrobial peptides (AMPs) are an essential part of the tobacco hornworm immune response and the mammalian innate immune response. A tobacco hornworm colony was reared, and tobacco hornworms of similar instars were chosen for inoculation. These tobacco hornworms were inoculated with *Escherichia coli*, *Staphylococcus epidermidis,* and a mixture of both bacteria, in an attempt to initiate an immune response. The hemolymph of the immune challenged tobacco hornworms was extracted and a microplate assay was performed to test the tobacco hornworm immune response. The infected tobacco hornworm hemolymph showed a noticeable immune response in *E. coli* (Gram-negative) broth culture up to 13 hours and in *S. epidermidis* (Gram-positive) broth culture up to 7 hours.

Figure 1. *Manduca sexta*

Introduction

There is concern among the scientific community that the current effort to develop new antibacterial drugs to treat multidrug resistant bacteria will soon be insufficient (Freire-Moran *et al*. 2011). The discovery of novel drugs may help solve this problem, and antimicrobial peptides produced by insects may present a large and diverse group of new drugs that can combat multidrug resistant bacteria.

Antimicrobial peptides are not present in large quantities in the hemolymph of a normal insect, but the rapid production and onset of antimicrobial peptides is induced after injury or an injection of microbes. The bacteria used to inject the tobacco hornworms (*Manduca sexta*, Figure 1), in this study was *Escherichia coli*, *Staphylococcus epidermidis*, and a mixture of the 2 bacteria, respectively. The aim of this research was to evaluate the immune response of the tobacco hornworm for antimicrobial benefit, by inoculating the tobacco hornworm with bacteria and using a microplate reader to test the hemolymph for antimicrobial properties.

Figure 2. Transferring *Manduca sexta* into new rearing chambers containing tobacco hornworm food

Methods

Rearing the Tobacco Hornworm Colony

Tobacco hornworm eggs were ordered from Carolina Biological Supply Company, along with tobacco hornworm food. Clear plastic cups were used as rearing chambers (Figure 2). The tobacco hornworms were allowed to grow until they reached their fourth instar.

Immune Challenge

A group of 30 tobacco hornworms of similar size and instar were selected for treatment. The 30 tobacco hornworms were then randomly assigned to 1 of 2 treatment groups, uninfected or infected. The tobacco hornworms in the "infected" treatment group were randomly assigned to be inoculated by 1 of the following solutions: *E. coli*, *S. epidermidis*, or a mixture of *E. coli* and *S. epidermidis*. The bacterial cultures of *E. coli* and *S. epidermidis* were 1:100 dilutions. The tobacco hornworms in the "uninfected" treatment group were randomly assigned to receive one of the following treatments: inoculation with phosphate buffer saline (PBS), mechanical injury via 23-gauge needle behind the third proleg, or no treatment.

Hemolymph Extraction

The tobacco hornworms were placed on ice and chilled for 15 minutes. Scissors and forceps were then sterilized by dipping them in ethanol. One tobacco hornworm at a time was removed from the ice using forceps and surface sterilized by dipping it into ethanol and quickly drying. The third proleg was clipped and hemolymph was pooled into labeled micro-centrifuge tube. All hemolymph from each test group was pooled into one micro-centrifuge tube and kept on ice.

Microplate Assay

A 96-well plate containing a negative and positive control as well as the experimental groups was set up and allowed to incubate in a microplate reader for 24 hours at a temperature of 37°C while continuously shaking at 200 rpm. The negative control contained antibiotics. The positive control contained just bacteria. The optical density was measured hourly to evaluate growth curves.

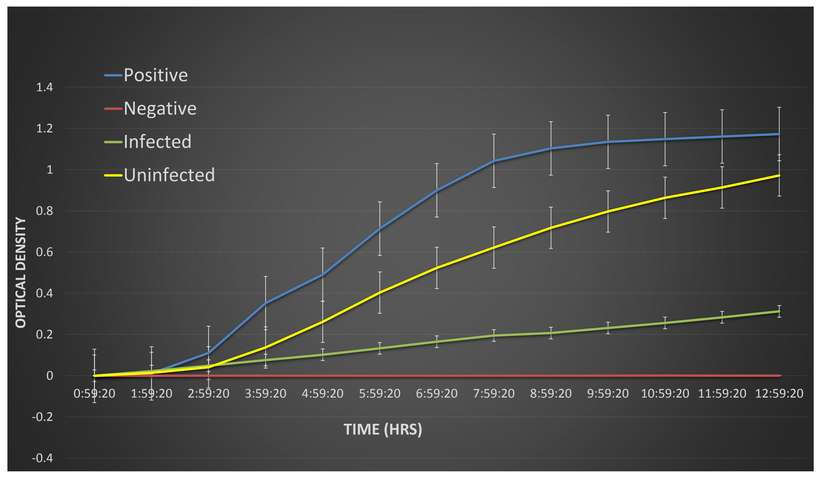

Figure 3. Optical density measurements of *E. coli* broth culture (positive) in the presence of uninfected and infected tobacco hornworm hemolymph and antibiotics (negative)

