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Research

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Behavioral Responses of Thrips (Thysanoptera: Thripidae) and Tarnished Plant Bug (Hemiptera: Miridae) to a New Bt Toxin, Cry51Aa2.834_16 in Cotton

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Abstract

Thrips (Thysanoptera: Thripidae) and tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae), are among the most important insect pests of cotton, *Gosssypium hirsutum*, in the mid-southern United States. These pests are currently managed primarily by insecticides; however, a new Bt toxin, Cry51Aa2.834_16 is under evaluation for control of thrips and tarnished plant bug. Experiments were conducted to evaluate the behavioral response of thrips and tarnished plant bug to Bt Cry51Aa2.834_16. Adult thrips avoided Bt Cry51Aa2.834_16 cotton in field choice tests and in separate field tests of Bt and non-Bt cotton not treated with insecticides. In a greenhouse choice test, approximately twice as many adult thrips and eggs were found on non-Bt compared with Bt Cry51Aa2.834_16 cotton. Similarly, in a field test of nontreated Bt Cry51Aa2.834_16 and non-Bt cotton, 68% of adult thrips collected were found on non-Bt cotton. In cotton that was not sprayed with insecticides, Bt Cry51Aa2.834_16 did not affect the distribution of tarnished plant bug within the canopy, although more square and flower injury was caused by tarnished plant bug in non-Bt cotton. Adult tarnished plant bug exhibited a nonpreference for diet containing lyophilized Bt Cry51Aa2.834_16 leaves and for excised Bt Cry51Aa2.834_16 squares in choice tests with non-Bt squares. The behavioral responses of these pests when exposed to this new Bt toxin will play a key role in the efficacy and potential resistance management strategies if this new technology is incorporated in an overall cotton insect pest management system.

Key words: thrips, tarnished plant bug, Bt cotton, Cry51Aa2.834_16, behavior

Thrips (Thysanoptera: Thripidae) are the most important insect pests of seedling cotton in the Mid-Southern United States (Cook 2018). The tobacco thrips, *Frankliniella fusca* (Hinds) (Thysanoptera: Thripidae), is the dominant species found on cotton in the Mid-South (Reed and Jackson 2002, Layton and Reed 2002, Cook et al. 2003, Reed et al. 2006), often composing more than 90% of all thrips collected on seedling cotton (Reed et al. 2006, Stewart et al. 2013). When left untreated, thrips injury can lead to stunted growth, delayed maturity, reduced stands, and yield loss (Layton and Reed 2002, Stewart and Lentz 2010). Thrips are typically managed using neonicotinoid seed treatments or prophylactic in-furrow insecticide applications. Imidacloprid and thiamethoxam are the primary seed treatments used by cotton growers (Cook et al. 2011). In recent years, failures of thrips control with neonicotinoid seed treatments have occurred across the Mid-South (Darnell et al. 2018) and the Southeast (Huseth et al. 2016, 2017), leading to the need of new ways to manage this pest.

The tarnished plant bug, *Lygus lineolaris* (Palisot De Beauvois) (Hemiptera: Miridae), is the key insect pest of cotton in the Mid-South. Economic damage from tarnished plant bug occurs from the beginning of squaring (flower buds) and continues through bloom (Layton 2000). Injury results from the abscission of squares and bolls, often leading to substantial yield losses (Scales and Furr 1968, Scott et al. 1985, Layton 1995, Russell 1999). Tarnished plant bug infestations are managed using foliar insecticide applications; however, the tarnished plant bug has developed resistance to many of the insecticides commonly used for management (Cleveland and Furr 1979; Snodgrass and Scott 1988, 2000; Snodgrass 1996, 2006; Snodgrass et al. 2009; Parys et al. 2017). Resistance issues have made tarnished plant bug infestations in cotton difficult to manage

and new ways to combat this pest are required to improve the profitability of the crop.

Cotton varieties expressing *Bacillus thuringiensis* (Bt) have been widely adopted for controlling key lepidopteran pests (Siebert et al. 2008). Although no commercial cotton varieties expressing Bt toxins for control of hemipteran or thysanopteran pests are currently available, Bayer CropScience (Monsanto Company, St. Louis, MO) has developed a new Bt protein, Cry51Aa2.834_16 (referred hereafter as Bt Cry51Aa2) with activity against thrips and tarnished plant bug (Baum et al. 2012, Gowda et al. 2016, Bachman et al. 2017, Graham and Stewart 2018). Graham and Stewart (2018) reported that cotton expressing Cry51Aa2 provided as good or better thrips management than an insecticide based approach. The authors also found that Bt Cry51Aa2 cotton produced similar yields to non-Bt cotton with fewer insecticide applications when both were sprayed based on current economic thresholds for tarnished plant bug. While these studies suggest that this technology could be an effective management strategy, no studies have been published on the behavioral effects of Cry51Aa2 on thrips or tarnished plant bugs in cotton.

Tobacco thrips have been reported to avoid potential resources based on insecticide use or plant types. When given a choice, they prefer leaves not treated with imidacloprid or cyantraniliprole (Groves et al. 2001, Joost 2003, Joost and Riley 2005, Jacobson and Kennedy 2011, 2013). When given a choice, tobacco thrips have shown a preference of chickweed, *Stellaria media* (L.), over tomato, *Solanum lycopersicum* (L.), for oviposition, even though tobacco thrips laid significantly more eggs in tomato plants than chickweed in a no choice test (Chaisuekul and Riley 2005).

The distribution of tarnished plant bugs within cotton plants is an important factor for both sampling techniques and management of this pest. On average, 75% of both nymphs and adults are found on the mainstem terminal and fruit and vegetative structures of the upper six nodes of the cotton plant (Snodgrass 1998). Prior to bloom, nymphs are found on fruiting structures, while adults tend to be on vegetative structures. As the crop matures to bloom, adults tend to become more dispersed throughout the reproductive and vegetative structures of the plant (Snodgrass 1998). Studies conducted to determine behavioral responses of tarnished plant bug to insecticide applications within the cotton canopy have shown mixed results. Graham (2016) showed no effect of insecticide treatment on the distribution of tarnished plant bug. Fontenot (2009), however, found that significantly more tarnished plant bugs were found in the middle third of the canopy in acephate-treated cotton plants compared with untreated cotton plants. The effects on tarnished plant bug distribution within the plant canopy from transgenic cotton plants expressing Bt Cry51Aa2 have not been reported. The distribution of some insects can be affected by the expression of Bt cry toxins. The cotton bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), has been shown to avoid structures that express high levels of Bt proteins (terminals and squares) and tend to feed more on structures with lower expression (flowers and bolls) (Greenplate 1999, Adamczyk et al. 2001, Akin et al. 2002, Gore et al. 2002). Gore et al. (2002) showed that bollworm larvae were more likely to move from the plant structure they were placed on in Bt cotton plants than non-Bt plants. While expression levels of Bt Cry51Aa2 have not been reported for individual structures of the cotton plant, the protein is present throughout the cotton plant (Baum et al. 2012).

