



Behavior and development of *Tetranychus ludeni* Zacher, 1913 (Acari: Tetranychidae) and physiological stress in genetically modified cotton expressing Cry1F and Cry1Ac proteins

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(With 2 figures)

Abstract

Genetically modified plants are one of the tactics used in integrated pest management - IPM. There is great concern about the impact of these plants on non-target organisms. On the other hand, there is little information in the literature on the effects of transgenics (*Bacillus thuringiensis*) Bt on populations of phytophagous mites, and the physiological responses that this attack promotes on plants. The objective of this work was to evaluate the biology of the *T. ludeni* mite in Bt cotton, expressing the Cry1F and Cry1Ac proteins. To evaluate the behavior of food and oviposition preference of the *T. ludeni* with Bt cotton and isohybrid. Verify if the physiological stress caused by *T. ludeni*'s attack is differentiated in Bt cotton. The mites were reared in Bt cotton and isohybrid, in a total of 40 replicates in the completely randomized design and the biological cycle was evaluated. The food preference and oviposition analysis were done with 10 replicates, with choice. The physiological stress was evaluated through chlorophyll fluorescence, under greenhouse conditions. The data of the *T. ludeni* biology were analyzed by Student's t-test, for food and oviposition preference the chi-square test was performed. Regression models were fitted for the fluorescence parameters. The model identity test was used to evaluate the differences between Bt and isohybrid treatments. Cry1F and Cry1Ac proteins have not affected the biology of *T. ludeni*. The photosynthetic parameters in Bt cotton plants were less influenced by *T. ludeni* infestation.

Keywords: cotton, transgenic, spider mite.

Comportamento e desenvolvimento de *Tetranychus ludeni* Zacher, 1913 (Acari: Tetranychidae) e estresse fisiológico em algodoeiro geneticamente modificado expressando as proteínas Cry1F e Cry1Ac

Resumo

O uso de plantas geneticamente modificadas é uma das táticas utilizadas no manejo integrado de pragas - MIP. Observa-se grande preocupação com o impacto dessas plantas sobre organismos não alvos. Por outro lado, existe pouca informação na literatura sobre efeitos dos transgênicos (*Bacillus thuringiensis*) Bt em populações de ácaros fitófagos, e as respostas fisiológicas que esse ataque promove nas plantas. Objetivou-se com esse trabalho avaliar a biologia do ácaro *T. ludeni* em algodoeiro Bt, expressando as proteínas Cry1F e Cry1Ac. Avaliar se há comportamento de preferência alimentar e postura de *T. ludeni* em relação ao algodoeiro Bt e seu iso-híbrido. E verificar se o estresse fisiológico causado pelo ataque de *T. ludeni* é diferenciado em algodoeiro Bt. Os ácaros foram criados em algodoeiro Bt e iso-híbrido, em um total de 40 repetições no delineamento inteiramente casualizado, onde foi avaliado o ciclo biológico. A análise de preferência alimentar e de posturas foi feita com 10 repetições, com escolha. O estresse fisiológico foi avaliando através da fluorescência da clorofila, em casa de vegetação. Os dados da biologia de *T. ludeni* foram analisados pelo teste t Student, para preferência alimentar e postura foi realizado o teste qui-quadrado. Para os parâmetros da fluorescência,

foram ajustados modelos de regressão. Para testar as diferenças entre Bt e iso-híbrido foi utilizado o teste de identidade de modelos. As proteínas Cry1F e Cry1Ac não afetaram a biologia de *T. ludeni*. Os parâmetros fotossintéticos em plantas de algodoeiro Bt foram menos influenciados pela infestação de *T. ludeni*.

Palavras-chave: algodão, transgenia, ácaro.

1. Introduction

Cotton cultivation is one of the main activities for agriculture in Brazil, being the fifth largest producer in the world, with a cultivated area of approximately 1.6 million hectares (CONAB, 2019). The national production of cotton is primarily aimed at the textile industry, and the main concern is fiber quality, to meet the requirements of domestic industries and external customers.

Several species of mites can cause significant reductions in cotton fiber quality and production. The red mite - *Tetranychus ludeni* Zacher, 1913 (Acari: Tetranychidae) is widely distributed to the producing countries. Mites are specialized in feeding the cytoplasmic content of cells, destroyed them (Fadini et al., 2012). Herbivorous mites are among the most important pests across the world (Castro et al., 2019). The use of transgenic plants expressing insecticidal proteins could affect or alter the biology and development of non-target organisms, such as mites. This is a concern often associated with the use of plants (*Bacillus thuringiensis*) Bt, their possible negative influence on non-target organisms (Becker et al., 2014).

