POTENTIAL NEW MECHANISM FOR INSECT CROSS RESISTANCE TO DIFFERENT PROTEIN/dsRNA TOXINS

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Abstract

Studies were conducted to evaluate the potential of changes in caterpillar behavior on resistance to protein toxins like that from Bt expressed in transgenic cotton. Evidence is provided that there are natural variations of feeding rates in caterpillars, that reductions in Bt susceptibility in caterpillars can be correlated with an increased feeding rate, that a laboratory strain of the tobacco budworm resistant to Bt has an increased feeding rate, and that temperature increases which increases the feeding rate reduces caterpillar susceptibility to Bt toxin. First studies are reported on the microbiome of the tobacco budworm where temperature effects on Bt susceptibility could not be explained by changes in the bacteria microbiome. Potential mechanisms for resistance to protein toxins expressed in cotton are discussed, a mechanism by which feeding rate could affect toxin susceptibility is provided, and a rationale explained on how this same mechanism could affect other large molecular weight, biological polymers like dsRNA.

Introduction

The adoption of insect-resistant transgenic crops is expanding at double-digit rates (Christou et al., 2006), and this technology has become an essential aspect of integrated pest management for a variety of reasons. These include its level of safety to consumers and the environment, its effectiveness in insect control (especially for caterpillars), and simplification of pest management. The insect protein toxins most often used in transgenic plants are derived from the endotoxin of the bacterium, *Bacillus thuringiensis* (Bt), of which there are several types. All existing evidence indicates that these proteins are safe to the environment, wildlife and human health. Additionally, transgenic plants expressing vegetative insecticidal proteins (VIPs) have been developed and will soon be commercially available in cotton varieties. Nucleic acid-based insect toxins (double stranded RNA, dsRNA) also seem a plausible option in the future for plant expressed insecticides. The pyramiding of these different insect resistant traits, representing different target site modes of action and different biopolymer chemistries (proteins versus nucleic acids), is a proven method for delaying the evolution of insecticide resistance. However, the one common feature of this technology which theoretically could be significant in terms of resistance is the large molecular weight of these toxins, which is the focus of this paper.

The development of resistance to insecticides has been documented since 1914 (IRAC, 2005). Resistance in some species to inorganic insecticides, organic insecticides, Bt sprays, and Bt traits has developed within 2–20 years of being applied to populations of target insects (IRAC, 2005; van Rensburg, 2007; Storer et al., 2010). Looking at the tobacco budworm, *Heliothis virescens*, in cotton as one example, this insect has developed resistance to a succession of four classes of insecticides since the 1960s (Sparks, 1981; Elzen et al., 1992). The focus of our work is the potential for selecting insect populations with changes in behavior that reduce access of toxins to the midgut epithelium and result in broad resistance to large molecular weight compounds in general.

History of Insect-Plant Evolution, Insect Digestive System Structure and Function, and Potential Mechanisms of Insect Resistance to Insect Resistant Transgenic Cotton

Phytophagous insects and plants have been in conflict for millions of years prior to the introduction of modern insecticidal sprays and transgenic crops. The insect would feed on a plant, plant traits would be selected which would prevent insects from successfully feeding on the plant, and then insects would be selected that had traits that could allow them to overcome the new plant defenses. The plant defenses have included a variety of strategies to prevent insect feeding, including but not limited to morphological features preventing access to plant tissues and the production of defensive chemicals. The interesting thing to note in this competition, a common plant defensive strategy was to attack the insect system through its digestive system, very similar to the approached being used today in

the application of transgenic cotton for insect control. This evolutionary process may be continuing with the recent introduction of GMO plants into an environment shared with phytophagous insects.

The insect digestive system is composed of three regions: the foregut, midgut and hindgut (Fig. 1, top). Most digestion and the absorption of nutrients are limited to a relatively short region of the digestive system, the midgut. As illustrated in Fig. 1, this midgut region is only about a third of the length of the entire digestive tract. Because of this short length, the rate of food movement through the midgut can affect the degree of enzymatic digestion (the rate of conversion of polymers like protein to amino acids) and the rate of absorption of both digested and undigested food. Furthermore, the insect midgut and hindgut is lined with peritrophic membrane (shown in Fig. 1, bottom). This membrane moves along with the food toward the hindgut as the insect eats. The peritrophic membrane also is optimized to allow the small molecular weight products of digestion (e.g., amino acids, fatty acids and glucose) to diffuse across the membrane to the brush boarder of the midgut where they are absorbed into the insect, and it excludes larger molecular weight biological polymers which have not been digested. Large polymers are impeded from passing through the peritrophic membrane and being absorbed by the insect.

