Anais da Academia Brasileira de Ciências (2017) 89(4): 2955-2969
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201720150525
www.scielo.br/aabc | www.fb.com/aabcjournal

Effects of temperature on the feeding behavior of *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) on Bt and non-Bt cotton plants

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Manuscript received on July 17, 2015; accepted for publication on March 1, 2016

ABSTRACT

The host acceptance behavior and environmental factors as temperature affect the feeding behavior of Lepidoptera pests. Thus, they must be considered in studies about the risk potential of resistance evolution. The current study sets the differences in the feeding behavior of neonate *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) larvae exposed to Bt and non-Bt cotton plants, under different temperatures and time gap after hatching. Two cotton cultivars were used: the Bt (DP 404 BG - bollgard) and the non-transformed isoline, DP 4049. We found that the feeding behavior of neonate *A. argillacea* is significantly different between Bt and non-Bt cotton. Based on the number of larvae with vegetal tissue in their gut found on the plant and in the organza as well as on the amount of vegetal tissue ingested by the larvae. *A. argillacea* shows feeding preference for non-Bt cotton plants, in comparison to that on the Bt. However, factors such as temperature and exposure time may affect detection capacity and plant abandonment by the larvae and it results in lower ingestion of vegetal tissue. Such results are relevant to handle the resistance of Bt cotton cultivars to *A. argillacea* and they also enable determining how the cotton seeds mix will be a feasible handling option to hold back resistance evolution in *A. argillacea* populations on Bt cotton, when it is compared to other refuge strategies. The results can also be useful to determine which refuge distribution of plants is more effective for handling Bt cotton resistance to *A. argillacea*.

Key words: cotton leafworm, feeding behavior, transgenic cotton, blended seed refuge.

INTRODUCTION

The cotton plant (*Gossypium hirsutum* Linné) hosts a set of Lepidoptera that defoliate the plant. Whenever they are in high density populations, Lepidoptera cause great loss in crop yield. Cotton leafworm *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) stands out among such species, due to its destructive capacity (Ramalho et al. 2014). Destruction may occur from the initial phase to the crop maturation in all cotton producer regions in Brazil (Ramalho et al. 2014). Thus, cotton pest control is a limiting factor to the cultivation of this malvaceae, since they represent high production cost due to the countless spraying needed to control insect pests (Ramalho et al. 2014).

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The use of cotton cultivars resistant to cotton leafworm has been suggested as an alternative to chemical control (Schnepf et al. 1999). Bt cotton cultivars are comprised by plants genetically modified by genes of *Bacillus thuringiensis* var. *kurstaki* bacteria. These cultivars express protein crystals (Cry) that are lethal if digested by the larvae (Schnepf et al. 1999, Vachon et al. 2012). However, the inserting of a transgene in cotton cultivations may lead to non-expected changes in the plant-insect interaction (Thu Cuc et al. 2008).

Since the commercial liberation of Bt plants in the USA, farmers have adopted such technology in order to get an effective production cost reduction in sustainable agriculture (Klumper and Qaim 2014). However, Lepidoptera monofage as that shown by *A. argillacea* and the continuous expression of Cry toxins in Bt plants put high selection pressure over target insect populations and, consequently, change the host selection behavior by the larva. Therefore, it favors resistance evolution, since dispersion and/or the feeding behavior of *A. argillacea* is different between Bt and non-Bt cotton plants (Ramalho et al. 2014). *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) resistance to the Cry1F toxin found in corn (*Zea mays* Linné) was observed in Puerto Rico (Storer et al. 2010) as well as *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) resistance to cotton plants singly expressing Cry1Ac or Cry1Ac in combination with Cry2Ab2, in India (Dhurua and Gujar 2011). On the other hand, Gassmann et al. (2011), also got to a field finding: a *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) strain resistant to Bt corn plants expressing Cry3Bb1 or Cry34/35Ab1. Thus, the main seed production multinational companies have discussed the possibility of mixing a percentage of non-transgenic seeds in bags with resistant seeds (Zancanaro et al. 2012) as a way to hold back the adaptation evolution of pest insects to the Cry toxin (Agi et al. 2001, Onstad et al. 2011). With regards to refuge in bag "RIB" strategy, a defined percentage of non-Bt plant seeds are mixed with Bt plant ones in each bag. The companies follow the aforementioned procedure so the bags can be sold to producers. However, some implications of this seed mixing technique must be observed, since *A. argillacea* larvae that emerge on Bt plants may do some tasting and get dispersed (Ramalho et al. 2014). Larvae that emerge on non-Bt plants may also do tasting, stop feeding and move to a transgenic plant (Gould 2000). Therefore, *A. argillacea* larvae stimulated by Bt cotton cultivars may migrate to non-Bt cotton plants.

The larval movement of target pest populations between Bt plants and non-Bt plants is the most important skill in the use of refuge strategies based on seeds mix (Wangila et al. 2012). Lepidoptera neonate pre and post feeding scattering is common when the host has toxins such as those derived from *B. thurigiensis* (Zalucki et al. 2002). The movement of *A. argillacea* larvae from a Bt to a non-Bt cotton plant may lead them to ingest sufficient toxin to make this strategy effective against the pest, and therefore, it helps to stop these toxins to emerge in *A. argillacea* populations (Ramalho et al. 2014). With regard to refuge in the bag "RIB" strategy, the biggest "concern" lies on the fact that the larval movement between Bt and non-Bt plants may booster the resistance evolution in target pest populations (Wangila et al. 2012). According to Wangila et al. (2012), the seeds mix may be able to give refuge to *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) and it can be compared with the structured refuge. Besides the host acceptance behavior, environmental factors such as relative humidity and temperature must be considered in studies about the risk potential to resistance evolution (Chen et al. 2012).

