University of São Paulo
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Approaches based on insect behavior and plant electrophysiology to evidence aphid- and imidacloprid-mediated stress in non-Bt and Bt cotton

Jéssica Karina da Silva Pachú

Thesis presented to obtain the degree of Doctor in Science.
Area: Entomology

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Bachelor in Agroecology

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DEDICATION

With love
I dedicate

To my family, especially to my parents José and Mª Zélia for their dedication, love and support renewed every day of my life.

My boyfriend José Bruno, for his friendship, support, motivation and unconditional love all the time.

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My dear family, even geographically distant, was always present, for their emotional support, love and affection.

"Pequenos passos podem não fazer muita diferença numa jornada curta, mas na longa jornada da vida são capazes de colocar você num lugar completamente diferente"

James C. Hunter

CONTENTS

RESUMO

Abordagens baseadas no comportamento do inseto e na eletrofisiologia da planta para evidenciar o estresse mediado por afídeos e imidacloprid em algodão não Bt e Bt

Plantas são capazes de emitir, processar e transmitir sinais elétricos quando em condições desfavoráveis. Diante disso, esse estudo teve por objetivo caracterizar a produção de sinais elétricos em plantas de algodão Bt e não-Bt (*Gossypium hirsutum* L.) infestadas com *Aphis gossypii* e, também caracterizamos o comportamento de dispersão desses afídeos para relacionar esse comportamento às respostas de sinalização das plantas. Adicionalmente, evidenciamos as respostas fotossintéticas e elétricas da planta ao estresse causado pela herbivoria de *A. gossypii* combinado ao estresse gerado pelo uso de imidacloprid e avaliamos também como esse estresse pode influenciar a ecologia comportamental do predador *Cycloneda sanguinea* e do afídeo. Os resultados mostraram que tanto as variedades de algodão Bt quanto as não-Bt, quando atacadas por *A. gossypii*, emitem sinais elétricos do tipo potencial de variação e mostram claramente a presença de respostas distintas quanto à percepção e comportamento dos pulgões. Além do registro da geração de potenciais de variação nas plantas, ocorreram alterações na taxa respiratória do algodoeiro mediadas pela aplicação do inseticida. O imidacloprid também causou intensa locomoção nos machos de *C. sanguinea*. Os resultados aqui obtidos motivam estudos futuros que visem elucidar os fatores envolvidos nos processos de resistência ao estresse e defesa das plantas e, assim, podem ser utilizados como ferramenta para a implementação de programas de manejo integrado, na busca pela conservação e conservação. maior eficiência e sustentabilidade do agroecossistema, e evidenciar os possíveis impactos do uso do imidaclopride na eletrofisiologia das plantas mesmo nos casos de não ocorrência do pulgão *A. gossypii*.

Palavras-chave: Fisiologia, Bioeletricidade, Algodoeiro, Pulgão do Algodão.

ABSTRACT

Approaches based on insect behavior and plant electrophysiology to evidence aphid and imidacloprid-mediated stress in non-Bt and Bt cotton

Plants are capable of emitting, processing, and transmitting electrical signals under unfavorable conditions. Therefore, this study aimed to characterize the production of electrical signals in Bt and non-Bt cotton plants (*Gossypium hirsutum* L.) infested with *Aphis gossypii* and characterize the dispersal behavior of these aphids to relate this behavior to the responses of plant signaling. Additionally, we evidenced the photosynthetic and electrical responses of the plant to the stress caused by the herbivory of *A. gossypii* combined with the stress generated by the use of imidacloprid, and we also evaluated how this stress can influence the behavioral ecology of the predator *Cycloneda sanguinea* and its prey. The results showed that both the Bt and non-Bt cotton varieties, when attacked by *A. gossypii*, emitted electrical signals of the variation potential type and clearly showed the presence of distinct responses regarding their perception and behavior of aphids. In addition to recording the generation of plant variation potentials, there were changes in the cotton respiration rate mediated by the application of the insecticide. Imidacloprid also caused high locomotion in *C. sanguinea* males. The results obtained here motivate future studies that aim to elucidate the factors involved in the processes of resistance to stress and plant defense and, thus, can be used as a tool for the implementation of integrated management programs in the search for conservation and greater efficiency and sustainability of the agroecosystem and to highlight the possible impacts of the use of imidacloprid on the electrophysiology of plants even in cases of nonoccurrence of the aphid *A. gossypii*.