The behavioral responses of thrips and tarnished plant bug to Bt Cry51Aa2 may play an important role in how cotton is managed for these important pests. Avoidance of Bt Cry51Aa2 expressing plants or of higher expressing parts of a plant could impact the efficacy of this toxin on thrips or tarnished plant bug. Previous field studies by Graham and Stewart (2018) showed that this toxin reduced thrips densities on seedling plants. Further, plant protection that is at least partly based upon avoidance has potential impact on resistance management strategies. The objective of this study was to evaluate the effects of cotton plants expressing Bt Cry51Aa2 on thrips and tarnished plant bug behavior.

Materials and Methods

Thrips

Choice Test (Field)

Choice tests were done in 2016 and 2017 at the West Tennessee Research and Education Center (WTREC) in Jackson, TN, to determine whether field populations of adult thrips showed a preference for non-Bt cotton compared with cotton expressing Bt Cry51Aa, in part because previous field studies showed that this toxin reduced thrips densities on seedling plants (Graham and Stewart 2018). In both years, greenhouse trays with 36 cells in each tray were planted with Bt Cry51Aa2 and non-Bt near-isogenic lines of Deltapine 393 (Monsanto Company, St. Louis, MO). Each tray had a total of 40 seeds planted, 20 Bt Cry51Aa2 and 20 non-Bt with Bt Cry51Aa2 seeds on one side and non-Bt on the other. Seeds were planted in potting soil roughly 2.0 cm below the soil surface and watered. Seeds were planted on 20 May in 2016 and on 15 May in 2017. Trays were placed in an incubator set at 29°C and 40–60% RH with a 14 h light and watered as needed. When seedlings reached the first true leaf stage, eight trays containing cotton seedlings were randomly oriented on bare soil in the alleys between replicates of the field trial described by Graham and Stewart (2018) for 24 h to allow natural infestation and oviposition by thrips. Treatments (Bt Cry51Aa2 and non-Bt) were organized in a randomized complete block (RCB). In each year, trays were considered replications with eight replications per year. After 24 h, 10 Bt Cry51Aa2 and non-Bt seedlings were collected from each tray by cutting the seedlings at the soil surface and placing in jars containing a 70% ethyl alcohol and water mixture to collect adult thrips from them. Each plant was removed from the jar and rinsed with 70% ethyl alcohol over a glass container topped with a sieve (150 µm) to collect adult thrips. Jars were then rinsed with 70% ethyl alcohol over the sieve to collect any remaining thrips left inside. The sieve was then rinsed with 70% ethyl alcohol into a gridded 100 mm × 15 mm petri dish and adult thrips were counted underneath a microscope using 10–20× magnification, and sight identified as either tobacco thrips, soybean thrips *Neohydatothrips variabilis* (Beach) (Thysanoptera: Thripidae) or other thrips (i.e., not tobacco thrips or soybean thrips).

The remaining 10 Bt Cry51Aa2 and non-Bt seedlings in each tray were placed in an insect rearing room. Seedlings were cut at the soil level, and three plants of the same isoline were placed in 50 ml centrifuge tubes suspended over a bowl of water and modified to allow plant stems to reach water to prevent desiccation. The bottoms of the tubes were covered with plumber's putty and the tops were capped to keep newly emerged thrips in tubes and to keep water from seeping into the tubes. Samples were left in the insect rearing room at a temperature of ~27°C, at 40–60% relative humidity, and 14 h of light per day for 5 d, allowing eggs to hatch and immature thrips to emerge. Seedlings were removed from tubes and both tubes and seedlings were rinsed with 70% ethyl alcohol over a glass container topped with a sieve to collect immature thrips. Finally, the sieve was rinsed with ethyl alcohol into a gridded petri dish, and the numbers of immature thrips were counted in order to evaluate any ovipositional preferences of adult thrips for Bt Cry51Aa or non-Bt cotton.

Choice Test (Greenhouse)

To further evaluate the ovipositional response of tobacco thrips to Bt Cry51Aa2 in cotton, a greenhouse study using laboratory-reared tobacco thrips was conducted in 2017 at WTREC. The tobacco thrips colony was reared at Mississippi State University on pieces of cabbage in a rearing room at a temperature of ~27°C, at 40–60% relative humidity, and 14 h of light per day in 5.1-liter Berry Thinwall Containers (PFS Sales Co., Raleigh, NC). Each pot was planted with two non-Bt seeds and two Bt Cry51Aa2 seeds for a total of four seeds per pot and six pots per treatment. Pots used were similar 5.1-liter Berry Thinwall Containers with modified lids to allow for ventilation. We prevented thrips from escaping by cutting a circular hole, approximately 10 cm diameter, and covering the hole with 100 µm nylon mesh (Midwest Filter Corporation). Similar holes were cut in the bottom of the pots and covered with the same mesh to keep soil from being water logged. Seeds were planted in potting soil about 2.0 cm below the soil surface and grown in a greenhouse. Upon full expansion of the first true leaf, pots were infested with 10 adult tobacco thrips from the laboratory colony described. Caged thrips were kept in the greenhouse for 5 d to allow for oviposition. Treatments (Bt Cry51Aa2 and non-Bt) were organized as an RCB with six greenhouse pots. Each pot was considered a replication. To determine the influence of Bt Cry51Aa2 on oviposition, leaves were cut from the stems and were decolorized by boiling 3–5 min following the lacto-phenol acid fuschin staining technique detailed by Nuessly et al. (1995) and Parella and Rob (1982). Stained leaves were cooled for 3–5 h and examined under a dissecting microscope as described by Chitturi (2005) after excess stain was removed with warm water. The total number of eggs was recorded for each treatment.