The use of genetically modified plants is one of the tactics of integrated pest management - IPM, allows the reduction of the number of applications of chemical-synthetic insecticides and promotes more effective pest control (Sharma and Ortiz, 2000). Among the transgenic crops cultivated in Brazil, we highlight the Bt cotton, event 281-24-236/3006-210-23 (WideStrike™). In addition to insect resistance through the action of the *cry1F* and *cry1Ac* genes, the event also shows resistance to the herbicide glufosinate-ammonium due to the presence of two copies of the pat gene (*Streptomyces viridochromogenes*) (CTNBIO, 2016).

There is little information in the literature on the effects of Bt transgenics on mite populations and the stress caused on that plant due to the damage caused by the attack. These factors affect plant growth, and in genetically modified organisms can prevent gene expression (Kalaji and Guo, 2008).

This study aimed to evaluate the biology of *T. ludeni* in genetically modified cotton, investigating whether the Bt proteins associated with this product have any effect on these non-target organisms. To evaluate the behavior of food and oviposition preference of the *T. ludeni* with Bt cotton and isohybrid. To verify the physiological stress caused by *T. ludeni*'s attack is differentiated in Bt cotton.

2. Material and Methods

The experiments were conducted in a greenhouse and at the Entomology Laboratory of the Federal University of the Jequitinhonha and Mucuri Valleys - UFVJM. The mites

were removed from laboratory mass rearing and submitted to two treatments, the Bt cotton 281-24-236/3006-210-23 and the isohybrid cotton.

The cotton seeds were planted in polyethylene containers of 5L, with soil fertilized according to recommendations for containers (Malavolta, 1980), maintaining two plants per container, in a total of 30 replicates for each treatment, to obtain leaves. The cotton plants were cultivated in the greenhouse.

2.1. Obtaining the biological data of *T. ludeni*

The biological cycle, the viability of the eggs and the survival of the stages of development for *T. ludeni* were evaluated. A total of 40 replicates were used in a completely randomized design. The evaluations were made in incubator type B.O.D. at 25 ± 1 °C, $60 \pm 5\%$ relative humidity and 12h photophase.

The development and reproduction of *T. ludeni* on leaf discs with 5cm in diameter of Bt cotton and the isohybrid were observed for one generation. For the preparation of the discs, leaves from the median part of the cotton plants were used, with 35 to 60 days old (Esteves-Filho et al., 2010).

The discs were placed on a moistened polyethylene sponge saturated with distilled water and kept in gerbox type plates (Esteves-Filho et al., 2010). To avoid the escape of the mites, the discs were surrounded by cotton moistened with water.

Each disc received five adult females of *T. ludeni*, which remained in the arenas (leaf of the cotton plant) during 16 hours, for oviposition. Then, the females were removed from the discs, the eggs were quantified, and only two eggs were left in the arena (Silva et al., 2005).

Eggs viability was calculated by the difference between the number of eggs left in the arenas and the number of eggs not hatched. For the incubation and the development phase duration, daily evaluations were performed.

After emergence, the females were mated with males obtained from the mass rearing, and the number of eggs produced and the adults' longevity was evaluated daily.

2.2. Food preference and oviposition behavior test of *T. ludeni*

The food preference and oviposition behavior of *T. ludeni* were evaluated in incubator type B.O.D. at 25 ± 1 °C, $60 \pm 5\%$ relative humidity and 12h photophase. For the evaluation, leaf discs with 5cm diameter were obtained from the central part of leaves of Bt cotton and the isohybrid. The discs were placed side by side and connected by a plastic cover. This set was placed on a sponge saturated with water, inside plastic plates (gerbox).

Six females of *T. ludeni*, obtained from the laboratory mass rearing, were released in the medial portion of the

plastic cover. After the mites releasing, the gerbox plates were incubated in the B.O.D. and after 24h, the number of females and laid eggs were evaluated. To evaluate the food preference of *T. ludeni* the treatments Bt vs Bt, Bt vs isohybrid and isohybrid vs isohybrid were proposed. Were evaluated 10 repetitions per treatment (Esteves-Filho et al., 2010).