Keywords: Physiology, Bioelectricity, Cotton, Cotton aphid.

General Introduction

For years, researchers have focused their efforts on studying chemical and hydraulic signals, neglecting the fact that plants also use electrical signaling. A growing body of literature has shown that electrical signaling is present not only in sensitive plants but also in the vegetable kingdom, supporting hypotheses about the role of bioelectricity as a fundamental 'model' for the regeneration activities of these organisms (Hanson, 2021). These signals are physical phenomena capable of transmitting information faster than chemical signals (Trebacz et al. 2006; Yan et al. 2009). The reason why plants have developed ways to use this type of communication probably lies in the need to respond quickly to environmental stressors (Volkov & Haack, 1995; Sukhov et al. 2019). Furthermore, a large number of important biological and physiological phenomena are accompanied by these cellular electrical manifestations.

Plants respond constantly and simultaneously to various environmental factors, such as light, gravity, soil water content, predators, nutrient availability, and others, and they need to transmit environmental information to adjacent cells over long distances along the apical-basal axis. This requires a sophisticated signaling mechanism to integrate perception, transmission, and response (Baluska & Volkmann; Menzel, 2005; Brenner et al. 2006; Pelagio-Flores et al. 2011). Therefore, the ultimate goal of this signaling is to activate response mechanisms in remote tissues, improving the ability of the entire plant to prepare its tissues for future challenges (Gilroy et al. 2014).

The study of electrical signals in plants has advanced over time, and several helpful discoveries have motivated further studies on different environmental stimuli and their triggered physiological responses. On the other hand, little is known about the electrophysiology of genetically modified plants that express *Bacillus thuringiensis* (Bt) and even more about the possible effects of insecticide molecules that are used in this crop. Therefore, the general objective of this study was to characterize electrical signaling in Bt and non-Bt cotton plants (*Gossypium hirsutum* L.) through the stress produced by *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae) and by the insecticide imidacloprid. Additionally, the dispersal behavior of the aphid and its predator living in transgenic plants and the insecticide was analyzed. Due to its importance for their producing countries, we chose as model plants a variety that expresses endotoxins produced by *B. thuringiensis* (Bt) and its isoline (non-Bt). According to the risk of facilitating the infestation of sucking insects in Bt cotton crops (Stewart et al. 2001; Malaquias et al. 2014), the plants were subjected to stress induced by the attack of

A. gossypii and application of the neonicotinoid imidacloprid – commonly used to control aphids on cotton cultivation. Some hypotheses were stated about the physiological effects triggered in this process. The effects of cotton varieties, as well as insecticides, were also discussed from the behavioral point of view of the insect pest and the natural enemy *Cycloneda sanguinea* (Linnaeus, 1763) (Coleoptera: Coccinelidae). Additionally, a brief review of how electrical signals are generated and propagated is presented, as well as a description of how these electrical potentials are measured. The mini-review (chapter II) has been submitted for publication in the journal Plant Signaling & Behavior, and chapters III and IV have been published in Plos One and Chemosphere, respectively.

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2. A short review: Electrical signaling and plant response to biotic stresses[1]

The ability to respond to environmental stimuli is a basic ability for an organism to live and thrive. When dealing with sessile beings such as plants, this ability is even more crucial considering that the environment changes constantly and as plants need to respond quickly to these changes, as under certain conditions, they can cause reversible and/or irreversible damage to the plant organism. Understanding how plants perceive and react to the most diverse environmental stimuli, especially harmful stimuli, has been the object of study by many researchers around the world. The main justification for this interest is that environmental changes can cause biotic and abiotic damage or stresses, which limit plant growth and development. Understanding how plants behave under unfavourable conditions is, therefore, of great importance and involves the effort of several areas of plant sciences, such as biochemistry, physiology, genetics, molecular biology, ecology and electrophysiology.