Field Test

Small-plot replicated field tests were established to evaluate the influence of Bt Cry51Aa2 on thrips. Cotton was planted in May 2016 and 2017 at WTREC and at the Research and Education Center at Milan, TN (MREC). Near-isogenic lines of DP393, either Bt Cry51Aa or non-Bt cotton, were planted and treatments were arranged as described by Graham and Stewart (2018). Treatments included Bt Cry51Aa2 and non-Bt cotton with a base fungicide seed treatment only and with an insecticide seed treatment. Thrips were sampled at the 1.5 and 3.5 true leaf stage from plots that were not treated with seed or foliar applied insecticides. Thrips were collected from seedlings and counted as previously described.

Tarnished Plant Bug

Field Behavior

An additional field experiment was conducted at WTREC in 2017 to determine the effects of cotton varieties expressing Bt Cry51Aa2 on the distribution behavior of tarnished plant bug. Plots were planted with near-isogenic lines of DP393, one expressing Bt Cry51Aa2 and one non-Bt isoline. The experiment was planted in 9 May at a seeding rate of 13.2 seeds/m. The experiment was designed as an RCB with four replications. To prevent the confounding effects of thrips damage, all cotton was treated with a commercial rate of imidacloprid (0.375 mg/seed) and thiodicarb (0.375 mg/seed) as an insecticide seed treatment, (Aeris, Bayer CropScience, Raleigh, NC). Individual plots were 10.7 m long and four rows wide with 0.97 m row spacing. Beginning at first-square and until first flower, plots were scouted and managed for tarnished plant bug based on the Insect Control Recommendations for Field Crops for Tennessee (Stewart and McClure 2017). No insecticide applications were made after flowering began.

During the second week of bloom, three sampling methods were used to collect data about tarnished plant bug density, locations within plants, and damage. The location of tarnished plant bugs was mapped on plants, by fruiting structure and node through visual examination until 25 bugs were found in each plot. Visual sampling of each individual plant began at the terminal and moved down each node and across to each fruiting structure on the respective nodes. Numbers of tarnished plant bug adults and nymphs were recorded by the node, position of fruiting structure, and type of fruiting structure (square, flower, or boll) as well as the total number of plants per plot required to find 25 bugs. Nymphs were classified based on size as either small (first and second instar), medium (third and fourth instar), or large (fifth instar). The relative density of tarnished plant bugs and various stink bug species (Hemiptera: Pentatomidae) were counted in two drop cloth samples taken in each plot by laying a black cloth between two rows of cotton near the center of the plot and vigorously shaking the plants from each row. In each sample, the number of hemipterans per 3.02 m row was counted, and species were totaled separately.

Damage caused by tarnished plant bug infestations was assessed by visually sampling 25 random squares and 25 random flowers. A 'dirty square' showed signs of feeding from tarnished plant bug as a yellow staining and a 'dirty flower' showed signs of damaged anthers, petals, and/or staining from tarnished plant bug excrement (Bagwell et al. 2007). Visual ratings of dirtiness were characterized based on subjective qualitative ratings as either low, medium, or high based on intensity of the injury. The total number and life stages of tarnished plant bugs found in the squares and flowers were also recorded in each plot. A second rating was done 2 wk after the initial rating and 25 flowers and thumb-sized bolls (≈2.3–2.8 cm diameter) were examined for tarnished plant bug injury (Bagwell et al. 2007). Bolls were assessed for puncture marks (stains) on the outside of bolls, stains on the inside of bolls, warts on the inside of bolls, and a visual estimate of percent damage of the developing lint. The severity of external boll staining was also rated on a 0–3 scale, with 0 indicating no injury and 3 indicating high injury. Because boll injury caused by stink bugs could not be differentiated from that caused by tarnished plant bug (Greene et al. 2006), stink bug population densities were also estimated. Two drop cloth samples were taken in each plot to estimate tarnished plant bug and stink bug densities. The center two rows of each plot were harvested to determine the level of yield protection from Bt Cry51Aa2 when no insecticide applications are made for tarnished plant bug after the initiation of bloom.

Ovipositional Cage Test

A cage test was conducted at WTREC in 2017 to determine whether tarnished plant bug oviposition was effected by Bt Cry51Aa2. Cotton seed, Bt Cry51Aa2, and non-Bt near-isogenic lines of DP393 were provided by Monsanto Co. (St. Louis, MO). Seeds were planted in a potting soil mix with one plant per pot. Plants were grown inside a greenhouse at WTREC until they were 8–9 nodes in size, having multiple squares but no flowers or bolls. Two plants, one Bt Cry51Aa2 and one non-Bt, were randomly placed in opposite corners in each of the eight cages. Cages were 61 cm × 61 cm × 121 cm and covered with a 24 × 20 mesh polyester netting (BioQuip Products, Rancho Dominguez, CA) and each cage was considered a replicate. In total, 15 tarnished plant bug adult females and five tarnished plant bug adult males were introduced into each cage. Insects were obtained from a laboratory colony reared at Mississippi State University and maintained on an artificial diet developed by Cohen (2000). Adults were approximately 7-d old when placed in cages so that females were likely mated. Insects were left in cages for 5 d, after

which, plants were removed, taken to the laboratory, and examined for eggs. The upper five nodes of plants were examined under a dissecting microscope so that the number of eggs, which are typically embedded into the plant tissue (Fleischer and Gaylor 1988), could be counted. The location of eggs was categorized by nodal location and plant part (stem, leaf petioles, leaf, leaf veins, and squares).

Laboratory Tests

A laboratory test was also conducted in 2017 to determine whether tarnished plant bugs exhibited an avoidance response when exposed to Bt Cry51Aa2. Tarnished plant bugs were placed in 2.12-liter plastic rectangular containers with self-sealing lids (S.C. Johnson & Son, Inc., Racine, WI). Insects were from the same laboratory colony as described previously. The containers were modified by removing the lid, except for the sealing frame and replacing the removed portion with a tulle fine mesh screen. Containers were considered replications. Lyophilized leaf tissue of near-isogenic DP393 Bt Cry51Aa2 and non-Bt cotton were pulverized into a fine powder prior to being incorporated into the artificial diet developed by Cohen (2000) at a rate of 5.11 g leaf tissue per 350 ml diet. In order to have a biologically relevant rate of Cry51Aa2, for tarnished plant bug, we tried to emulate a field rate of Cry51Aa2 toxin in the artificial diet present in leaf tissue based on the LC_{50} for nymphs of *Lygus hesperus* (Knight) as reported by Bachman et al. (2017). Diet packs containing Bt Cry51Aa2 and non-Bt lyophilized leaves were placed on opposite sides of the containers on top of the mesh screen. Thus, tarnished plant bugs in each container had the option of feeding on one Bt Cry51Aa2 or one non-Bt diet pack filled with equal parts of lyophilized plant tissue. The orientation of the containers was randomized within a rearing room maintained at 27 ± 2°C, 60 ± 5% RH, and a photoperiod of 14:10 (L:D) h. Tests were done with first and third instars and 6-d-old adults, with three or four replications (containers) for each test. Each container had a total of 25 tarnished plant bugs. The number of adult tarnished plant bugs on diet packs were examined at 1, 3, 18, and 24 h intervals, first instars were examined at 1, 3, and 5 h intervals and third instars were examined at 1, 4, 8, and 24 h intervals. Diet packs given to adult tarnished plant bugs were taken at the end of the 24-h period and examined for the number of eggs laid into each pack.