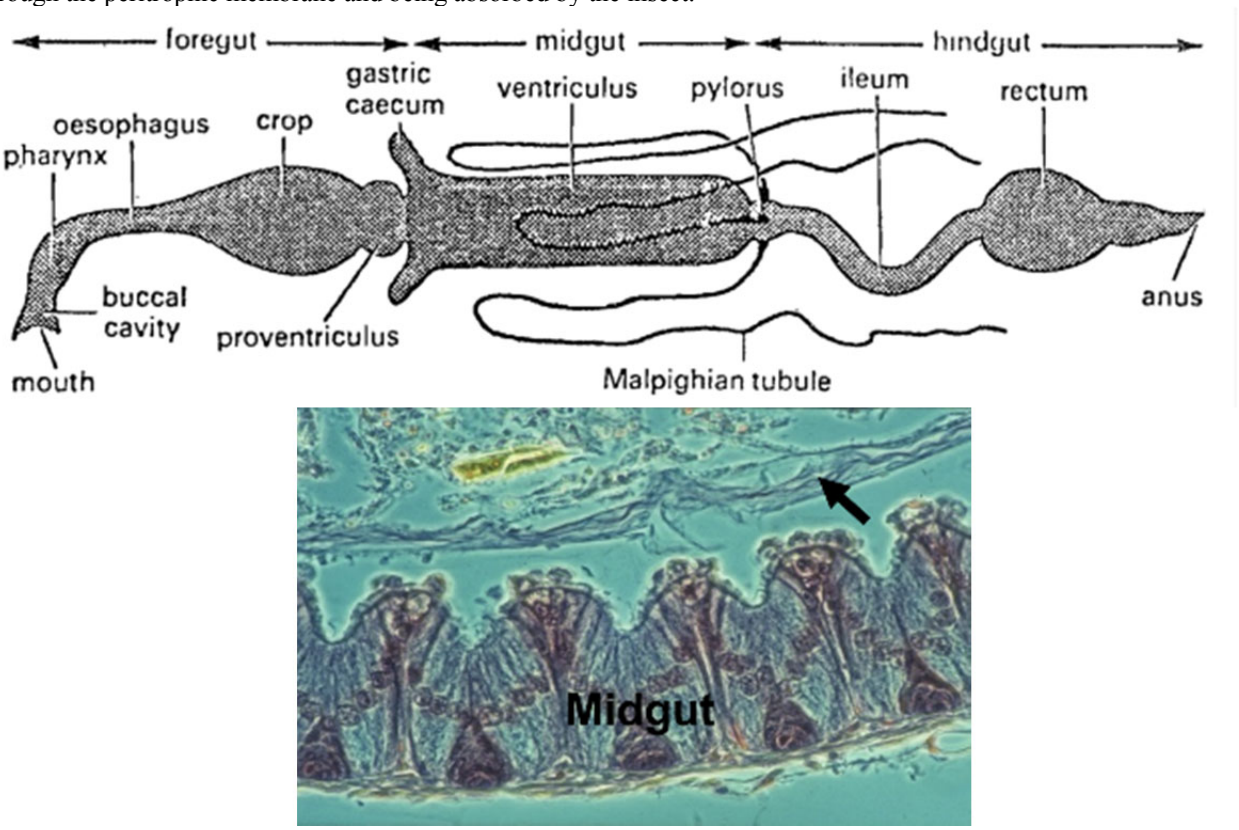

Figure 1. TOP diagram, basic structure of the insect digestive system; and BOTTOM photo, the midgut epithelium consisting of columnar cells with a brush border lining the gut lumen, muscles on the hemocoel side of the midgut and the peritrophic membrane (labeled) containing food material. Diagram and photo from a model insect to show basic features of the digestive system.