High temperatures lead to lower effect on Bt toxin production (Chen et al. 2005). It does not happen under low temperatures, because, in this case, there is significant increase in the production of this toxin (Chen et al. 2005). Temperature is one of the environmental factors that affect the bollworm control efficacy in Bt cotton. Dong and Li (2007) reported in greenhouse studies that Bt cotton plants significantly lose their insect resistance under high (above 37 ºC) and low temperatures (below 18 ºC). According to Chen et al. (2005), high temperatures cause reduction in Bt protein concentrations during the boll filling stage. The percentage of *A. argillacea* larvae recovered from the cotton plants after 24 h, regardless of the temperature, was significantly higher for the non-Bt cotton cultivar than for the Bt cotton cultivar (Ramalho et al. 2014). However, the percentage of neonate *A. argillacea* larvae recovered on cotton plants was lower at 31 and 34 °C than at 22, 25 and 28 °C, with no differences among the other temperatures. According to Medeiros et al. (2003), *A. argillacea* larvae reached thermal stress at 33 °C; it was assumed that at 35 °C and above, the production of enzymes in *A. argillacea* larvae was partially inhibited. In addition to the *A. argillacea* larvae's low acceptance of the host plant, the heat stress, regardless of the cultivars, may stimulate the dispersion behavior of the neonate *A. argillacea* larvae (Ramalho et al. 2014). In general, the percentage of *A. argillacea* larvae found on Bt cotton plants is less than on non-Bt cotton plants (Ramalho et al. 2014).

Results from previous studies, Ramalho et al. (2014) suggested that further researches were needed to help disclosing neonate *A. argillacea* larvae feeding behavior when they emerge and find Bt cotton plants as well as how such behavior is affected by temperature throughout time. Thus, the current study aimed to determine differences in neonate *A. argillacea* larvae feeding behavior when they are exposed to Bt and non-Bt cotton plants, under different temperatures and time gaps, after their hatching. We have hypothesized that a1) the percentage of *A. argillacea* larvae found on the plants, with vegetal tissues in their gut is lower in Bt cotton plants when it is compared with the number of larvae found on non-Bt plants; a2) the percentage of larvae found outside the plants (larvae that have escaped the Bt and non-Bt plants) without food in their gut is lower in Bt cotton plants when it is compared with larvae found in non-Bt. Such difference changes depending on the environmental temperature and time gap they are exposed to the cotton plants. Knowledge generated by the current study will be useful to the development of more effective management programs for Bt cotton resistance to *A. argillacea*.

MATERIALS AND METHODS

LOCATION, INSECTS AND COTTON CULTIVARS

Alabama argillacea rearing as well as the bioassays were performed at Unidade de Controle Biológico da Embrapa Algodão (Biological Control Unit of Embrapa Cotton), Campina Grande, PB, Brazil. *A. argillacea* larvae from the rearing stock were kept in a BOD type acclimatized chamber at 25 °C, 70 ± 10% RH and 12 h photophase. Two cotton cultivars were used in the study: a Bt cultivar DP 404 BG (Bollgard) and the other was its non-transformed isoline, cultivar DP 4049. The cultivars were separately grown in plastic pots (20 cm diameter and 30 cm high) and kept in greenhouse under 35 ± 10 °C, 70 ± 10% RH and 12 h photophase.

BIOASSAYS

Neonate A. argillacea feeding behavior under different temperature and time gap

The experiment was installed in 2x4x5 factorial system in randomized blocks in which two cotton cultivars (Bt and non-Bt) were used as well as four time gaps. These time gaps were represented by evaluation done at 6, 12, 18 and 24 h after the plant was infested with neonate *A. argillacea* larvae under five temperatures (22, 25, 28, 31 e 34 ºC).

The experimental unit consisted of a cotton plant from the Bt or non-Bt cultivar which had reached the eight leaves stage. The plant received 30 recently-hatched larvae *A. argillacea* (0 to 24 h) that were left on a leaf at the plant's apical region. Next, each plant was dressed with an organza bag and randomly conditioned in a BOD type acclimatized chamber at 22, 25, 28, 31 and 34 ºC, 70 ± 10% relative humidity and 12 h photoperiod.

The bioassay used neonate *A. argillacea* larvae because they present greater mobility and have easier accepted the host plant than larvae in other stages of life (Zalucki et al. 2002). Once a day the plants were moved randomly to minimize the effects of their position within the chamber. After each time gap, the cotton plants and the organza bags were inspected and the larvae were removed using a paintbrush. The larvae were gathered in two categories: found on the plant and in the organza bag (outside the plant). In order to check if the larva had fed, each larva was assembled on microscope slide in Karo® syrup solution diluted in water (Johansen 1940). The larvae were examined through light transmission over their intact bodies to check the presence of vegetable tissue in their gut (Razze et al. 2011). Such fact would be the evidence that the larvae had fed. Subsequently, the material found in the gut of each larva was quantified by means of an ocular micrometer linked to the microscope's ocular with contrast phase. The vegetable tissue identified and quantified in the gut of each larva were: chlorenchyma and tracheary elements (Fig. 1). Chlorenchyma is a specialized parenchyma in charge of photosynthesis and it presents an abundance of visible chloroplasts; whereas the tracheary elements are conducting cells found in the xylem (Evert 2006).

DATA ANALYSES

The obtained data were subjected to variance analysis (PROC GLM) (SAS Institute 2006) to check if there was cultivar (C), temperature (T) and time gap (t) effects on neonate *A. argillacea* larvae. PROC GLM was also used to check the interaction between cultivar and temperature, cultivar and time gap, temperature and time gap; and cultivar, temperature and time gap, with regard to the percentage of larvae that had fed and were found on the plant or on the organza bag (outside the plant). The variance analysis was also used to measure the amount of vegetable tissue (chlorenchyma and/or tracheary elements) (Fig. 1) observed and quantified (μm^2) in the gut of *A. argillacea* larvae in Bt and non-Bt plants as well as in the organza bag (outside the plant). The comparison of treatment means was performed using the Student-Newman-Keuls test ($P = 0.05$).