Another laboratory study was conducted in 2018 using excised squares from Bt Cry51Aa2 and non-Bt cotton plants grown in a greenhouse at WTREC. Adult tarnished plant bugs were collected locally from wild hosts, mainly *Amaranthus palmeri* (S. Watson). Tarnished plant bugs were starved for 2 h prior to the start of the study. A total of four adult tarnished plant bugs were placed into 0.94-liter plastic containers with self-sealing lids (S.C. Johnson & Son, Inc., Racine, WI). Each container had two Bt Cry51Aa2 and two non-Bt squares placed in the container corners. Containers were randomly arranged with a total of seven replications. Tarnished plant bugs were left in containers for 24 h, and the number of tarnished plant bugs on Bt and non-Bt squares was recorded at 1, 4, 8, and 24 h intervals. The number of eggs deposited in squares was also recorded after 24 h.

Statistical Analyses

Thrips

Adult thrips preference data (number and species of adult thrips on Bt Cry51Aa2 or non-Bt cotton), immature emergence data (number of larvae on Bt Cry51Aa2 or non-Bt cotton), and ovipositional preference data (number thrips eggs on Bt Cry51Aa2 or non-Bt cotton) for choice tests were analyzed using a normal generalized linear mixed model analysis of variance PROC GLIMMIX of SAS (Version 9.4, SAS Institute, Cary, NC). Bt trait (Bt Cry51Aa2) was a fixed effect in the model. Sample date, replication, and replication nested within sample date were random effects in the model to test whether significantly more numbers of adults were found on either variety, and to examine how many immatures resulted from eggs laid during the exposure period. Replication was included as the random effect for ovipositional preference data.

For the field test, a Pearson χ^2 analysis was done to test if the distribution of adult thrips differed between the Bt Cry51Aa2 and non-Bt seedlings when not treated with an insecticide seed treatment by using PROC FREQ of SAS (Verison 9.4, SAS Institute, Cary, NC). Adult thrips numbers were analyzed using a general linear mixed model of analysis of variance PROC GLIMMIX of SAS (Version 9.4, SAS Institute). Bt trait was considered a fixed effect. Year, location, year by location, and replication nested within year by location were designated as random effects to allow inferences to be made over a range of environments (Carmer et al. 1989, Blouin et al. 2011).

Tarnished Plant Bug

All field data were analyzed using a general linear mixed model of analysis of variance PROC GLIMMIX of SAS (Version 9.4, SAS Institute). Bt trait was considered a fixed effect in all models. Bt trait, node, fruiting position, fruit structure, and their interactions were fixed effects and plant and plant nested within replication were included as random effects for tarnished plant bug plant distribution data. A separate analysis was done to analyze the number of plants scouted in Bt Cry51Aa2 and non-Bt plots to find 25 tarnished plant bugs. Sample date and replication were included as random effects for analyses on injured cotton squares, flowers, and drop cloth data.

In laboratory assays, the proportions of tarnished plant bugs on diet packs and squares were calculated at each rating interval. Data over the 24-h rating period were analyzed using a mixed model analysis of variance for repeated measures (PROC MIXED, Littell et al. 1996). Bt trait was considered a fixed effect and replication was considered random with time as the repeated measure. Covariances were structured using the standard Variance Components in PROC MIXED.

In all statistical analyses, degrees of freedom were estimated using the Kenward–Rogers method (Kenward and Roger 2009). Means were estimated using LSMEANS and were separated using Fisher's protected least significant difference ($\alpha = 0.05$).

Results

Thrips

Choice Test (Field)

The Bt trait reduced the number of adult thrips found per 10 plants ($F = 28.00$; df = 1, 29; $P < 0.001$) in the field choice test. On average, 8.44 ± 0.82 (mean ± SEM) thrips were found on non-Bt seedlings and 3.81 ± 0.67 were found on Bt Cry51Aa2 seedlings. The Bt trait also reduced the number of tobacco thrips ($F = 24.55$; df = 1, 22; $P < 0.001$), as more tobacco thrips were found on non-Bt seedlings than on Bt Cry51Aa2 seedlings (Table 1). No difference was observed in the number of soybean thrips ($F = 2.48$; df = 1, 15; $P = 0.14$) or other thrips ($F = 1.57$; df = 1, 22; $P = 0.22$) (Table 1). The Bt trait did not affect the number of immature thrips that emerged from cotton naturally infested by adult thrips ($F = 2.75$; df = 1, 15; $P = 0.12$). On average, 10.88 ± 4.82 and 3.38 ± 1.65 immature thrips were found per 10 non-Bt plants and 10 Bt Cry51Aa2 plants, respectively.

Choice Test (Greenhouse)

On a per plant basis, more eggs (F = 25.29; df = 1, 10; P < 0.001) were found in non-Bt (157.17 ± 12.24) than in Bt Cry51Aa2 cotton (72.67 ± 11.51) in the greenhouse choice test.

Field Test

The proportions of adult tobacco, soybean, and other thrips were not different in Bt Cry51Aa2 and non-Bt cotton (χ^2 = 2.34; df = 2; P = 0.84) (Table 2). However, the Bt trait affected the number of adult thrips per five plants (F = 29.53; df = 1, 187; P < 0.001), as more adult thrips were found on non-Bt cotton than on Bt Cry51Aa2 cotton. The Bt trait had a similar effect on all thrips species monitored (tobacco thrips F = 23.79; df = 1, 165; P < 0.001, soybean thrips F = 11.47; df = 1, 152.5; P < 0.001, and other thrips F = 12.51; df = 1, 152.6; P < 0.001) (Table 2).