Among the various biotic stress factors, herbivory is one of the most affecting plant lives. Herbivory is caused by mammals or phytophagous insects, and in addition to damaging plant tissue, it also opens "doors" for the attack of pathogenic microorganisms that cause disease, which in turn characterizes another stress factor. The plant-herbivore interaction, although widely studied, is very complex and still has many obscure aspects. However, it is known that plants are not passive organisms and have several defense strategies against herbivory, including the presence of mechanical barriers such as thorns and chemical barriers, such as the production of alkaloids, terpenoids, steroids, phenolic compounds, among other secondary metabolites that can be toxic to animals, have an unpleasant taste or even serve as a signal to warn neighboring plants about the attack (Mittler & Blumwald, 2015). However, for stress responses to be effective, plants need to perceive the stress and then activate mechanisms that lead to a local or systemic defense response; that is, there needs to be a signal to link perception and response. A key actor in these signaling mechanisms is jasmonic acid, a lipid-derived plant hormone, which rapidly accumulates in organs remote from the herbivore's feeding site. Electrical signals also act in this signaling process (Wildon et al. 1992; Mousavi et al. 2013).

Bioelectrical activity due to stimulation in plants was first discovered by Burdon-Sanderson in 1873. He measured an action potential that propagated at a speed of 200 mm s^{-1} in *Dionaea muscipula* and occurred under the strong influence of temperature. Sanderson also observed that action potential propagation occurs in the central portion of the leaf blade and

[1] The mini-review has been submitted for publication in the journal Plant Signaling & Behavior

faster in the abaxial face and directed perpendicularly to the midrib (Pickard, 1973). Later, electrical signals in plant cells were discovered and studied in various plants.

It is known that at resting membrane potential, living cells present an electrical potential difference of several tens of millivolts across the plasma membrane, with the intracellular medium negative in relation to extracellular fluids. The genesis of this membrane potential is associated with ion transport mechanisms, which create an intracellular ionic medium with a different composition from that of the extracellular ionic medium. Therefore, diffusion processes and active transport represent the basic mechanisms responsible for the polarization of the plasma membrane. Ion diffusion in favor of concentration gradients is the most important cause of electrical manifestation in biological systems (Lacaz-Vieira, 1981).

Intracellular electrical signals serve as a mode of information transmission in plant cells (Fromm & Lautner, 2007). It has been shown that long-distance electrical signaling is involved in several physiological processes, such as photosynthesis (Koziolek et al. 2003), cell elongation (Shiina & Tazawa, 1996), respiration (Dziubinska et al. 1989), water absorption (Davies et al. 1991), gas exchange (Fromm et al. 1995), phloem transport (Fromm & Bauer, 1994), polinization (Clarke et al. 2013) and many other vital processes (Sukhov et al. 2019). Furthermore, studies have shown that plants have greater electrical excitability under unfavorable conditions, certainly due to the need to respond quickly to environmental stresses, both from biotic (Maffei et al. 2006; Pachu et al. 2021) and abiotic factors (Silva et al. 2020; Dolfi et al. 2021). In this brief review, we address general aspects of plant electrophysiology, such as types of electrical signals and methods for recording the electrical activity of plants, and highlight the role of electrical signals in plant responses to biotic stresses.

2.1 Types of electrical signals in plants

The main electrical signals in plants are the action potential (AP), the variation potential (VP) and the system potential (SP). The action potential transmits at constant speed and maintains constant amplitude; it follows the all-or-nothing law, in which stimuli weaker than a certain threshold can generate a change in this potential. After AP is generated, the cell membrane enters absolute and relatively refractory periods in succession (Trebacz et al. 2006). Action potentials are induced by nonharmful stimuli, e.g., cold, mechanical and electrical stimuli (Opritov et al. 2004; Krol et al. 2006; Degli Agosti, 2014; Sevriukova et al. 2014), and are a signaling phenomenon that can transmit information quickly over long distances. APs are based on the activity of voltage channels, with calcium, chlorine and potassium being the main

ions involved in the mechanisms of generation of this signal in plants (Opritiv et al. 2002; Vodeneev et al. 2006).