RESULTS

With regards to the percentage of the larvae that had fed and were found on the cotton plant (Table I), it was found that there was no significant interaction among cotton cultivar (C), temperature (T) and the exposure time (Et) *A. argillacea* larvae were exposed to on cotton plants ($F_{(CxTxEt)\ 12,\ 117} = 1.15$, $P = 0.3254$). However, with regards to the percentage of *A. argillacea* larvae that had fed and were found on the cotton plant ($F_{(TxEt)\ 12,\ 117} = 2.93$, $P < 0.0014$) (Table I), the interaction between temperature (T) and exposition time (Et) was significant. With respect to the percentage of larvae that had fed and were found on the cotton plants, there were significant differences between cotton cultivars (Bt and non-Bt) as well as the tested temperatures and the time gap in which the larvae were exposed to on cotton plants. Ninety four percent (94%) of the larvae on the non-Bt cotton plants presented food in their gut, whereas just 70% of the larvae on Bt cotton plants presented food in their gut (Fig. 2a). The average of vegetable tissues found in the neonate *A. argillacea* larvae was 9.27 μm^2/larva and 4.67 μm^2/larva in those on non-Bt and Bt cotton plants (Fig. 2b). The mean percentage of neonate *A. argillacea* larvae got from cotton plants, under

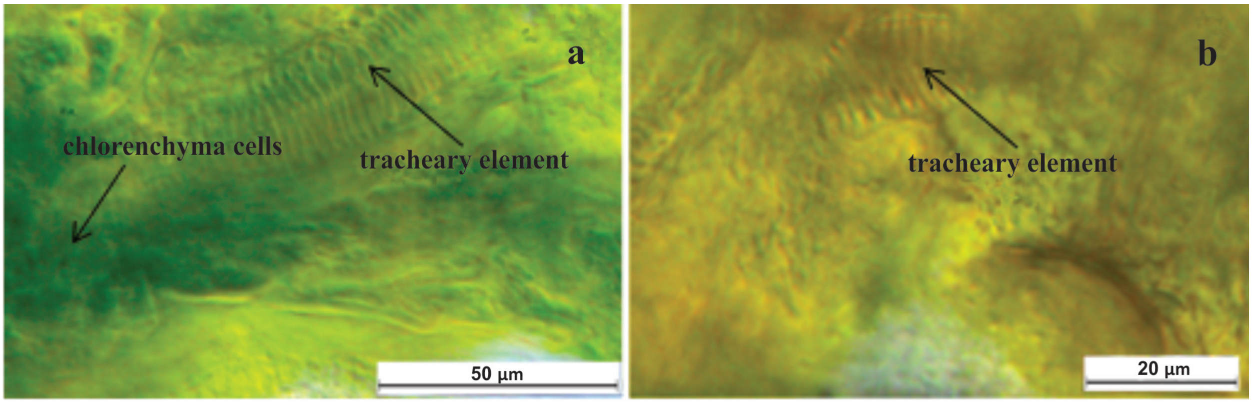

Figure 1 - Plant tissue inside the gut of *A. argillacea*. **a**: chlorenchyma cells and tracheary elements in the gut of a neonate larva of *A. argillacea* that had fed on non-Bt cotton for 24 h, and **b**: tracheary elements in the gut of a neonate larva of *A. argillacea* that had fed on Bt cotton for 24 h.

the tested temperatures and that had fed, changed depending on the exposure time the larvae were exposed to cotton plants (except for exposure to 28 °C) (Table II).

With regards to the percentage of *A. argillacea* larvae that had fed and were found in the organza bag ($F_{(CxTxEt)12, 117} = 32.09$, $P < 0.0001$) (Table I), there was a significant interaction between cotton cultivar (C), temperature (T) and the exposure time (Et) *A. argillacea* larvae were exposed to cotton plants. However, the effect of cotton cultivars (Bt and non-Bt) on the percentage of larvae that had fed and were found in the organza bags depends on the temperature and the exposure time. It was seen that the percentage of larvae, got in the organza bags, that had fed and came from non-Bt cotton plants at 25 °C and 31 °C, did not change with the exposure. However, at 22 and 34 °C, the percentage of larvae found in the organza bag and that had eaten was higher when they were exposed for 6 h on the non-Bt cotton plant (Table III). In case of larvae from the Bt cotton plants, the percentage of larva got in the organza bags and that had fed was higher after 24 h exposure on Bt cotton plants at 31 °C (Table III). On the other hand, respectively 52% or 41% of the lavae found in the organza bags from non-Bt or Bt cotton plants presented vegetal tissues in their guts (Fig. 2c). The average of vegetable tissue in the guts of neonate *A. argillacea* larvae found in organza bags from non-Bt cotton plants (Fig. 2d) was respectively 4.16 μm^2/larva and 2.03 μm^2/larva.