Table 1. Mean number (SEM) of tobacco, soybean, and other adult thrips per 10 plants collected from a choice test between Bt Cry51Aa2 and non-Bt seedling cotton averaged across years (2017 and 2018) in Tennessee

Thrips	Bt	Non-Bt
Tobacco thrips	2.31 (0.54)b	6.13 (0.59)a
Soybean thrips	0.56 (0.27)a	0.88 (0.30)a
Other thrips	0.88 (0.24)a	1.44 (0.42)a

Means, within rows, followed by a common letter are not significantly different (Fisher's Protected LSD, α = 0.05).

Tarnished Plant Bug

Field Behavior

The Bt trait did not affect the distribution of tarnished plant bugs within the cotton canopy (F = 0.26; df = 1, 177.7; P = 0.61). Tarnished plant bugs were found on a mean of 6.2 nodes from the terminal on Bt Cry51Aa2 cotton and 6.0 nodes on non-Bt cotton. The Bt trait also did not affect average fruiting position where tarnished plant bugs were found (F = 1.23; df = 1, 177.4; P = 0.27) (Fig. 1). No interaction of Bt trait by fruiting structure was observed on the number of adult tarnished plant bugs found on fruiting structures (F = 2.03; df = 5, 185.1; P = 0.08). However, an interaction of Bt trait by fruit structure was observed for the number of nymphs found per structure (F = 5.06; df = 5, 184.6; P < 0.001). More nymphs were found in non-Bt flowers than were found on Bt Cry51Aa2 squares and Bt Cry51Aa2 bolls (Fig. 2). No difference was observed between the number of nymphs found on non-Bt flowers, non-Bt bolls, non-Bt squares, and Bt Cry51Aa2 flowers (Fig. 2). Also, no difference was found in the numbers of tarnished plant bug nymphs observed on non-Bt squares, Bt Cry51Aa2 flowers, Bt Cry51Aa2 squares. Fewer nymphs were found on Bt Cry51Aa2 bolls than all other structures. Finally, Bt trait had no effect on the size of plant bug nymphs observed (F < 0.71 and P > 0.40 for all). More plants (F = 4.11; df = 1, 189.1; P = 0.044) were required in Bt Cry51Aa2 plots (8.96 ± 0.91) than in non-Bt plots (7.59 ± 0.91) to locate the 25 tarnished plant bugs per plot.

Table 2. Total number (percentage) of tobacco, soybean, and other adult thrips per 30 plants collected from field tests on Bt Cry51Aa2 and non-Bt cotton that was not treated with insecticides for thrips

Plants	Tobacco thrips	Soybean thrips	Other thrips	Total no. collected
Bt	292 (76.2%)b	32 (8.4%) b	59 (15.4%)b	383
Non-Bt	629 (76.2%)a	76 (9.2%) a	120 (14.5%)a	825
Total no. collected	921	108	179	1208

Data were averaged across years (2017 and 2018) and locations (Jackson, TN and Milan, TN). Means, within columns, followed by a common letter are not significantly different (Fisher's Protected LSD, α = 0.05). Data from Graham and Stewart (2018). Relative distribution (%) of thrips species between Bt Cry51Aa2 and non-Bt cotton were statistically similar (X^2 = 2.34; df = 2; P = 0.84).

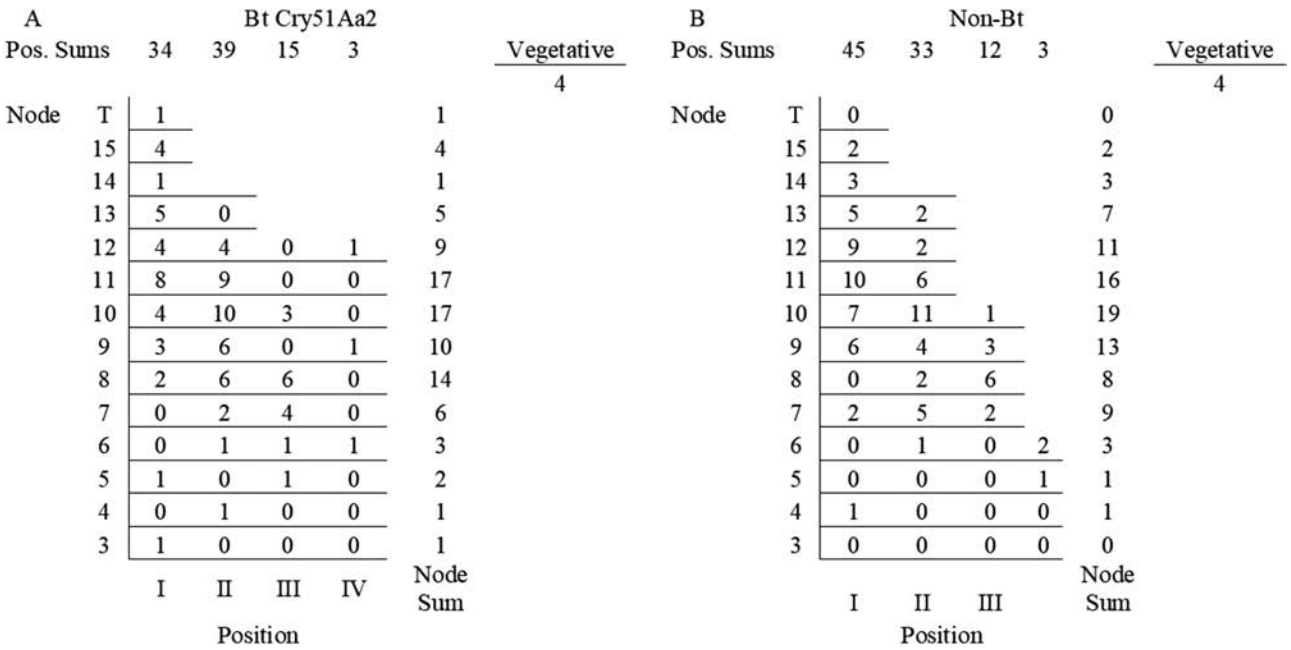

Fig. 1. Total number of tarnished plant bugs observed by node and reproductive structure for (A) Bt Cry51Aa2 and (B) Non-Bt cotton. Position = any reproductive structure found at a given position; vegetative = leaves.