The variation potentials, also called slow wave potentials, are not constant and decrease in speed and amplitude as they move away from the stimulus site. They are induced by harmful stimuli (e.g., burning and cutting) (Fromm et al. 2007), and they are characterized by long-term depolarization whose duration can be tens of minutes or longer (Trebacz et al. 2006; Sukhova et al. 2018). VP generation occurs after activation of mechanosensitive or ligand-dependent Ca^{2+} channels induced by hydraulic waves or wound substance propagation (Vodeneev et al. 2011), which leads to increased intracellular calcium concentrations and consequent inactivation of H^+-ATPase in the plasma membrane (Vodeneev et al. 2015).

SP is a systemic signal induced by abiotic and biotic factors, self-propagating and transmitted via a hyperpolarization event, related to the activation of H+-ATPase in the plasma membrane (Zimmermann et al. 2009). However, the participation of Ca^{2+} and K^+ channels in SP propagation is likely, since this signal was suppressed in plants deficient in these nutrients (Lautner et al. 2005). Zirmmermann et al. (2016) also observed the propagation of SP in the stimulation zone after the induction of VP and/or AP. This means that both depolarization and hyperpolarization in the stimulated zone must induce some similar processes that participate in the propagation of SP (Sukhov et al. 2019).

2.2 Methods for recording electrical activity in plants

Approaches to the study of electrical activities in plants include intracellular and extracellular measurements. Extracellular measurement is a noninvasive and physically stable technique, and measurements may be performed simultaneously with other physiological methods, such as gas exchange and plant turgor; among others, it applies to tests to observe the variation in electrical potential in the long term (> 24 h) (Li et al. 2006; Macedo et al. 2021). Measurements are made using electrodes that consist of an Ag/AgCl lead wire moistened with 0.1% KCl (w/v) in agar and wrapped in cotton to promote proper contact with the plant surface (Fromm & Spanswick, 1993), or the electrodes can be connected to the plant surface using a conductive aqueous gel, commonly used in electrocardiography (Mancuso, 1999).

A four-channel data acquisition interface and software (Lab-Trax 4 / 24T, World Precision Instruments and LabScribe® version 3, iWorx Systems Inc.) is required. Each channel is independent, with its own 24-bit analog-to-digital converter and equipped with the appropriate filters. The electrical signals in the plants need to be amplified, and the recording device must have a high input impedance. The electrodes can be connected by cables to a

computer screen, and an identical electrode must be connected in the distal region of the plant or to the ground to serve as a reference electrode. When the various channels show stabilized potentials, the plant can be electrically stimulated (3 V for 2 s) or by another stimulus (heat, cold, cut) applied to the leaf. Signals from the electrodes are then amplified and recorded, and usually, the electrical response can be verified in all electrodes, indicating that the transmission of the electrical signal occurs throughout the plant (Fromm & Lautner, 2007; Macedo et al. 2021).

The intracellular measurement technique applies to the observation and study of bioelectricity at the cell level and typically uses a glass microelectrode with a tip diameter of less than 1 µm inserted into the cell. These are very accurate measurements but punctual because, as this is an invasive technique, the measurement is done in a short time because the electrolytes present in the electrode can diffuse into the cell, changing the original bioelectrical condition (Huang 2006; Fromm and Lautner, 2007; Xiaofei et al. 2009). The microelectrodes were filled with KCl, fixed to an Ag/AgCl wire and connected to an amplifier. After the amplifier has been reset with both electrodes outside the cell, a microelectrode is inserted into the cytoplasm (or vacuole) of a cell with a micromanipulator, and the reference electrode is placed in the solution around the cell (Fromm & Lautner, 2007).