The interaction among cultivar (C), temperature (T) and the exposure time (Et) was significant with regard to the amount of vegetable tissue found in the gut of larvae got on cotton plants ($F_{(CxTxEt)\ 12, 117} = 1.91$, $P = 0.0395$) (Table IV) or in the organza bags ($F_{(CxTxEt)\ 12, 117} = 32.86$, $P < 0.0001$) (Table IV). It means that the ingestion of vegetable tissue (tissue of cotton plants) by neonate *A. argillacea* larvae depend on the cotton cultivar (Bt and non-Bt cotton), environmental temperature and exposure time. It was found that after 6 h of exposition on the cotton plants (Bt and non-Bt), the amount of vegetable tissue in the guts of larvae got on non-Bt cotton plants was 1.72 (at 22 °C), 1.98 (at 25 °C), 4.20 (at 28 °C), 1.58 (at 31 °C) and 1.86 (a 34 °C) times higher than that on Bt cotton plants (Table V). A similar behavior was seen in larvae exposed on cotton plants under other exposure times (Table V). More expressive results were recorded for the amount of vegetable tissue in the gut of *A. argillacea* larvae got in the organza bags, i.e., the amount of vegetable tissue in the gut of larvae got

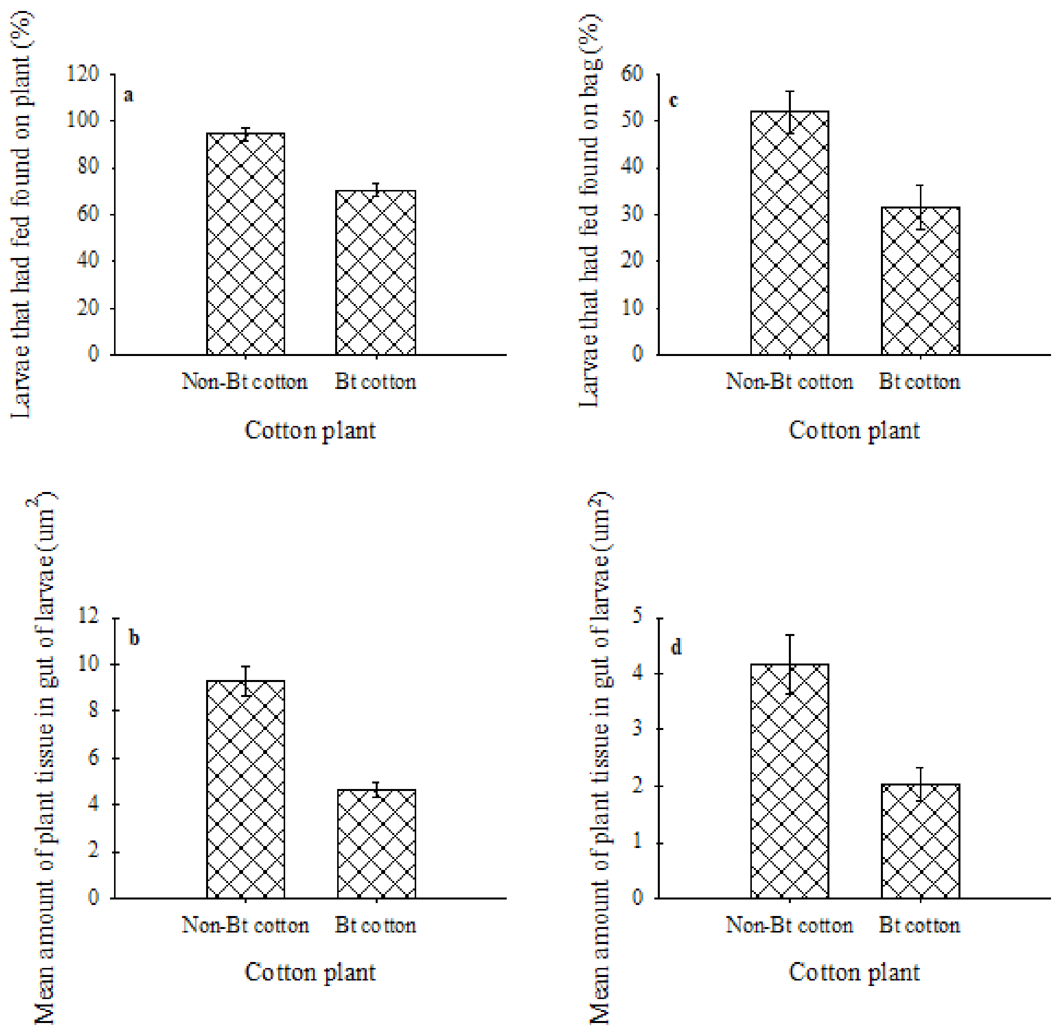

Figure 2 - a: Mean percentage of larvae that had fed that were found on the cotton plant (Bt or non-Bt) (F = 8.64, df = 1, 117, *P* = 0.0040), **b**: mean plant tissue amount found in the gut of neonate larvae of *A. argillacea* from cotton plant (Bt or non-Bt) (F= 170.75, df = 1, 117, *P* = 0001), **c**: mean percentage of larvae that had fed (Bt or non-Bt) that were found on the bag (F= 32.49, df = 1, 117, *P* = 0.0001), and **d**: mean plant tissue amount found in the gut of neonate larvae of *A. argillacea* from cotton plant (Bt or non-Bt) (F = 123.12, df = 1, 117, *P* = 0.0001), and were found on the bag.

in the organza bags, regardless of temperature, was 2.65 (at 6 h), 6.56 (at 12 h) and 11.91 (at 18 h) times higher in larvae got on non-Bt cotton plants than that on Bt cotton plants (Table VI).

DISCUSSION

Most neonate lepidopterans have a pre-feeding movement phase. This phase may take place within the feed substrate itself or may happen in the long distance dispersion (Berger 1992, Saxena and Onyango 1990) so called *Ballooning* (Zalucki et al. 2002). Larvae that hatch on Bt plants may do tastings, stop feeding and disperse to other hosts (Razze et al. 2011). The neonate larvae's capacity to detect the presence of toxin in Bt plants is associated to the ingestion of the toxin after the tasting (Yan et al. 2004). However, this pre-feeding behavior of the larvae will depend on contact time and/or the plant's exposure to the larva. This

TABLE I
Summarized model of the three-way analyses of variance (ANOVA) for the effects of cultivar[1], temperature[2] and exposure time[3] of neonate larvae to Bt cotton or non-Bt cotton on the percentage of neonate larvae of *A. argillacea* that had fed and were found on the plant or bag (Bt or non-Bt near isoline)[4].