Fig. 2 lists all of the possible mechanisms associated with resistance to insecticides. The mechanisms include changes in the target site to prevent insecticide activity, increased metabolism of the pesticide to reduce its toxicity, reduced rate of penetration, and increased rates of sequestration and/or excretion. Insects can also demonstrate changes in behavior that might reduce access of a pesticide to its target site. One additional mechanism which has been understudied is the importance of the microbiome which could include bacteria, fungi, viruses and protozoa. There is an increased interest in the microbiome in the last few years because of new DNA sequencing techniques allowing the global characterization of microorganisms associated with animal and plant systems. Later in this paper, we summarize a recent examination of the microbiome of the tobacco budworm associated with environmentally induced changes in Bt toxin susceptibility (also see Dhammi et al., 2014). However, much more work is needed in this area.

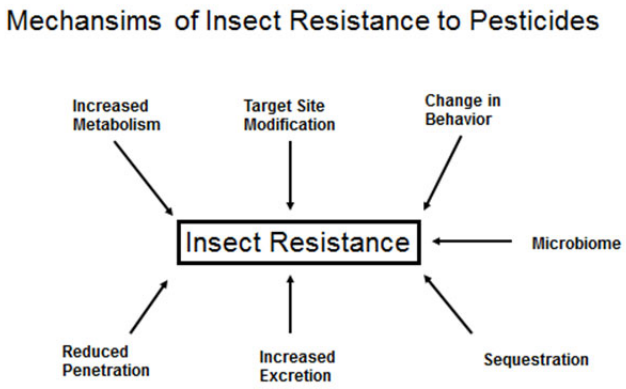

Figure 2. Potential mechanisms of insect resistance to pesticides.

One resistance method used by insects to avoid plant toxins in their diet is simply by increasing the rate of food movement through the digestive system (referred to as feeding rate hereafter). One common characteristic of the use of proteins and possibly in the future nucleic acids expressed in plants for insect control *per os* is their expected poor penetration of the peritrophic membrane compared to small molecular weight compounds. An increased feeding rate might reduce any enzymatic processing of toxins and result in reduced toxin access to the midgut epithelium beyond the peritrophic membrane. Our group has been interested in examining the rate of food consumption and environmental and population factors that might affect feeding rates on the susceptibility of the tobacco budworm to Bt toxins.

Natural Variations in Food Consumption and Environmental Factors that Affect Feeding Rate and Bt toxin Susceptibility in the Tobacco Budworm

Cabrera et al. (2011) examined the feeding rate of three different populations of the tobacco budworm collected directly as eggs from the field in three North Carolina counties. The eggs were collected on tobacco not treated with insecticides, were separated from the tobacco tissue in the laboratory, and allowed to hatch in the laboratory. Within 12 h of hatching, the neonates were placed on artificial diet with no Bt. The number of fecal pellets produced over a 24 h period was used as a convenient measure of the rate of food consumption (the more they eat, the more feces are produced). Among the three populations examined, there was a 2.5-fold variation in feeding rate as measured in terms of fecal production. Whether these differences in feeding rate are a result of heritable traits and/or environmental factors is unknown, although the experimental design was aimed at eliminating the latter. These data suggested that there are natural variations in insect feeding rates, and these feeding rates might affect Bt toxin susceptibility for the reasons discussed earlier.

There are also methods to artificially change an insect's feeding rate by changing the insect's rearing temperature. It is not uncommon for an increase of 10 °C to increase the feeding rate as well as the metabolic rate of an insect by two fold. Van Kretschmar et al. (2013) reported that fecal production at 20 °C for neonates of the tobacco budworm was 42% of fecal production at 30 °C for a 24 h incubation period on artificial diet. These experiments were repeated with a dose of MVPII in the diet that reduced fecal production at 30 °C to 54% of the non-Bt control. Based on the observed effect of temperature alone on the feeding rate, the expected fecal production for neonates of the same strain fed diet containing Bt at 20°C was 15 fecal pellets per larva. However, the actual fecal production rate observed was lower, 60% of the expected rate. The reduced rate of food ingestion and passage rate through the gut may have increased the insect's susceptibility to the Bt toxin. If gut processing of the toxin to an active form is important in its insecticidal activity, it would be expected that a reduced temperature would reduce this activation step and hence toxicity. It is possible then that an even greater impact of changes in feeding rate on Bt toxin susceptibility is occurring in our temperature studies. Also if gut processing is an inactivation mechanism for the active toxin, a lower temperature would be expected to increase toxic activity.