2.3. Evaluation of the physiological stress of cotton

After the occurrence of a natural infestation of *T. ludeni* in greenhouse cotton plants, the physiological stress was evaluated using a fluorometer (Mini-Pam-2014, Heinz-Walz), with 30 repetitions. The tweezers of the fluorescence measuring device were placed in the middle of the third fully expanded cotton leaves, where the initial fluorescence, maximum fluorescence, the ratio between variable and maximum fluorescence, and the rate of electron transport were obtained. Measurements were taken after 30 minutes of adaptation to the dark (Ferreira et al., 2015).

The severity of *T. ludeni* infestation was evaluated. According to the number of adult mites present in the leaves, different damage scores were awarded. Score 1 was assigned to non-infested leaves (zero mites), score 2 to infested leaves (≤ 100 mites), and score 3 to heavily infested leaves (> 100 mites).

2.4. Statistical analyzes

The biological data were analyzed by Student's T-test. For food and oviposition preference, the chi-square test was performed, both using the computer program R through the functions `t.test` and `chisq.test`, respectively. Survival curves for the juvenile and adult phases were determined using the Kaplan-Meier estimator. For the analysis of chlorophyll fluorescence data, the simple linear regression model was adjusted. To verify if the adjusted regressions

for Bt cotton and its isohybrid are statistically equal, the model identity test was used. In this test, the equality of the regression coefficients (B0 and B1) was assumed as the null hypothesis. Details on this statistical methodology are presented by Cruz et al. (2014).

3. Results

There was no difference observed on development of larva, protonymph, deutonymph, longevity of adults and duration of egg to adult for *T. ludeni* reared in Bt cotton and isohybrid (Table 1).

No differences were observed for the characteristics evaluated in the adult phase: number of eggs per female, egg viability, incubation period, number of eggs per female of first-generation, pre-oviposition, oviposition and post-oviposition period for both treatments (Table 2).

Kaplan-Meier survival curves showed the same behavior for both varieties studied (Figure 1). It was found a high mortality rate in the immature phase, individuals that passed into adulthood had a lower mortality rate until the cycle was completed, characterizing a type III curve (Ricklefs, 2011). The adults reared on Bt cotton discs survived for six days longer than isohybrid.

In the tests of food and oviposition preference, there was also no difference for Bt vs. Bt, Bt vs. isohybrid and isohybrid vs. isohybrid (Table 3). Thus, no food and oviposition preference were observed for *T. ludeni* to Bt cotton and isohybrid.

The model identity test did not detect differences between Bt cotton and isohybrid as a function of *T. ludeni* infestation for initial fluorescence (Fo), maximum fluorescence (Fm), variable fluorescence/maximum fluorescence ratio (Fv/Fm), and electron transport rate (ETR) (Table 4).

Table 1. Mean duration, in days, of the stages of development and Student's t-test (t-Estimate) of *Tetranychus ludeni* reared on Bt cotton and isohybrid.

Stages	Bt	Isohybrid	t-Estimate	p-value
Larva	2.800 \pm 0.140	2.775 \pm 0.098	0.147	0.884
Protonymph	2.000 \pm 0.136	1.920 \pm 0.162	0.378	0.707
Deutonymph	3.000 \pm 0.277	2.867 \pm 0.256	0.353	0.727
Longevity of adults	11.000 \pm 3.246	9.714 \pm 2.447	0.316	0.756
Egg - Adult	14.350 \pm 3.525	13.500 \pm 2.490	0.197	0.847

Note: p-value= evidence against a null hypothesis.

Table 2. Number of eggs per female, egg viability, incubation period, number of eggs per female of the first-generation, pre-oviposition, oviposition, post-oviposition period and Student's t-test (t-Estimate) of *Tetranychus ludeni* reared on Bt cotton and isohybrid.

Feature	Bt	Isohybrid	t Estimate	p-value
N. of eggs/♀	1.415 \pm 0.112	1.225 \pm 0.100	1.266	0.210
Egg viability	64.000 \pm 5.491	60.000 \pm 5.591	0.488	0.627
Incubation period	4.486 \pm 0.093	4.391 \pm 0.083	0.763	0.448
Total eggs/♀ 1ª G	24.857 \pm 4.383	29.000 \pm 1.080	-0.929	0.408
Pre-oviposition	1.333 \pm 0.333	1.500 \pm 0.289	-0.378	0.715
Oviposition	14.167 \pm 4.061	10.500 \pm 2.327	0.783	0.458
Post-oviposition	0.833 \pm 0.307	1.000 \pm 0.408	-0.326	0.755

Note: p-value= evidence against a null hypothesis.