2.3 Electrical signaling in response to biotic stress

It has been shown that different environmental stimuli evoke specific responses in living cells, which are rapidly transmitted over long distances (Lautner et al. 2005). Numerous physiological effects of electrical signaling have been described in recent years (Fromm & Lautner, 2006; Trebacz, Dziubinska & Krol, 2006; Silva et al. 2020; Pachu et al. 2021a).

Electrical signals have been elucidated as one of the main responses to herbivory, occurring within seconds to minutes after the injury suffers and followed by a cascade of chemical signaling (Maffei et al. 2007; Zebelo & Maffei, 2015). Volkov & Haack (1995) were the first to measure insect-induced action and variation potentials in long-distance plant communication. The experiment was carried out with potato plants (*Solanum tuberosum* L.) In the presence of Colorado potato beetle larvae (*Leptinotarsa decemlineata* (Say); Coleoptera: Chrysomelidae), the larvae consumed the upper leaves of the plants, and after 6-10 h, action potentials with amplitudes of 40 ± 10 mV were recorded every 2 ± 0.5 h during a 2-day test period. In studies with *Spodoptera littoralis* in *Phaseolus lunatus*, both direct herbivory and oral secretions of the insect induced a rapid depolarization of Vm (Maffei et al. 2007; Bricchi

et al. 2010; Bricchi et al. 2012). Bricchi et al. (2012), studying the aphid *Myzus persicae*, observed a 12 Vm depolarization in response to feeding this aphid.

Plants have developed the ability to respond to herbivores, producing toxic weapons (such as many secondary metabolites), producing new defense components (Heil & Ton, 2008; Mittler & Blumwald, 2015) and through molecular interactions that can attract predators or parasitoids of these herbivores (Wu & Baldwin, 2010; Baldwin, 2010). Green & Ryan (1972) reported that, for example, tomatoes (*Lycopersicon esculentum*) respond to insect feeding by producing defensive proteins, such as proteinase inhibitors, that is, compounds that reduce protein digestion by insects and are induced in damaged leaves. Systemic transport signals are also involved when translocation of defensive compounds contributes to systemic resistance, for example, when nicotine is produced in tobacco roots (*Nicotiana sylvestris*) when leaves are attacked (Baldwin, 1997).

The electrophysiological signals from plants triggered by attack by herbivores are complex and can lead to the activation of multiple defenses (Maffei et al. 2004) and, consequently, morphological, physiological, biochemical and molecular changes that affect plant growth and productivity (Wang et al. 2004). Therefore, knowing which signaling pathways are involved in this regulation makes it possible to establish strategies to improve physiological performance and improve the development capacity and productivity of plants (Abid et al. 2018). In addition, the knowledge of stress-induced alterations in the membrane's electrical potential and their effects allow the emergence of new stress monitoring tools, which is of paramount importance to elucidate the factors involved in these processes.

In recent studies, Pachu et al. (2021a; 2021b) characterized the production of electrical signals in Bt and non-Bt cotton plants (*Gossypium hirsutum* L.) infested with *Aphis gossypii* (Glover, 1877) (Hemiptera: Aphididae). The dispersal behavior of aphids to correlate this behavior with plant signaling responses. In their studies, the photosynthetic and electrical responses of the plant to the stress caused by the herbivory of *A. gossypii* combined with the stress generated by the use of imidacloprid were evidenced.

The results obtained by Pachu et al. (2021a) showed that both the Bt and non-Bt cotton varieties, when attacked by *A. gossypii*, emitted electrical signals of the variation potential type and clearly showed the presence of distinct responses as to your perception and behavior of aphids. Bt cotton plants propagated VP signals faster; however, they produced signals in a smaller amount with a higher density of aphids, also promoting greater dispersion of aphids within the plant. Despite this, there was a delay in terms of the number of signals propagated on Bt cotton plants with 60 aphids per plant, which produced the fewest number of signals

between 0 and 36 h. Another important result was the greater dispersion behavior related to this same treatment, especially during and after 48 h of infestation.