Bt Resistant Tobacco Budworms Have a Higher Feeding Rate that the Parent Susceptible Strain

Van Kretchmar et al. (2013) also examined the feeding rate of neonates of the YHD2 Bt resistant tobacco budworm strain compared to a Bt susceptible (YDK) strain. These experiments were conducted using adults of similar age reared and allowed to oviposit under identical laboratory conditions and only examined the neonates from eggs produced during a narrow window of peak egg production. The feeding rates for the resistant strain on artificial diet containing no Bt toxin was 37% greater than for the susceptible strain on the same diet. One explanation for these results is that the increased rate of food movement through the gut may be one component of the resistant mechanism for Bt in this strain. The relative importance of the feeding rate versus other possible mechanisms on Bt resistance in the YHD2 strain was not determined.

Materials and Methods

Temperature Effect on Bt Susceptibility

Neonates of a susceptible strain of the tobacco budworm were reared at two different temperatures on a diagnostic dose of Bt toxin from either MVPII or leaf extract from Bollgard I. The preparation of leaf extracts and the incorporation of toxin into artificial diet were conducted using hydrateable meal pads as described before from our laboratory (Cabrera et al., 2011 and the references and patents cited therein). The appropriate no Bt controls were also conducted. The impact of temperature on the bacteria microbiome was also examined.

DNA Extraction

DNA was extracted from TBWs by a method described previously (Ponnusamy et al. 2014). Four TBW larvae were transferred to a 1.5 ml eppendorf tube and 160 µl of lysis buffer 1 was added along with 20 µl lysozymes and 20 µl proteinase K to the samples. Samples were then homogenized and incubated at 37 ^{o}C for 1h. Subsequently, 200 µl of pre-warm lysis buffer 2 was added with further incubation at 56°C for 1 h. DNA was recovered through phenol/chloroform extraction and ethanol precipitation, and the resulting DNA pellet was resuspended in 100 µl ultrapure water. Subsequently, crude DNA was purified with the WIZARD DNA Cleanup System (Promega, Madison, WI, USA).

DNA Amplification

Purified DNA was used as template to amplify the V3 region of the 16SrRNA gene (specific to bacteria) with the universal primers F357-GC (5′-GC-clamp+ CCTACGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′). The GC-clamp was added at the 5’ end of the forward primer to prevent a complete denaturation of the double-stranded fragments.

Denaturing Gradient Gel Electrophoresis

DGGE was performed using the *DCode System* (*Bio-Rad*) as described by Muyzer el al. (1993). Samples (15 µl) were loaded onto 8 % polyacrylamide/bis (37.5:1) gels with denaturing gradients from 45–55 % [where 100 % is 7 M urea and 40 % (v/v) deionized formamide] in 1× TAE electrophoresis buffer. Electrophoresis was performed at 50 V at a temperature of 60°C for 18 h. The gel was stained with SYBR green I in 0.5X TAE buffer and digitally photographed with a ChemiDoc-It^{TS2} Imager (UVP, LLC Upland, CA).

Results and Discussion

Impact of Temperature and Feeding Rate on Tobacco Budworm Mortality on Bt Artificial Diet

Previous research is suggesting that neonate budworm susceptibility to Cry1Ac toxin in artificial diet is decreased with increased temperature, that temperature increases the feeding rate, and that Bt resistant budworms have an increased feeding rate over susceptible budworms using fecal production as a measure of Bt susceptibility. We have published a number of papers previously to show the end point of fecal production can be used as an accurate measure of Bt toxin susceptibility (see Cabrera et al., 2011 and the references and patents cited therein). To further demonstrate the impact of temperature and feeding rate changes on Bt susceptibility, we examined the impact of these parameters using mortality as an end point (Fig. 3). Neonates of the Bt susceptible tobacco budworm strain (YDK) were placed on artificial diet containing a dose of MVPII that would produce mortality greater than 0 and less than 100% for the two temperatures tested (20 and 30 °C). The incubation time was 7 days. As shown in Fig. 3, a 10 degree increase in temperature which increases the feeding rate by approximately 100% reduced the percentage mortality 2 fold. This change in incubation temperature had no effect on mortality in the absence of Bt toxin in the diet (Fig. 4).