More dirty squares (F = 21.24; df = 1, 6; P < 0.001) were found in non-Bt cotton (8.0 ± 0.71) than Bt Cry51Aa2 cotton (3.3 ± 0.75). However, no differences were observed in the mean number of adult (F = 0.20; df = 1, 6; P = 0.67) or immature tarnished plant bugs (F = 3.25; df = 1, 6; P = 0.12) found on squares (Table 3). Significantly more tarnished plant bug adults (F = 12.70; df = 1, 14; P = 0.003) and nymphs (F = 23.17; df = 1, 14; P < 0.001) were observed in non-Bt flowers than in Bt Cry51Aa2 flowers (Table 3). As a result, the Bt trait had an effect on the number of injured flowers (F = 30.31; df = 1, 14; P < 0.001), flowers with no injury (F = 31.59; df = 1, 14; P < 0.001), low injury (F = 4.94; df = 1, 14; P = 0.04), and high injury ratings (F = 90.43; df = 1, 14; P < 0.001). Flowers from Bt Cry51Aa2 cotton had less tarnished plant bug injury and less severe injury than those from non-Bt cotton (Table 4).

More adults (F = 5.09; df = 1, 13; P = 0.04) were observed per two drop cloth samples in non-Bt than in Bt Cry51Aa2 cotton (Table 5). However, nymphs composed the vast majority (≈97%) of the overall tarnished plant bug population, and there was no effect of the Bt trait on the mean number of tarnished plant bug nymphs (F = 0.69; df = 1, 13; P = 0.42) or total tarnished plant bugs observed (F = 0.36; df = 1, 13; P = 0.59) (Table 5). The green stink bug, *Chinavia hilaris* (Say), and brown stink bug, *Euschistus servus* (Say), were the primary stink bug species observed, but the total numbers of stink bugs observed in non-Bt cotton and the Bt Cry51Aa2 cotton was similar (Table 5).

Similar to flower injury, the Bt trait reduced the level and severity of boll injury compared with non-Bt cotton. Thumb-sized bolls from Bt Cry51Aa2 plants had a lower number of stains on the outside of bolls and a lower severity of boll staining (Table 6). Similarly, more inner boll stains, more warts, and more lint staining were observed in non-Bt bolls compared with Bt Cry51Aa2 bolls (Table 6). Differences in square, flower, and boll injury were reflected by seed cotton yield (F = 35.38; df = 1, 3; P = 0.009), with higher yield in Bt cotton (2,598 ± 175 kg/ha) compared with non-Bt cotton (1,654 ± 126 kg/ha).

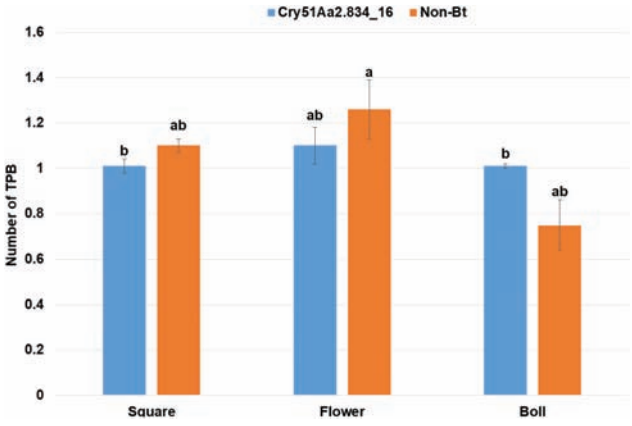

Fig. 2. Influence of Bt Cry51Aa2 and non-Bt cotton on the distribution of tarnished plant bug nymphs on cotton reproductive structures when not sprayed with insecticides. Error bars represent 95% confidence intervals. Common letters above bars indicate treatments are not different (Fisher's protected LSD, α = 0.05).

Ovipositional Caging Test

In the adult tarnished plant bug cage test, no interaction of Bt trait by plant structure was found on the number of tarnished plant bug eggs laid per plant (F = 2.37; df = 4, 99.75; P = 0.06), nor was there an effect of the Bt trait (F = 3.12; df = 1, 99.75; P = 0.08). However, an effect of plant structure (F = 58.66; df = 1, 99.75; P < 0.001) was observed for the average number of eggs per plant, with the most tarnished plant bug eggs found in petioles (29.9 ± 3.2) followed by leaves (8.7 ± 1.3). No differences were observed between the average number of tarnished plant bug eggs found in leaf veins (3.9 ± 0.6), squares (3.2 ± 0.6), or main stems (1.9 ± 0.6). On average, 54.1 ± 6.3 tarnished plant bug eggs were found in non-Bt cotton and 42.1 ± 6.2 tarnished plant bug eggs were found in Bt Cry51Aa2 cotton; however, this difference was not significant (F = 1.86; df = 1, 14; P = 0.19). In the study with excised squares, the Bt trait also did not affect the number of tarnished plant bug eggs laid (F = 2.64; df = 1, 6; P = 0.16), although there was a trend of more eggs in non-Bt squares (9.9 ± 5.1) than in Bt Cry51Aa2 squares (1.7 ± 0.9).

Laboratory Tests

Adult tarnished plant bugs preferred to feed on diet packs and excised squares that did not contain Bt tissue (Diet packs F = 28.34; df = 1, 84; P < 0.001 and Excised Squares F = 14.04; df = 1, 54; P < 0.001). There was no interaction of time by Bt trait on the proportion of adults observed on Bt diet packs or excised cotton squares (P > 0.05). More adults were observed on non-Bt diet packs than on Bt Cry51Aa2 diet packs, and on non-Bt squares than on Bt Cry51Aa2 squares (Fig. 3). Bt trait had no effect on the feeding choice of first instar (F = 0.94; df = 1, 16; P = 0.35), third instar (F = 0.53; df = 1, 230; P = 0.47), or nymphs overall (F = 0.53; df = 1, 48; P = 0.28) (Fig. 2). Similar to the difference in adult preferences, more tarnished plant bug eggs were found in non-Bt diet packs (44.0 ± 11.2) than Bt Cry51Aa2 diet packs (26.3 ± 8.9) (F = 30.87; df = 1, 2; P = 0.03).