Source	Models	DF	F ratio	Prob > F
Neonate larvae of *A. argillacea* that had fed that were found on the **plant** (%)	Model	42	4.65	0.0001
	Cultivar (C)	1	8.64	0.0040
	Temperature (T)	4	18.98	0.0001
	Exposure time (Et)	3	14.79	0.0001
	C x T	4	1.33	0.2643
	C x Et	3	2.26	0.0846
	T x Et	12	2.93	0.0014
	C x T x Et	12	1.15	0.3254
Neonate larvae of *A. argillacea* that had fed that were found on the **bag** (%)	Model	42	39.92	0.0001
	Cultivar (C)	1	32.49	0.0001
	Temperature (T)	4	97.34	0.0001
	Exposure time (Et)	3	10.23	0.0001
	C x T	4	14.08	0.0001
	C x Et	3	56.78	0.0001
	T x Et	12	50.88	0.0001
	C x T x Et	12	32.09	0.0001

[1]Cultivars: Bt cotton and non-Bt near isoline. [2]Temperature (ºC): 22, 25, 28, 31, and 34. [3]Exposure time: 6, 12, 18, and 24 h. [4]Data were square root transformed priori to statistical analyses.

TABLE II
Mean percentage (± SEM) of neonate larvae of *A. argillacea* that had fed that were found on the cotton plants (%) ($F_{(T\ versus\ Et)}$ = 2.93, df = 12, 117, *P* < 0.0014).

Temperature (ºC)	Exposure time (h)			
	6	12	18	24
22	77.68 ± 0.06 Bb	81.71 ± 0.02 Bb	82.24 ± 0.03 Bb	100.00 ± 0.00 Aa
25	97.03 ± 0.01 Ba	100.00 ± 0.00 Aa	100.00 ± 0.00 Aa	100.00 ± 0.00 Aa
28	100.00 ± 0.00 Aa	96.50 ± 0.03 Aa	95.83 ± 0.04 Aab	100.00 ± 0.00 Aa
31	80.55 ± 0.03 Bb	81.61 ± 0.02 Ba	82.03 ± 0.05 Bb	100.00 ± 0.00 Aa
34	84.33 ± 0.04 Bb	93.75 ± 0.02 Ab	95.68 ± 0.01 Aab	98.48 ± 0.01 Aa

Within rows, means with the same upper case letter do not differ significantly by the Student-Newman-Keuls test (*P* = 0.05); within columns, means with the same lower case letter do not differ significantly by the Student-Newman-Keuls test (*P* = 0.05). Original data.

TABLE III
Mean percentage (± SEM) of neonate larvae of *A. argillacea* that had fed that were found on the bag on non-Bt and Bt plant at different exposure times and temperatures ($F_{(C\ versus\ T\ versus\ Et)}$ = 32.09, df = 12, 117, P < 0.0001).

Temperature (ºC)	Exposure time (h)			
	6	12	18	24
		Non-Bt cotton		
22	75.00 ± 14.43 Aa	0.00 ± 0.00 Bb	0.00 ± 0.00Bc	50.00 ± 10.20Ab
25	100.00 ± 0.00 Ab	100.00 ± 0.00 Aa	100.00 ± 0.00Aa	100.00 ± 0.00Aa
28	37.50 ± 12.50 Aa	50.00 ± 10.20 Ac	50.00 ± 12.24Ab	0.00 ± 0.00Bc
31	75.00 ± 5.00 Aa	75.00 ± 8.66 Ab	75.00 ±8.66Aa	50.00 ± 20.41Ab
34	100.00 ± 0.00 Aa	0.00 ± 0.00 Bd	0.00 ± 0.00Bc	0.00 ± 0.00Bc
		Bt cotton		
22	50.00 ± 20.41Bb	0.00 ± 0.00 Cc	0.00 ± 0.00 Cc	100.00 ± 0.00 Aa
25	0.00 ± 0.00 Cc	80.00 ± 8.16 Bb	100.00 ± 0.00 Aa	100.00 ± 0.00 Aa
28	0.00 ± 0.00 Bc	0.00 ± 0.00 Bc	50.00 ± 0.00 Ab	50.00 ± 0.00 Ab
31	100.00 ± 0.00 Aa	100.00 ± 0.00 Ac	0.00 ± 0.00 Bc	0.00 ± 0.00 Bc
34	0.00 ± 0.00 Bc	0.00 ± 0.00 Ba	50.00 ± 17.67 Ab	50.00 ± 0.00 Ab

Within rows, means with the same upper-case letter do not differ significantly by the Student-Newman- Keuls test (P = 0.05); within columns, means with the same lower-case letter do not differ significantly by the Student-Newman- Keuls test (P = 0.05). Data transformed $\sqrt{x}$.

behavior was documented in neonate larvae of the Noctuidae species such as *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), which also had the capacity and/or the tendency to detect and avoid the Bt cotton plant (Zhang et al. 2004*,* Men et al. 2005*)*. During choice tests with neonate *H. armigera* larvae on Bt and non-Bt cotton plants, Zhang et al. (2004) observed higher percentage of *H. armigera* and higher consumption on non-Bt cotton plants in comparison to the consumption on Bt cotton plants. Similar results were found for *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Berdegué et al. 1996, Stapel et al. 1998), *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) (Gould and Anderson 1991, Parker and Luttrell 1999), *Helicoverpa zea* (Hübner) (Lepidoptera: Noctuidae) (Jyoti et al. 1996, Gore et al. 2002, 2005), *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Razze et al. 2011), and *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae) (López et al. 2013, Jyoti et al. 2013). According to Razze et al. (2011), more than 50% of neonate *O. nubilalis* larvae left the primary host plant (in which the hatching took place) during the first 48 h after hatching and dispersed to other plants. However, 21 days after infestation 85 to 94% of the *O. nubilalis* larvae remained on the corn plants (Ross and Ostlie 1990). *A. argillacea* larvae showed lesser acceptance to Bt cotton plants than to the non-Bt, especially, in the time gap between 18 and 24 h (Ramalho et al. 2014).