Pachú et al. (2021a) suggest that their results can be supported by two hypotheses and explained independently or combined. The first hypothesis is based on the possibility of a trade-off in terms of defense of the Bt plant; a high dispersal could reflect a greater exploitation of food resources by aphids and facilitate the penetration of mouthparts by aphids on Bt cotton plants, which may explain why Bt cotton plants emitted faster electrical signals than non-Bt cotton plants in the former moment, showing that Bt cotton plants may be more susceptible to aphid stress. The second hypothesis is that the greater dispersal of aphids in Bt cotton may indicate that the first signals emitted by Bt cotton, even in smaller numbers than non-Bt cotton, were sufficient to activate the defense of that variety, preventing or making it difficult for aphids to feed.

Pachú et al. (2021b) revealed that the application of imidacloprid in Bt and non-Bt cotton plants without the presence of the aphid led to variation potentials (VPs). These signals may have resulted in inactivation or low efficiency of photosynthesis in some specific periods. Non-Bt cotton plants exposed to insecticide + aphid resulted in low photosynthetic efficiency, indicating combined stress in this cultivar. The cotton respiration rate was also affected by insecticide + aphid. Bt cotton had a low respiration rate and low quantum yield, while non-Bt cotton had higher respiration and lower quantum yield. More details about the results obtained in the studies of Pachú et al. (2021a; 2021b) are presented in the next chapters of the current thesis.

2.4 Electrical signals and activation of defense genes

Mousavi et al. (2013) observed that *Arabidopsis thaliana* plants, when attacked by *Spodoptera littoralis* larvae, generate electrical signals that were evoked at the damage site and spread to neighboring leaves. Additionally, the authors found that in regions affected by electrical signals, gene expression mediated by jasmonic acid was triggered and defense responses initiated, which did not occur in leaves where electrical signal transmission was blocked.

In addition, they found that the loss of function of certain members of the glutamate receptor family (*GLR - GLUTAMATE RECEPTOR-LIKE*) – some of which form channels permeable to calcium ions – affects injury-induced electrical signal generation. More specifically, the combined disruption of the genes that encode two of these channels, *GLR3.3* and *GLR3.6,* results in the nonpropagation of electrical waves after injury. Thus, tissue damage triggers the generation of a local electrical signal through the activity of *GLRs*; this signal then spreads to neighboring organs where jasmonic acid biosynthesis is induced and in turn triggers defense responses dependent on this hormone. However, it is still unclear how electrical signals are interpreted by cells to trigger jasmonic acid biosynthesis, as both herbivory and mechanical injury appear to trigger similar response mechanisms.

Indeed, previous studies have shown that mechanical damage alters the hydraulic pressure in the xylem, which in turn triggers a wave of depolarization that propagates through the plant (Stankovic & Davies, 1998). This mechanism of electrical signal generation is called the hydraulic hypothesis. According to this hypothesis, changes in xylem pressure, caused by harmful stimuli (burning, cutting), trigger mechanosensitive channels present in the xylem parenchyma cells adjacent to the xylem vessels that trigger the generation of electrical signals, characterized as variation potentials (Stahlberg et al. 2006; Vodeneev et al. 2015). Stankovic & Davies (1998) also demonstrated that electrical signals induce proteinase inhibitor gene expression in tomato. Based on this information, it is possible to synthesize the activation of defense genes against herbivory in the image below.

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3. Electrical signalling on Bt and non-Bt cotton plants under stress by *Aphis gossypii*[2]

Abstract

Plants have developed various mechanisms to respond specifically to each biotrophic attack. It has been shown that the electrical signals emitted by plants are associated with herbivory stress responses and can lead to the activation of multiple defences. Bt cotton is a genetically modified pest-resistant plant that produces an insecticide from *Bacillus thuringiensis* (Bt) to control lepidopteran species. Surprisingly, no study has characterized the signaling mechanisms in transgenic cotton plants attacked by nontarget insects, such as aphids. In this study, we characterized the production of electrical signals on Bt and non-Bt cotton plants infested with *Aphis gossypii*, and in addition, we characterized the dispersal behaviour of aphids to correlate this behaviour to plant signalling responses. Electrical signalling of the plants was recorded with an extracellular measurement technique. Impressively, our results showed that both Bt and non-Bt cotton varieties, when attacked by *A. gossypii*, emitted potential variation-type electrical signals and clearly showed the presence of distinct responses regarding their perception and the behaviour of aphids, with evidence of delay, in terms of signal amount, and almost twice the amount of Cry1F protein was observed on Bt cotton plants at the highest density of insects/plant. In our article, we present some hypotheses based on plant physiology and insect behaviour to explain the responses found on Bt cotton plants under aphid stress.