Discussion

Thrips preferred non-Bt cotton over cotton expressing Bt Cry51Aa2, as evidenced by our choice studies done in the field and greenhouse where there was approximately a 2:1 preference for non-Bt cotton by adults and for oviposition. No previous research has reported the

Table 3. Impact of Bt Cry51Aa2 on mean number (SEM) of tarnished plant bugs (TPB) visually observed on squares and flowers of Bt Cry51Aa2 and non-Bt cotton in Tennessee

	Non-Bt	Bt	F	df	P
Squares					
Adults	0.25 (0.25)a	0.50 (0.50)a	0.20	1, 6	0.6704
Nymphs	5.00 (1.08)a	2.50 (0.87)a	3.25	1, 6	0.1210
Total TPB	5.25 (1.31)a	3.00 (0.91)a	1.98	1, 6	0.2095
Flowers					
Adults	3.13 (0.61)a	0.13 (0.13)b	12.70	1, 14	0.003
Nymphs	3.50 (0.57)a	1.13 (0.35)b	23.17	1, 14	<0.001
Total TPB	6.63 (0.91)a	1.25 (0.31)b	31.94	1, 14	<0.001

Means, within rows, followed by a common letter are not significantly different (Fisher's Protected LSD, α = 0.05).

behavioral response of tobacco thrips to Bt Cry51Aa2; however, it is known that thrips have behavioral responses to insecticides and plant types (Joost 2003, Chaisuekul and Riley 2005). Tobacco thrips have been shown to avoid imidacloprid-treated leaves (Joost 2003) and to have an ovipositional preference to tomato over chickweed (Chaisuekul and Riley 2005). Similar avoidance behavior could explain why imidacloprid seed treatments in cotton still provide better protection against thrips than thiamethoxam (Cook et al. 2016), despite assays indicating tobacco thrips are resistant to both insecticides (Huseth et al. 2016, Darnell et al. 2018). Thrips avoidance of cotton expressing Bt Cry51Aa2 appears to be a mechanism of plant protection that has previously been observed in field trials (Graham and Stewart 2018). If most of Bt Cry51Aa2 activity on thrips is related to avoidance, it may have implications on insecticide resistance management, although the specific impacts are not clear.

Based on our observations, the presence of Bt Cry51Aa2 did not impact the distribution of tarnished plant bug within the canopy of cotton, although we had to scout ≈15% more plants to find similar numbers of bugs. This finding differs from the response of bollworm to lepidopteran-active Bt toxins in cotton, which move to avoid structures with high expression of toxins (Greenplate 1999, Adamczyk et al. 2001, Akin et al. 2002, Gore et al. 2002). However, it is currently unknown if Cry51Aa2 is expressed differently in various structures of the cotton plant. In our study, nearly 66% of all tarnished plant bugs observed were on squares, regardless of whether they were on a Bt or non-Bt plant. This finding is similar to the results of Pack and Tugwell (1976), Snodgrass (1998), and Fontenot (2009). The majority of tarnished plant bugs were found on the upper six nodes of the cotton canopy, which is also consistent with studies by Snodgrass et al. (1998) and Graham (2016). Understanding the effect of Bt Cry51Aa2 on tarnished plant bug distribution is important because distribution plays a role in scouting techniques and insecticide efficacy. Because we found no effect of Bt Cry51Aa2 on the distribution of tarnished plant bug dispersal on the cotton plant, current sampling strategies should still be effective. Sumner and Herzog (2000) showed that foliar insecticide applications provided less control of tarnished plant bugs in the lower and middle parts of the canopy. Graham and Stewart (2018) found increased insecticide efficacy on cotton expressing Bt Cry51Aa2 compared with non-Bt cotton. For cotton sprayed weekly with insecticides, the number of tarnished plant bugs found in drop cloth samples was reduced by 88% in Bt Cry51Aa2 cotton and 77% in non-Bt cotton when compared with plots not treated with insecticide. This finding suggests that tarnished plant bugs exposed to Bt Cry51Aa2 might be more sensitive to insecticides because they are already somewhat stressed by the Bt toxin. It might also mean that despite a similar distribution within the canopy, tarnished plant bugs exposed to the Bt toxin are moving more within the canopy, and thus, are more likely to contact insecticide residues.

In this study, we found no difference in the size of tarnished plant bug nymphs observed on Bt Cry51Aa2 and non-Bt cotton. This finding is somewhat contradictory to a previous and more intense study (Graham and Stewart 2018) which found a significant reduction (≈50%) in the number of large tarnished plant bug nymphs observed. However, this study was similar to Graham and Stewart (2018) in that there was little effect of Bt Cry51Aa2 on numbers of smaller nymphs. A reduction in the number of large nymphs is important because they cause more damage than smaller nymphs (Cooper and Spurgeon 2013). A reduction in the level and severity of flower, and boll injury was observed in Bt Cry51Aa2 cotton compared with non-Bt cotton (Tables 4 and 6), and fewer tarnished plant bugs were found in the flowers of Bt Cry51Aa2 cotton (Table 3). The protection of these fruiting structures provided by Bt Cry51Aa2 is important, especially in the bottom of the plant canopy where there is the potential for reduced insecticide efficacy (Sumner and Herzog 2000).

Stink bugs were present in our field trial, and little is known about the potential impacts of Bt Cry51Aa2 on stink bugs or how it might affect boll injury caused by stink bugs. Stink bug populations observed in our drop cloth samples were below the suggested economic threshold in Tennessee (Stewart and McClure 2017), with no significant difference between Bt Cry51Aa2 and non-Bt cotton (Table 6). Tarnished plant bug nymphs found in drop cloth samples

Table 4. Impact of Bt Cry51Aa2 on average number (SEM) of injured flowers from tarnished plant bug feeding observed in Bt Cry51Aa2 and non-Bt cotton in Tennessee

Injury level	Non-Bt	Bt
No injury	7.50 (1.09)a	17.00 (1.29)b
Low injury	11.13 (1.13)a	7.50 (1.18)b
High injury	6.25 (0.56)a	0.38 (0.26)b
Total injured	17.38 (1.16)a	7.88 (1.27)b

Means, within rows, followed by a common letter are not significantly different (Fisher's Protected LSD, α = 0.05).

Table 5. Average number (SEM) of tarnished plant bugs (TPB) and total stink bugs (multiple species) found per 3.02 m row of Bt Cry51Aa2 and non-Bt cotton in Tennessee

Insects	Non-Bt	Bt
TPB adults	0.88 (0.39)a	0.13 (0.13)b
TPB nymphs	17.38 (3.67)a	20.25 (4.95)a
TPB total	18.25 (3.43)a	20.38 (4.89)a
Total stink bugs	1.25 (0.49)a	1.37 (0.71)a

Means, within rows, followed by a common letter are not significantly different (Fisher's Protected LSD, α = 0.05).