Results in the current study evidenced that, regardless temperature and exposure time, the percentage of larvae found on plants, with vegetable tissue in their guts was lower than that on Bt cotton plants when it was compared to that found on non-Bt (Fig. 2). Such information about the feeding behavior of *A. argillacea* larvae associated to their dispersion behavior when they are exposed to endotoxins produced by *B. thuringienesis* must be seen as an important tool to improve effective strategies in management programs of Bt cotton plants resistance to *A. argillacea,* such as the refuge in the bag "RIB". *A. argillacea* larvae dispersion is related to host acceptance (Ramalho et al. 2014). It indicates that in a few cases the larvae are more up to leave the Bt cotton plants than the non-Bt. It results in lower food consumption on Bt cotton plants (Ramalho et al. 2014*),* or they remain in a higher percentage on the plant, but on the other hand, they ingest less vegetable tissue, as it was observed in our study on *A. argillacea*. The occurrence of *A. Argillacea* larvae low mobility among plants within the same agroecosystem and the little ingestion of vegetable tissues are favorable to the maintenance of the refuge strategy effectiveness in Bt plants' plantations. Whenever under "RIB" conditions, the larval movement between plants helps decreasing larval survival of recessive homozygous and the relative increase in the surviving time of heterozygous, due to the higher probability of feeding on Bt plants. Such effect may be reversed within low larval mobility scenarios, thus showing resistance evolution (Glaum et al. 2012).

The physiological response of lepidoptera larvae to toxic or nutritionally inadequate substance found on host plants lead to increase in the movement of larvae looking for better nutritional sources (Cohen et al. 1987). Thus, the dispersion of neonate larvae is important to determine how a refuge will be set in a plantation (Razze and Mason 2012). The feeding behavior of larvae includes the ingestion of vegetable tissue in the tasting phase and it is highly influenced by microclimatic conditions. Therefore, many factors may integrate and generate additive or antagonist effects in host acceptance: food ingestion and subsequent mortality (Zalucki et al. 2002). Temperature differences within greenhouse conditions were enough to result in more consumption, faster growth and stronger larval movement of *D. saccharalis* (Wangila et al. 2012). Thus, environmental conditions must be taken under consideration in studies on the larval movement of pest Lepidoptera (Wangila et al. 2012). The percentage of *A. argillacea* larvae found on Bt and non-Bt cotton plants was low when they were exposed to temperatures between 31 and 34 °C (Ramalho et al. 2014). The current study shows that the environmental temperature interacts with the cotton cultivar (Bt or non-Bt cultivars) and with the exposure time, during food ingestion, by larvae found on the cotton plant and in the organza bags as well as with the percentage of larvae that had fed and were found in the organza bags. However, the capacity or tendency of *A. argillacea* to detect and avoid Bt cotton plants must be emphasized according to climatic factors such as environmental temperature and exposure time on Bt cotton plants. The interaction involving temperature, exposure time and cultivar was not significant in terms of the percentage of larvae found on the plant and the amount of vegetal tissue in their guts. However, the interaction temperature *vs* exposure time affected the percentage of larvae found on the plant and the amount of vegetal tissue in their guts. It was verified that at 28 °C and exposure time of 6 h there was high food preference, differently from 22 °C, temperature under which there was less preference. The lower host acceptance percentages were recorded during the 12 and 18 h period, at 22 and 31 ºC (Table I).

The activity pattern of larvae in different Lepidoptera species is associated to the favorite body temperature. There are modifications in response to changes in environmental conditions (Casey et al. 1988). Behavior changes measured by factors taken under consideration in the current study such as temperature, cultivar and exposure time may help understanding how to mitigate resistance evolution and to optimize the effectiveness of the refuge strategy. The ingestion of vegetable tissue by neonate *A. argillacea*

TABLE IV
Summarized model of the three-way analyses of variance (ANOVA) for the effects of cultivar[1], temperature[2] and exposure time[3] of neonate larvae to Bt cotton or non-Bt cotton on plant tissue amount in the gut of neonate larvae of *A. argillacea* that were found on the cotton plant or bag[4].

Source	Models	DF	F ratio	Prob > F
Plant tissue amount in the gut of neonate larvae of *A. argillacea* that were found on the cotton plant (μm^2)	Model	42	14.72	0.0001
	Cultivar (C)	1	170.75	0.0001
	Temperature (T)	4	58.53	0.0001
	Exposure time (Et)	3	45.88	0.0001
	C x T	4	7.51	0.0001
	C x Et	3	0.74	0.5329
	T x Et	12	1.61	0.0978
	C x T x Et	12	1.91	0.0395
Plant tissue amount in the gut of neonate larvae of *A. argillacea* that were found on the bag (μm^2)	Model	42	51.57	0.0001
	Cultivar (C)	1	123.17	0.0001
	Temperature (T)	4	103.28	0.0001
	Exposure time (Et)	3	61.44	0.0001
	C x T	4	38.82	0.0001
	C x Et	3	63.31	0.0001
	T x Et	12	51.74	0.0001
	C x T x Et	12	32.86	0.0001

[1]Cultivars: Bt cotton and non-Bt near isoline. [2]Temperature (°C): 22, 25, 28, 31, and 34. [3]Exposure time: 6, 12, 18, and 24 h. [4]Data were square root transformed priori to statistical analyses.

larvae consistently differed between temperatures and cultivars, since in Bt cotton plants the higher consumption happened at 34 °C. It is different from the non-Bt cotton plants, in which the higher ingestion rate of vegetable tissue by the larvae occurred at 28 °C (Table II). As some insects are able to selectively distinguish and feed on some parts of the cotton plant with low expressions of *B. thuringiensis* proteins (Gore et al. 2002), it is possible that most of the ingestion of Bt cotton plant vegetable tissue happens at 34 °C. It is linked to the synthesis of insecticidal protein, since the expression of Bt toxin may be affected by different factors, not just by the genetic constitution of cotton cultivars but also by environmental conditions (Adamczyk and Sumerford 2001, Zhang et al. 2004, Mahon et al. 2002, Shen et al. 2010), such as light, temperature, water availability and rain fall (Cui and Xia 1999, Xing et al 2001). However, in the current study, the cotton plants were kept under the studied temperatures during the 24 h evaluation time. Other studies that take under consideration the plant's exposure for a longer period, under different temperatures, might be necessary.