Figure 2a shows the curves referring to Fo, showing the behavior of each treatment concerning the severity of the infestation. No significance was observed for B0,

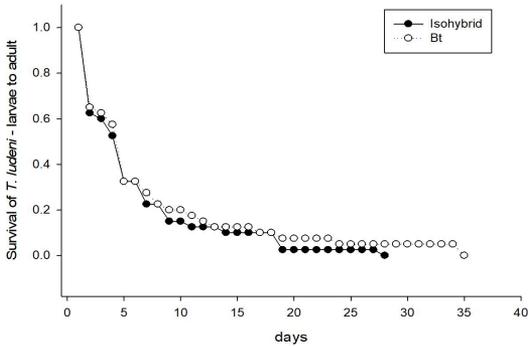


Figure 1. Survival curves, obtained by the Kaplan-Meyer estimator, of individuals of *Tetranychus ludeni* reared on leaf discs of Bt cotton and isohybrid.

Table 3. Result of Chi-square test (X^2) for food and oviposition preference of *Tetranychus ludeni* submitted to Bt cotton and isohybrid, with option of choice.

Trat.	Food		Oviposition	
	X^2	p-value	X^2	p-value
Bt vs. Bt	0.154	0.695	0.600	0.438
Bt vs. Isohybrid	1.000	0.317	0.053	0.818
Isohybrid vs. Isohybrid	0.571	0.449	3.769	0.052

Note: p-value= evidence against a null hypothesis.

indicating an absence of effect on Fo as a function of the increment of the mite infestation for the evaluated treatments. Although there was no significance for the angular coefficient of the line (B0), there was a tendency for an increase in the Fo due to the increase in the severity of *T. ludeni* infestation in the isohybrid.

When evaluating the variables maximum fluorescence (Figure 2b), variable fluorescence ratio and maximum fluorescence (Figure 2c) and electron transport rate (Figure 2d), a similar behavior was observed, with a tendency to decrease in the values with the increase of the *T. ludeni* infestation. Both Bt cotton and isohybrid presented lower values of Fv/Fm, in the high severity condition (Figure 2c). It was observed that there was a significant effect (B0) of *T. ludeni* infestation on the Fv/Fm and ETR ratio for the isohybrid (Figure 2c and 2d). However, Bt cotton was not affected by infestation levels (B0 not significant), presenting stable behavior for all photosynthetic parameters (Figure 2).

4. Discussion

Cry1F and Cry1Ac toxins produced by Bt cotton did not affect the duration of immature stages, adult longevity and egg to adult duration in *T. ludeni*. Similar results were found for *Tetranychus urticae* Koch, 1836 (Acari: Tetranychidae), with no differences for the immature stages, adult longevity and the egg to adult period for mites submitted to Bt cotton treatments expressing the Cry1Ac protein (Esteves-Filho et al., 2010). Guo et al. (2016) obtained the same conclusions in studies with

Table 4. Scheme of analysis of variance relative to the model identity test, considering as null hypothesis the equality of the two adjusted regression models for the variables initial fluorescence, maximum fluorescence, relation between variable and maximum fluorescence and electron transport rate in function of the degree of infestation of *Tetranychus ludeni*.

	Initial fluorescence (Fo)					
	FV	GL	SQ	QM	F	p-value
Model (Full)		4	2,791,302.180	-	-	-
Model (Reduced)		2	2,665,805.180	-	-	-
Reduction (Ho)		2	125,497.010	62,748.504	4.660	0.177
Residue		6	26,928.780	13,464.391	-	-
Maximum fluorescence (Fm)						
Model (Full)		4	37,638,964.800	-	-	-
Model (Reduced)		2	37,615,317.020	-	-	-
Reduction (Ho)		2	23,647.780	11,823.892	0.573	0.636
Residue		6	41,242.840	20,621.422	-	-
Variable fluorescence/maximum fluorescence (Fv/Fm)						
Model (Full)		4	3.196	-	-	-
Model (Reduced)		2	3.195	-	-	-
Reduction (Ho)		2	0.002	0.001	1.532	0.395
Residue		6	0.001	0.001	-	-
Electron transport rate (ETR)						
Model (Full)		4	6,540.549	-	-	-
Model (Reduced)		2	6,540.549	-	-	-
Reduction (Ho)		2	76.318	38.159	15.794	0.060
Residue		6	4.832	2.416	-	-

Note: FV= Source of variation; GL= degrees of freedom; SQ= sum of squares; QM= mean squares; F= test statistic, p-value= evidence against a null hypothesis.