Keywords: Biotic, Response, Plant-insect, Interaction.

[2] The current chapter has been written following the manuscript submission guidelines of the journal Plos one. The published version is avalaible at: https://doi.org/10.1371/journal.pone.0249699

3.1 Introduction

An organism's capacity to survive in an ecosystem depends on its ability to respond quickly and efficiently to external stimuli and to develop effective and sustainable defences [1]. For this reason, plants have developed numerous mechanisms to react specifically to each biotrophic attack, and cell-to-cell communication between distant tissues is essential to coordinate activities in response to the environment. Thus, plants need to produce a signaling mechanism to integrate perception, transmission, and response to biotic and abiotic actions that occur in the ecosystem [2–5]. Electrical signals have been shown to be associated with responses to herbivory [6], leading to the activation of multiple organism defences [7]. However, studies that better characterize the electrical signalling mechanisms in plants attacked by herbivores, such as aphids, are still scarce.

The aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) is a cotton-damaging pest [8] of cotton. and one of the most important nontarget species of Bt cotton, which is a genetically modified variety expressing proteins derived from Bacillus thuringiensis (Berliner) (Bt), which gives them high efficiency against some lepidopteran species [9, 10]. However, researchers have raised concerns about their potential impact on nontarget organisms such as aphids [11] and their stress on Bt and non-Bt plant physiology [12].

Different environmental stimuli cause specific responses in living cells that are capable of transmitting electrical signals [13, 14]. Among the signals involved in electrophysiological responses, variation potentials are characterized by rapid depolarization and subsequent slow repolarization. The amplitude and shape of the variation potentials (VPs) vary with the stimulus intensity. In addition, the magnitude and speed of responses decrease as the signal moves away from the stimulus site, and its induction depends on the type of damage sustained [15].

Although the methods used to produce transgenic crops are being continuously improved, the plant-insect relationship can be influenced. Thus, it is crucial to understand how plants produce different signs of stress and convert them into appropriate specific responses [12].

Therefore, it is important to characterize the type of electrical signalling of Bt and non-Bt cotton plants as a function of the stress caused by *A. gossypii* and provide insights to understand how plants convert these different signals into appropriate physiological reactions. In our study, we characterized the production of electrical signals on Bt [variety WideStrike1] cotton plants and their non-Bt isoline [variety FM 993] infested with *A. gossypii* in alternating light–dark cycles. The aphid *A. gossypii* was used as a model insect for the study because insect feeding occurs at the phloem level, and the biological interactions between the herbivore and

its host plant can be considered unique. Additionally, we characterized *A. gossypii* dispersal behaviour to relate it to plant signalling responses.

3.2 Methods

3.2.1 Characterization of the electrical signalling potential of Bt and non-Bt cotton plants

Aphis gossypii were reared at the Insect Ecology and Forestry Entomology Laboratory of the Department of Entomology and Acarology (LEA) of the Luiz de Queiroz College of Agriculture (ESALQ) at the University of São Paulo Paulo (USP), Piracicaba, São Paulo, Brazil. Adults of *A. gossypii* were collected in cotton plants in the experimental area from LEA. Specimens of *A. gossypii* were transported to the laboratory for the establishment of rearing at LEA. Insect-rearing stocks were kept for 2 generations in a phytotron chamber at 26±1°C, with a relative humidity of 60±10% and a photophase of 12 h.