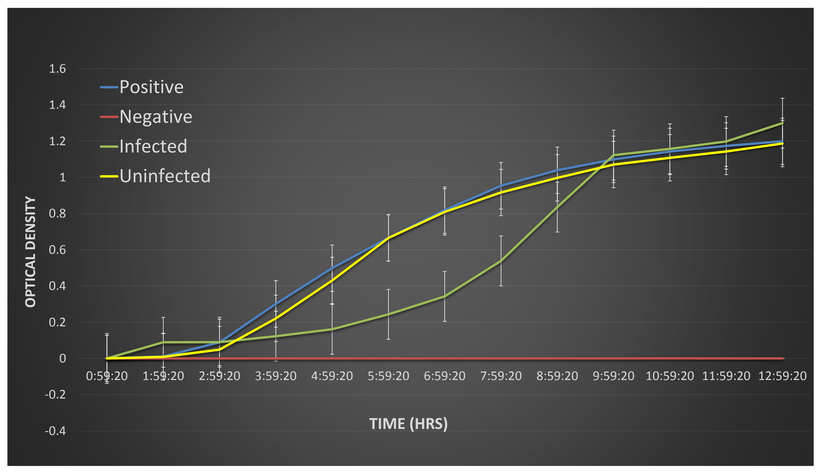

Figure 4. Optical density measurements of *Staph epidermidis* broth culture (positive) in the presence of uninfected and infected tobacco hornworm hemolymph and antibiotics (negative)

Results

The hemolymph of the infected tobacco hornworms produced a noticeable immune response to *E. coli* (Gram-negative) up to 13 hours (Figure 3), and to *S. epidermidis* (Gram-positive) up to 7 hours while in the microplate reader (Figure 4). The negative control group, containing antibiotics, showed no bacterial growth, and the positive control group grew bacteria exponentially. The uninfected group showed a lower optical density than the positive control and a higher optical density than the infected group in both the *E. coli* and *S. epidermidis* broth cultures. Melanization may have occurred at 13 hours in the *E. coli* broth culture and at 7 hours in the *S. epidermidis* broth culture, causing optical density readings to grow exponentially (Figure 5).

Discussion

The immune response produced by the tobacco hornworm contained antimicrobial properties and inhibited bacterial growth. The microplate reader can be used to test growth rate of bacteria against antibiotic activity (Stevenson *et al.* 2016). Lower average optical density levels compared to the unchallenged group of tobacco hornworms, suggests bacterial challenge and mechanical insult induce an immune response in the hemolymph of the tobacco hornworm. In future research, a zone of inhibition assay could be used along with the microplate assay to confirm the findings.

Melanization co-occurred with bacterial inhibition during incubation and caused the optical density readings to grow exponentially. This could be due to several factors. Melanization plays an important role in the cellular immune response of insects (Deel and Mulnix 1999), and this could explain the melanization reaction and sudden increase in optical density seen after 13 hours in the *E. coli* assay. Another factor that could cause the melanization is phenoloxidase activity, which produces indole groups that are polymerized to melanin (Gonzalez-Santoyo and Cordoba-Aguilar 2011) . It is this enzymatic reaction that produces intermediate products helpful in fighting bacteria during the immune response.

Future research efforts centered around preventing the melanization process from occurring would help ensure any increase seen in optical density while in the microplate reader is due to bacterial growth and not melanization in the hemolymph. The protocol used in this study was effective and can be used in future studies to test for immunity at varying instars and for transgenerational immunity.

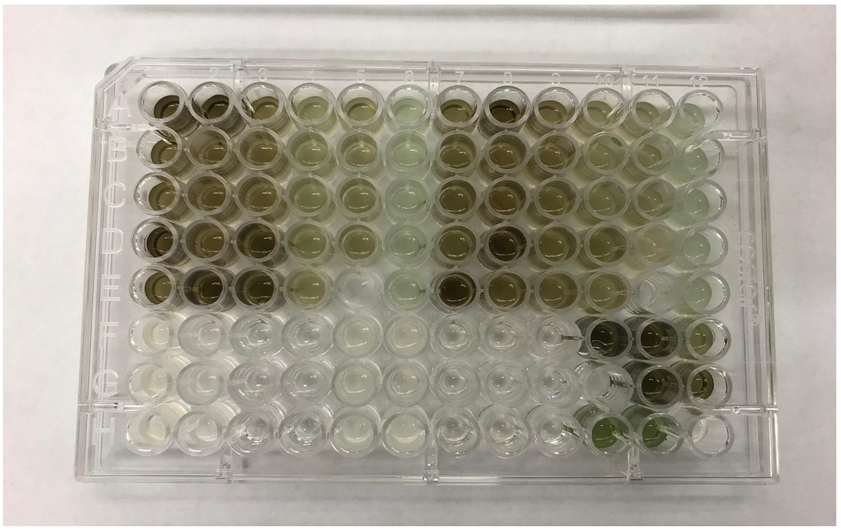

Figure 5. Melanization showing in the 96-well plate after 24 hours incubation in the microplate reader

Literature Cited

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•Gonzalez-Santoyo, Isaac, and A. Cordoba-Aguilar. 2011. Phenoloxidase: a key component of the insect immune system. *Entomologia Experimentalis et Applicata,* 142(1):1-16.

Spring 2018