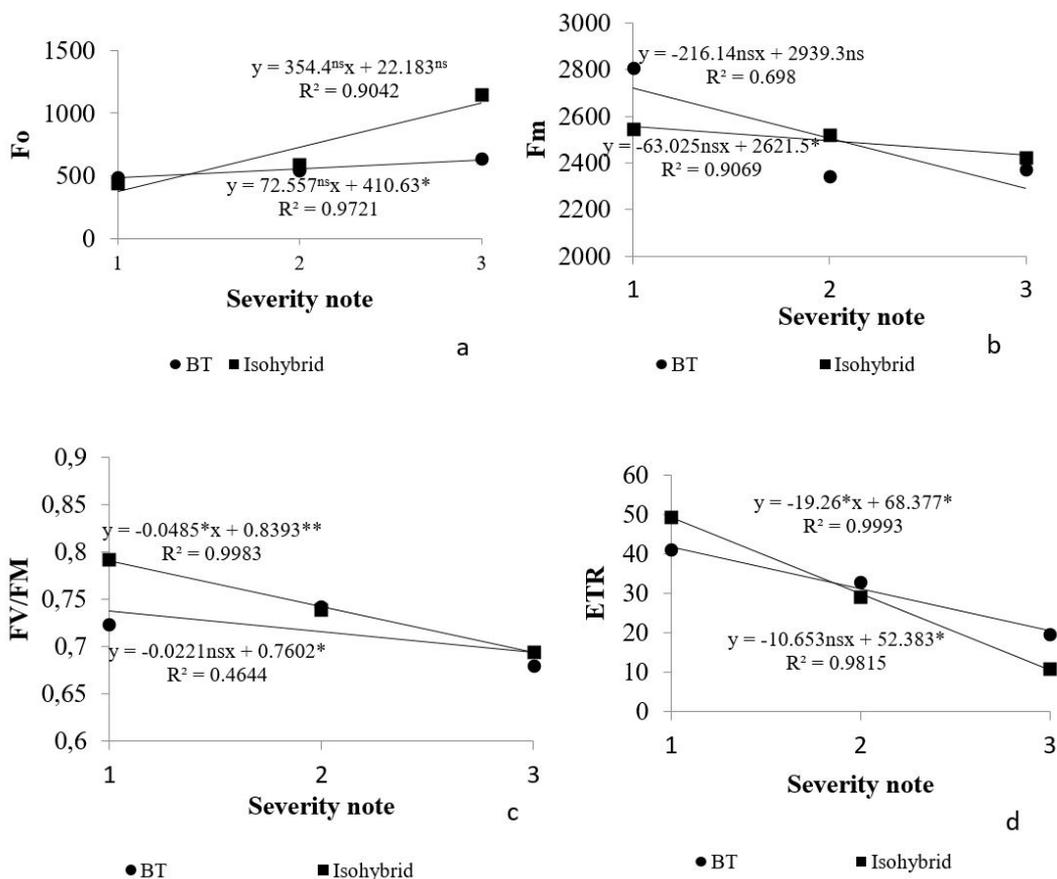


Figure 2. Regression model for initial fluorescence (F_o- Figure a), maximum fluorescence (F_m- Figure b), ratio between variable and maximum fluorescence (F_v/F_m- Figure c) and electron transport rate (ETR- Figure d) in Bt cotton and isohybrid as a function of grades of infestation severity of *Tetranychus ludeni*: 1 = zero; 2 = ≤ 100; 3 = > 100 mites/leaf.

the Cry1F toxin, finding no impacts for the biology of *T. urticae* mites. The results obtained in this work provide strong evidence that this technology, including pyramidal hybrids, is safe for the non-target organisms in question.

The averages found for the characteristics evaluated in the adult phase show that there is no effect of the Cry toxins in *T. ludeni*. Contrary results verified that alterations caused in the secondary metabolism of Bt cotton plants were responsible for the reduction in generation time and increase in the number of eggs of mites *Tetranychus cinnabarinus* Boisduval, 1987 (Acari: Tetranychidae) and *Tetranychus truncatus* Ehara, 1956 (Acari: Tetranychidae) (Ma et al., 2014).