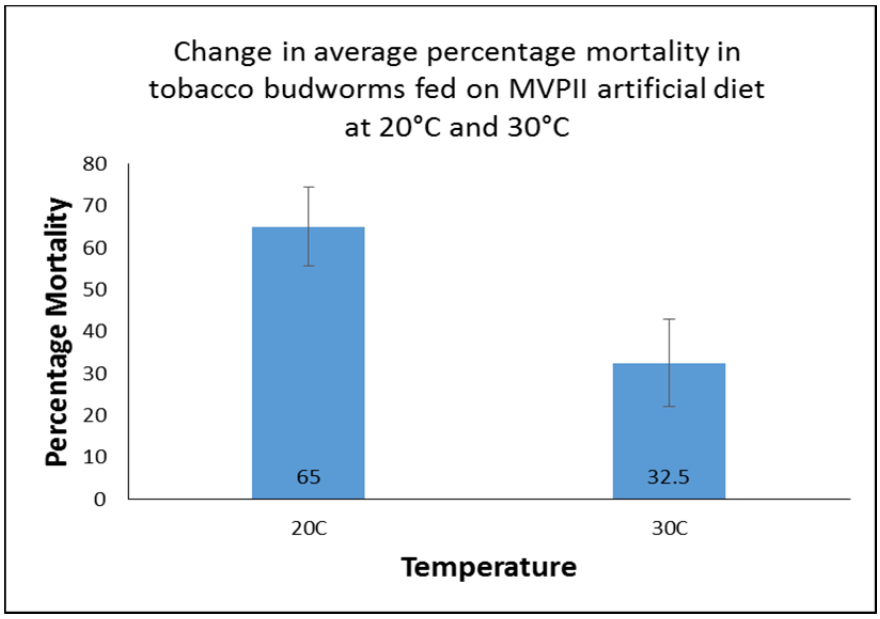

Figure 3. Impact of temperature increase on neonate Bt-susceptible (YDK) tobacco budworm percentage mortality on artificial diet containing a diagnostic dose of MVPII. The insects were maintained on the test diet for 7 days at one of the two temperatures shown and then percentage mortality determined. Mortality was defined as no movement when the insect was touched with a blunt probe. p-value = 0.0001 (significantly different); n= 64 (t-test). Error bar = 2 standard errors of the mean.

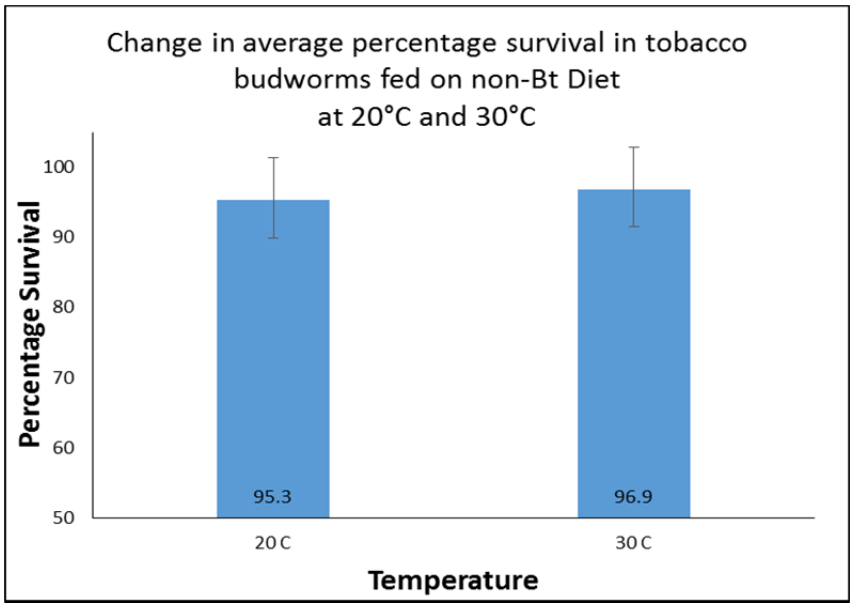

Figure 4. Impact of temperature increase on neonate Bt-susceptible (YDK) tobacco budworm percentage mortality on artificial diet with no Bt toxin. The insects were maintained on the test diet for 7 days at one of the two temperatures shown and then percentage mortality determined. Mortality was defined as no movement when the insect was touched with a blunt probe. p-value = 0.67 (no-significant difference); n=64 (t-test). Error bars = 2 standard errors of the mean.