The lower ingestion of vegetal tissue of Bt and non-Bt cotton plants by neonate *A. argillacea* larvae shows their non-preference for feeding the Bt in the first 6 h of exposure, since the amount of vegetable tissue in the guts of larvae got on non-Bt cotton plants vary from 1.58 (31 °C) to 4.20 (25 °C) times higher than that on Bt cotton plants (Table V). Similarly, the amount of vegetable tissue found in the gut of larvae got in the organza bags, regardless of temperature, vary from 2.65 (at 6

TABLE V
Plant tissue amount (± SEM) in the gut of neonate larvae of *A. argillacea* that had fed that were found on the plant Bt and non-Bt on the cotton plant ($F_{(C\ versus\ T\ versus\ Et)}$ = 1.91, df = 12, 117, *P* = 0.0395).

Temperature (ºC)	Exposure time (h)			
	6	12	18	24
		Non-Bt cotton		
22	1.88 ± 0.64 Bc	4.79 ± 0.81 Ac	5.26 ± 1.20 Ac	5.37 ± 0.47 Ad
25	7.54 ± 0.39 Bab	9.07 ± 0.52 ABab	10.69 ± 0.70 Ab	11.24 ± 0.77 Abc
28	10.34 ± 2.06 Ba	11.56 ± 0.91 Ba	16.67 ± 1.72 Ba	26.28 ± 3.17 Aa
31	4.62 ± 0.82 Bb	6.52 ± 1.09 ABbc	8.15 ± 0.67 Ab	8.92 ± 0.67 Ac
34	5.77 ± 0.52 Cab	6.87 ± 0.40 Cbc	8.56 ± 0.62 Bb	14.34 ± 0.56 Ab
		Bt cotton		
22	1.09 ± 0.38 Ab	1.73 ± 1.43 Ab	2.01 ± 0.59 Ac	4.59 ± 0.29 Ab
25	3.80 ± 0.65 Aa	4.04 ± 0.41 Aab	5.07 ± 0.49 Ab	5.47 ± 0.92 Ab
28	2.46 ± 0.64 Ba	5.72 ± 0.65 ABa	6.95 ± 1.38 Aab	8.19 ± 0.45 Ab
31	2.92 ± 0.50 Ba	4.05 ± 0.23 ABab	4.11 ± 0.19 ABb	5.34 ± 0.70 Ab
34	3.10 ± 0.70 Ba	4.56 ± 1.15 ABab	8.91 ± 1.47 Aa	9.22 ± 2.31 Aa

Within rows, means with the same upper-case letter do not differ significantly by the Student-Newman- Keuls test (*P* = 0.05); within columns, means with the same lower-case letter do not differ significantly by the Student-Newman- Keuls test (*P* = 0.05). Original data.

h) to 11.91 (at 18 h) times higher in larvae got on non-Bt cotton plants than that on Bt cotton plants. The antibiosis resistance mechanisms and/or the preference for food found in Bt cotton plants must be strictly faced in the time the refuge strategy is chosen (Halcomb et al. 2000). The selection of the host plant according to the food results from the specific complex chemical pattern of each insect species (Beck and Schoonhoven 1980). According to Beck and Schoonhoven (1980), in the presence of such stimuli and during starvation, the insect is induced to feed regardless the presence of the toxin; although the insect has the ability to respond to the toxin. According to Halcomb et al. (2000), there was not effect of the non-preference by the endotoxin found in Bt cotton plants in the 3rd instar *H. zea* and *H. virescens* larvae. The lowest amount of Bt cotton plant vegetable tissue in the gut of *A. argillacea* larvae, in comparison to that found in non-Bt cotton plants, may be attributed to the number of tasting tests done by the larvae exposed to Bt plants. It is common to find a leaf exploration phase in Lepidoptera larvae, the time when they look for more palatable plant tissues. This food selection phase implies the movement of the larvae (Wangila et al. 2012). According to Boiça Junior et al. (2012), the Bt cotton cultivar (NuOpal Bollgard I) expresses resistance to *A. argillacea* by antibiosis mechanisms rather than by food preference. Regarding olygophagous and/or polyphagous, genetic models indicate that cultivars of plants presenting both the antibiosis resistance mechanisms and the non-preference are less up to adapt to target insects when they are compared to

TABLE VI
Plant tissue amount (± SEM) in the gut of neonate larvae of *A. argillacea* that had fed that were found on the plant Bt and non-Bt on the bag ($F_{(C\ versus\ T\ versus\ Et)}$ = 32.86, df = 12, 117, P < 0.0001).