The observed behavior for survival curves is expected for mites since a type III survival curve results from high mortality in early life due to the vulnerability of young individuals (Ricklefs, 2011). The survival of phytophagous organisms is directly linked to the quality of the host plant. Survival curves show the same behavior for mites reared in Bt and isohybrid plants. Esteves-Filho et al. (2010) evaluating the biology and behavior of *T. urticae* mites obtained similar results, with no difference for survival in Bt cotton plants and isohybrid cotton. However, the *T. ludeni* adults reared on Bt cotton leaf discs survived longer than in

the isohybrid, although without statistical differences in the average longevity in the different treatments. The prolonged survival of some *T. ludeni* individuals in Bt treatment may be related to changes in secondary metabolism caused by the addition of genes. Considering that the insertion of Bt genes into cotton plants may promote changes in secondary compounds associated with plant-herbivore interaction (Yan et al., 2004).

Studies on the effects of Cry proteins expressed by transgenic plants on populations of phytophagous mites are still incipient but have shown that these organisms are generally unaffected, despite the high exposure through acquisition and accumulation of the protein in their body and the fact that the protein remains active after ingestion (Obrist et al., 2006).

Tetranychus ludeni did not present food and oviposition preferences with Bt cotton and isohybrid plants. According to Rovenská et al. (2005) *T. urticae* mites preferred transgenic plants for feeding and laying eggs relative to conventional plants. Li et al. (2014) reported that mites *Tetranychus turkestanii* Tetrk, 1937 (Acari: Tetranychidae) preferred to feed and lay eggs in conventional plants than in transgenics. Few studies and the complexity of adequacy of the trials may be the reasons for variations in results

and difficulty of comparisons. But it must be considered that the behavior of the mites can be influenced by the constitutive characteristics of the plants, which include their allelochemicals and the toxins expressed.

Thus, the results presented in this study provide evidence that Bt technologies containing the *cry1F* and *cry1Ac* genes do not affect *T. ludeni* populations. One of the great advantages of the use of genetically modified organisms - GMOs is the reduction in the use of insecticides, which allows the use of this tactic with biological control. Populations of phytophagous mites that develop in Bt cotton could be controlled and kept below the level of economic damage by their natural enemies, such as the predator mite *Phytoseiulus macropilis* Banks, 1904 (Acari: Phytoseiidae) already associated with *T. ludeni* (Reichert et al., 2017; Castro et al., 2014), and the cotton crop (Esteves-Filho et al., 2010) in the literature.

In the evaluation of the physiological stress, higher values of F_0 may indicate structural damage in the reaction centers of Photosystem II or compromise in the transport of excitation energy from the antenna complexes to the reaction centers (Cruz et al., 2009). The accessory pigments such as chlorophyll-a, b are part of the antenna complex and have as their main function to absorb the photons and transfer the energy to the reaction center complex (Bolh ar-Nordenkampf et al., 1989). Thus, damage to the photosynthetic apparatus may have occurred in the cotton plants of the isohybrid treatment, because of *T. ludeni* infestation. This is because feeding of mites damages directly the photosynthetic cell apparatus (Aldea et al., 2005).

The maximum quantum yield of photosystem II (F_v/F_m) is determinant for the identification of a biotic stress (Kalaji and Guo, 2008). It may vary from 0.75 to 0.85 in plants not subjected to stress (Bolh ar-Nordenkampf et al., 1989). Values below 0.75 indicate stress and reduced photosynthetic potential of the plant (Ferreira et al., 2015). Which was observed for the Bt cotton and isohybrid attacked by the mite. Huang et al. (2013) state that under low infestation of sucking insects, the photochemical reactions and the ETR are not very affected, however, when there is a high infestation and a long period of interaction between herbivore-plant, the fluorescence parameters are negatively influenced, which occurred with F_m , F_v/F_m , and ETR.

Bt plants were less affected by the severity of the mite infestation, presenting less biotic stress and this can be an advantage. Since mites feed on cellular cytoplasmic content, directly damaging the cell's photosynthetic apparatus and indirectly the gas exchanges, by interrupting the transport of nutrients and water (Aldea et al., 2005). This attack causes a reduction in the photosynthetic rate and lower leaf growth of the plant (Velikova et al., 2010), chlorosis and premature loss of leaves, with a consequent decrease in productivity (Li et al., 2013). The use of GMOs has increasing in agriculture, especially in countries like Brazil, which due to its extensive territorial area and favorable climate has the potential to increase the production efficiency.

5. Conclusions

The Cry1F and Cry1Ac proteins constitutively present in Bt cotton did not affect the biological and behavioral parameters of *T. ludeni* mites in this study.

The photosynthetic parameters in Bt cotton plants were less influenced by *T. ludeni* infestation. The isohybrid showed higher physiological stress in the high severity of infestation.

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