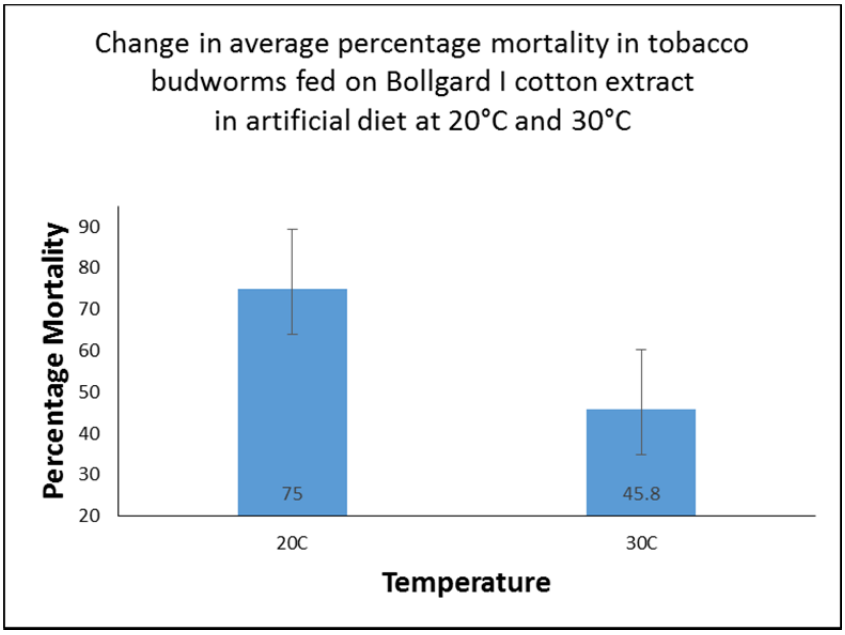

Figure 5. Impact of temperature increase on neonate Bt-susceptible (YDK) tobacco budworm percentage mortality on artificial diet containing a diagnostic dose of Bollgard I cotton leaf extract. The insects were maintained on the test diet for 7 days at one of the two temperatures shown and then percentage mortality determined. Mortality was defined as no movement when the insect was touched with a blunt probe. p-value = 0.0008 (significantly different); n=48 (t-test). Error bars = 2 standard errors of the mean.