Temperature (ºC)	Exposure time (h)			
	6	12	18	24
		Non-Bt cotton		
22	1.06 ± 0.29 Bc	0.00 ± 0.00 Cc	0.00 ± 0.00 Cc	5.04 ± 0.20 Ad
25	6.39 ± 0.05 Ba	8.43 ± 0.69 Aa	9.23 ± 0.53 Ab	11.00 ± 0.00 Acb
28	4.00 ± 0.91 Ba	4.50 ± 1.40 Bb	14.12 ± 1.65 Aa	0.00 ± 0.00B Ca
31	0.00 ± 0.00 Cc	1.51 ± 0.38 Bc	6.15 ± 0.42 Ab	6.34 ± 0.40 Ac
34	2.07 ± 0.01 Bb	7.53 ± 0.04 Aa	0.00 ± 0.00 Cc	0.00 ± 0.00 Cb
		Bt cotton		
22	3.60 ± 1.84 ABa	0.00 ± 0.00 Bb	0.00 ± 0.00 Bb	6.88 ± 3.03 Aa
25	0.00 ± 0.00 Cb	1.10 ± 0.05 Bab	4.56 ± 1.23 Aa	5.07 ± 0.58 Aa
28	0.00 ± 0.00 Bb	0.00 ± 0.00 Bb	3.65 ± 0.36 Aa	5.56 ± 1.62 Aa
31	1.50 ± 0.38 Ab	2.24 ± 1.13 Aa	3.20 ± 0.00 Aa	3.30 ± 0.05 Ab
34	0.00 ± 0.00 Bb	0.00 ± 0.00 Bb	0.50 ± 0.28 Bb	1.95 ± 0.57 Ac

Within rows, means with the same upper-case letter do not differ significantly by the Student-Newman- Keuls test (P = 0.05); within columns, means with the same lower-case letter do not differ significantly by the Student-Newman- Keuls test (P = 0.05). Original data.

events with antibiosis (Gould 1984). The evolution of the behavioral resistance may happen if the larvae are able to feed on Bt plants and to develop on the non-Bt ones (Goldstein et al. 2010). There is strong selection stress in *A. argillacea* continuous expression of the toxin in Bt cotton plants due to their monophagous feeding behavior. The plant-plant dispersion may lead to the ingestion of insufficient amounts of the toxin, to the probability of increased survival of heterozygous and it may also potentially speed up the resistance evolution. Similar results were reported by Goldstein et al. (2010), who stated that *O. nubilalis* presents the ability to detect the endotoxin in Bt corn plants when they are exposed for 24 h. According to Goldstein et al. (2010), the potential of *O. nubilalis* larvae with vegetable tissue in their guts from Bt corn was higher than that found in insects that have dispersed on non-Bt corn. However, the amount of vegetable tissue in Bt plants was lower when it was compared with that quantified in larvae kept on non-Bt corn (Razze et al. 2011).

Besides the classic feeding preference, other factors may be involved with the lowest percentage of *A. argillacea* larvae on Bt cotton plants such as larval intoxication, food reduction, inability of the larvae and mortality. According to Halcomb et al. (2000), larvae from the 3rd instar of *H. zea* and *H. virescens* move around cotton plants and indiscriminately feed on Bt and non-Bt cotton. Some behavioral effects of the interaction between larvae and the Bt cotton plants may be the result of δ-endotoxin after it is ingested by the larvae (Halcomb et al. 2000). On the other hand, the resistance evolution is faster when the larvae move and indiscriminately feed just on Bt or just on non-Bt plants when it is compared with larvae that remain sedentary or that separate Bt plants from the non-Bt ones (Heuberger et al. 2011). In comparison to the structured refuge, the seed mix may increase mortality among susceptible insects, if the larva randomly moves around (Mallet and Porter 1992). As per such scenario, the movement/migration of susceptible *A. argillacea* larvae from non-Bt cotton plants to Bt ones, in RIB, may cause higher mortality among susceptible insects than among that in structured refuge. Consequently, it may result in lower population of *A. argillacea* in the refuge; although the effect of the larval movement on the resistance evolution may change according to the size of the refuge and the prevalence of the resistance (Heuberger et al. 2011). Together, the genic flow mediated by the seeds and the pollen transportation has important effect on the resistance evolution. The indiscriminate movement of *A. argillacea* larvae among cotton plants may consistently increase the association between genic flow and resistance if compared with the behavior of sedentary larvae. Even with the 20% to 50% increase in refuge, the resistance evolution is faster when the larvae are in an indiscriminate larval movement scenario, high genic flow and in a refuge with host plants (Heuberger et al. 2011). Besides the larval movement of *A. argillacea* among Bt and non-Bt cotton plants, the differential susceptibility among instars, may cause sublethal exposure and build-up resistance in populations of the target pest, due to survival increase of resistant heterozygous or of individuals that conduct resistance alleles (Wangila et al. 2012). It happens because the feeding behavior of Lepidoptera larvae may be modified between instars; however, the size and physical ability to consume certain diets may influence changes in the larvae's feeding behavior (Zalucki et al. 2002). Thus, the differential feeding behavior among *A. argillacea* instars must be emphasized in further studies.

In conclusion, we found that the feeding behavior of neonate *A. argillacea* is significantly different between Bt and non-Bt cotton cultivars. Based on the percentage of larvae found on the plant and in the organza bags as well as on the amount of vegetal tissue ingested by the larvae, *A. argillacea* shows stronger feeding preference for non-Bt cotton plants when compared to that for Bt cotton plants. However, factors such as temperature and exposure time may affect detection capacity and the abandonment of the plants by the larvae. It results in lower ingestion of vegetal tissue. Information about the feeding behavior and the hosting acceptance of *A. argillacea* on Bt cotton plants are relevant for the handling of its resistance to Bt cotton cultivars. It allows determining how the cotton seeds mix may be a feasible handling option to hold back the resistance evolution of *A. argillacea* populations to Bt cotton cultivars when it is compared with other refuge strategies. The results can also be useful to determine the most effective plants' refuge distribution to manage the resistance of Bt cotton to *A. argillacea*.

ACKNOWLEDGMENTS

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarships granted to the first author, and also thank Embrapa Algodão for their technical support.

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