Table 6. Impact of Cry51Aa2 on mean (SEM) ratings of outer boll stains, boll stain severity, inner boll stains, warts, and percent lint staining of cotton bolls sampled from Bt Cry51Aa2 and non-Bt plots related to damage from tarnished plant bugs in Tennessee

Boll injury	Non-Bt	Bt	*F*	df	*P*
Outer stains	17.28 (1.62)a	9.78 (0.88)b	17.65	1, 187	<0.0001
Boll stain severity[a]	1.38 (0.06)a	1.14(0.03)b	11.63	1, 186	0.0008
Inner stains	1.68 (0.24)a	0.74 (0.19)b	10.30	1, 192	0.0016
Warts	3.28 (0.34)a	2.13 (0.27)b	8.12	1, 193	0.0048
% Lint staining	19.42 (2.59)a	9.91 (1.87)b	8.81	1, 198	0.0034

Means, within a row, followed by a common letter are not significantly different (Fisher's protected LSD, α = 0.05).
[a]Relative outer boll staining severity rating from 0 to 3 (0 = no staining, 3 = severely stained).

were also similar between Bt Cry51Aa2 and non-Bt cotton; however, boll injury ratings were far less in Bt Cry51Aa2. Prior to this study, it was unknown that adult tarnished plant bugs avoided this Bt toxin, and it is possible that stink bugs may respond similarly when feeding on Bt Cry51Aa2 cotton. It is likely that at least some boll injury observed in our test was caused by stink bugs, and thus, the reduced boll damage in plots of Bt Cry51Aa2 could be at least partly attributed to stink bugs. However, tarnished plant bug infestations were about three times the recommended treatment threshold. Although the size of nymphs was not recorded in this study, Graham and Stewart (2018) reported a nearly 52% reduction of large nymphs in Bt Cry51Aa2 compared with non-Bt cotton when no insecticide was applied. This reduction of large nymphs is similar to the reduction of stains on the outside of bolls (≈44%). Furthermore, when examining stains made on the outside of bolls, the injury was more characteristic of tarnished plant bug feeding than stink bug (Fig. 4). Thus we believe treatment effects on boll injury in the field can be mostly attributed to tarnished plant bugs.

In our choice tests, tarnished plant bug adults avoided diet containing lyophilized Bt Cry51Aa2 leaves, and they also preferred non-Bt cotton squares over squares from Bt Cry51Aa2 plants. Egg deposition in the whole-plant cage study, in the diet pack assay, and on excised squares followed a similar trend. It is not surprising that oviposition would be lower if tarnished plant bugs avoided cotton expressing Bt Cry51Aa2. The reduction of oviposition associated with exposure to Bt Cry51Aa2 was probably related to the adult avoidance behavior rather than sublethal effects of the toxin of adult fecundity because our assays were short in duration (24 h).

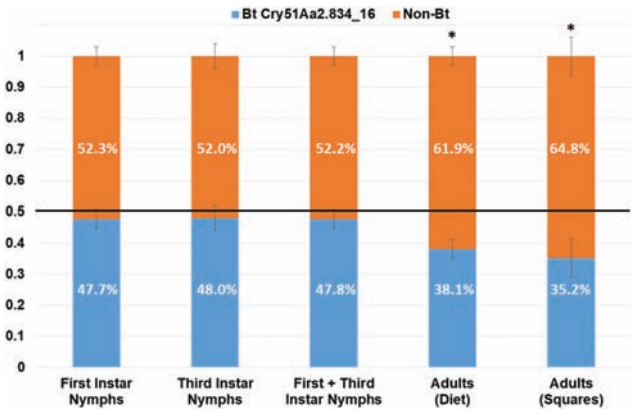

Fig. 3. Overall proportion of tarnished plant bugs observed on diet packs containing lyophilized Bt Cry51Aa2 and non-Bt leaf tissue over 24-h period. Error bars represent 95% confidence intervals. An asterisks above bars indicate treatments are different (Fisher's protected LSD, $\alpha = 0.05$).

No preference in diet-pack assays with first or third instar tarnished plant bugs was observed. A lack of response in first instars is not unexpected because they feed little, if at all and are not as mobile as adults. Stewart et al. (1992) found that although first instar *L. hesperus* caused feeding punctures in diet packs, they did not ingest a significant amount of diet. The host plant on which an immature plant bug develops is largely determined by the ovipositional preference of adult females. Indeed, nymphs may have less ability than adult females in discerning the quality of food sources or detecting toxins. If nymphs do have less ability to detect toxins, it could help with the efficacy of the trait, because if nymphs are not detecting Cry51Aa2 enough to elicit avoidance, they will be more likely to continue feeding on cotton expressing the toxin. A study by Graham et al. (2016) found that third instar tarnished plant bugs avoided green beans treated with field rates of various insecticides, suggesting that nymphs can have an avoidance response if the stimulus is strong enough. Because it is known that Cry51Aa2 is relatively slow (~6 d) to kill nymphs (Baum et al. 2012, Bachman et al. 2017), it is possible that third instars were not exposed to enough toxin to elicit a behavioral response in 24 h, but a longer assay may have come to a different conclusion.

The efficacy of cotton varieties expressing Bt Cry51Aa2 could be reduced in large fields if efficacy is partly based on avoidance and alternative food sources are less readily available. For example, small plot research showed significant reductions of tarnished plant bugs in nectariless cotton varieties compared with nectaried cotton varieties (Meredith et al. 1973, Adjei-Maafo and Wilson 1983, Bailey et al. 1984). However, Scott et al. (1988) reported that no reduction in tarnished plant bug populations would be seen in nectariless cotton varieties at a field size of 31 ha. Further research needs to be conducted to determine how deployment of Bt Cry51Aa2 cotton in large fields might affect populations of thrips and tarnished plant bug. The yield increase reported in this paper (e.g., 57% yield increase above non-Bt plots) was likely the result of differences in

Fig. 4. Photographs of representative boll injury observed in (A) non-Bt and (B) Bt Cry51Aa2 for plots not sprayed with insecticides in Jackson, TN in 2017.

tarnished plant bug injury. Thrips injury was managed with insecticides and did not reach levels expected to cause yield loss, and unlike tarnished plant bug, stink bug infestations were below the recommended treatment threshold. Similar numbers of tarnished plant bugs were found on Bt Cry51Aa2 and on non-Bt cotton in drop cloth samples, suggesting that the mode of action of Bt Cry51Aa2 on tarnished plant bug extends beyond mere avoidance of Bt Cry51Aa2 plants. This suggestion is supported by our data showing a substantial reduction in injury to fruiting structures despite similar numbers of tarnished plant bugs on Bt Cry51Aa2 and non-Bt cotton. While Bt Cry51Aa2 cotton appears to provide substantial protection from thrips, previous research indicates that supplemental applications of insecticides will still be needed at times to control tarnished plant bugs (Graham and Stewart 2018).

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