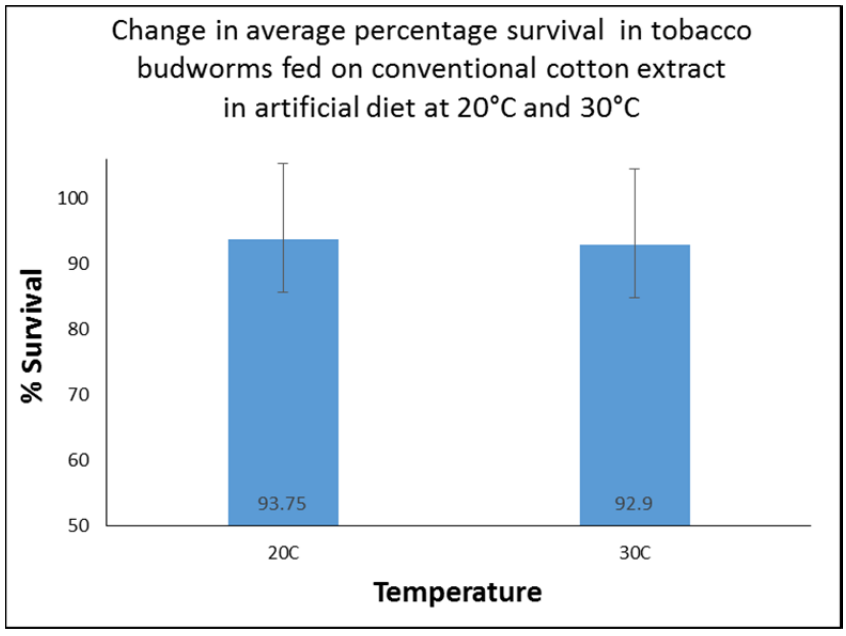

Figure 6. Impact of temperature increase on neonate Bt-susceptible (YDK) tobacco budworm percentage mortality on artificial diet made with cotton leaf extract from conventional cotton leaves (with no Bt toxin). The insects were maintained on the test diet for 7 days at one of the two temperatures shown and then percentage mortality determined. Mortality was defined as no movement when the insect was touched with a blunt probe. p-value = 0.6 (no-significant difference); n=48 (t-test). Error bars = 2 standard errors of the mean.

Impact of Temperature and Feeding Rate on Tobacco Budworm Mortality on Bollgard I Cotton Extract

The studies summarized in Figs. 3-4 using MVPII toxin were repeated under the same experimental conditions but using an aqueous extract of Bollgard I cotton leaves prepared and tested as described by Cabrera et al. (2011). As shown in Fig. 5, a 10 degree increase in temperature which increases the feeding rate by approximately 100% reduced the percentage mortality at 30 °C to 61% of that at 20 °C. This change in incubation temperature had no effect on mortality in the absence of Bollgard I extract in the diet (Fig. 6).

Potential Impact of Temperature on the Tobacco Budworm Microbiome

As discussed earlier and illustrated in Fig. 2, changes in the insect microbiome could potentially impact insecticide susceptibility or explain the effects of temperature change on Bt toxin susceptibility in our tobacco budworm experiments. Dhammi et al. (2014) provides additional background on microbiomes and budworm-cotton interactions and the methods used here to examine the global bacteria microbiome of the tobacco budworm. Fig. 7 shows that changes in temperature from 20 to 30 °C had no measurable effect on the community of bacterial species found and their relative abundance. Thus, changes in Bt susceptibility due to temperature change appears not to involve changes in the insect's microbiome. This result could be different under field conditions.

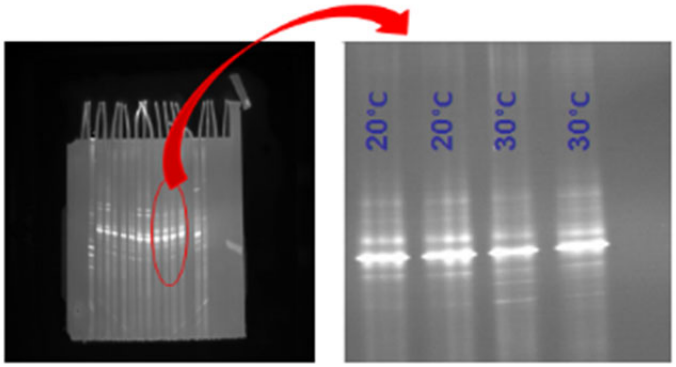

Figure 7. Bacteria microbiome of two neonates (n = 2) of the tobacco budworm (YDK strain) reared at two different temperatures for 7 days on artificial diet.

Summary

We present evidence that increased feeding rates in tobacco budworm neonates could be a mechanism for reduced susceptibility and resistance to Bt toxins. Increased insect rearing temperature reduced Bt susceptibility suggesting that environmental changes could potentially impact the performance of transgenic cotton in the field relative to caterpillar control. Temperature change had no impact on the budworm bacteria microbiome. A possible mechanism for a decreased susceptibility to Bt toxin with an increased feeding rate is reduced absorption of the protein toxin by the midgut epithelium. This may be possible due to the peritrophic membrane, designed for the differential retention of large molecular weight biological polymers like protein (and insoluble food material) and the enhanced absorption of the smaller molecular weight products of digestion across the insect midgut epithelium into the hemocoel. This is occurring at the same time as an increased backward movement of the peritrophic membrane due to increased feeding rates. If this model is correct, then the level of activity of any protein toxin or other biological polymer like dsRNA or siRNA could be affected by this mechanism. Furthermore, if this is being used as a mechanism of Bt resistance by insects in the field, the application of transgenic plant technologies could potentially be selecting for caterpillar populations with increased feeding rates.

Acknowledgments

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