Cotton plants expressing Cry1Ac/Cry1F [variety FM 975 (WideStrike1)] and their non-Bt isoline [variety FM 993] were used in this study. The cotton plants were planted in plastic pots (one plant per pot) 25 cm in diameter and 40 cm in height containing soil conditioning substrate (Forth®) and kept in a phytotron chamber at 26±1°C with a relative humidity of 60 ±10% and a photophase of 12 h.

Bioassays to record electrical signalling of cotton plants were conducted in the laboratory. The experimental design was a randomized block design, and each treatment was repeated 10 times = 10 plants per treatment. The measurement of electrical signals was made on the Bt or non-Bt cotton plant surface that reached the six-leaf stage. A technique was used to detect electrical signaling potential differences over long periods. At the time of the electrical signal measurements, the cotton plants were placed in a Faraday cage to ensure electromagnetic isolation of the environment at 26±1˚C, with a relative humidity of 60±10% and a 12-h photophase.

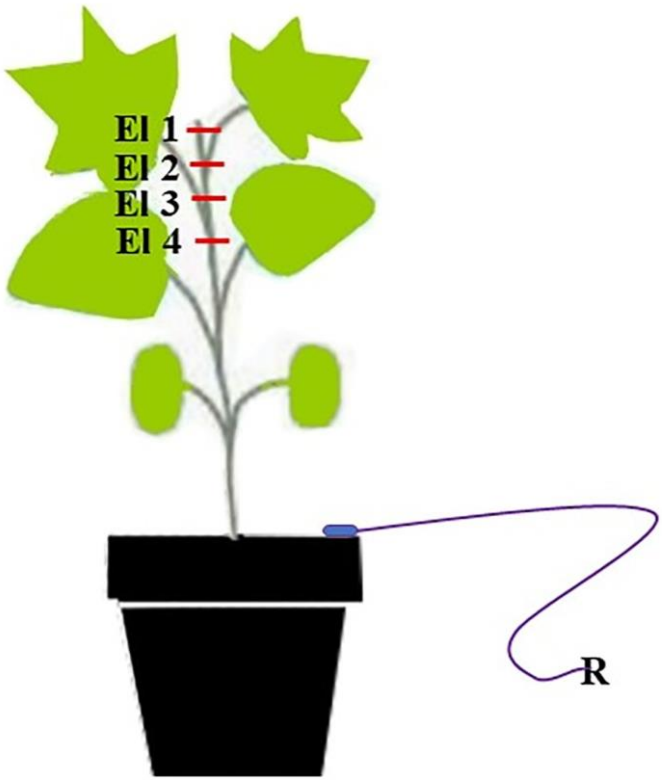

Fig 1. Scheme of the electrodes (El, E2, E3 and E4) in the cotton plant inserted into the stem; and R (reference electrode) inserted into the soil. The published version is available at: https://doi.org/10.1371/journal.pone.0249699.g001

Measurements were made using electrodes consisting of a 0.25–0.5 mm diameter silver lead wire chlorinated in 3 M KCl solution. After the acclimatization period, five electrodes were used. Four were inserted in different arrangements along the stem of cotton plants.

The reference electrode (fifth electrode), which has the same composition of the recording electrodes, was inserted in the ground (Fig 1). The electrodes are connected to a four-channel data acquisition system with a built-in amplifier (World Precision Instruments Lab-Trax-4 / 24T model) that is connected to a computer with LabScribe® version 3.0 software that decodes the signal [13, 16–18].

The recordings of electrical activities in cotton plants started one hour after electrode insertion. The records were performed continuously for three days. The following variables were obtained: amplitude of the signal, number of signals generated, time after insect infestation to emission of the signals and frequency of signals generated by cotton plants. The electrical signalling profile was contrasted between Bt and non-Bt cotton plants infested with those not infested with *A. gossypii* (control).

Bt and non-Bt cotton varieties were planted in plastic pots containing soil conditioning substrate (Forth1) and kept separately in cages under the same climate conditions mentioned

before. The plants were infested with the aphids with a paintbrush. We used the following 6 treatments: a1. Bt cotton plants infested with 30 aphids/plant; a2. Bt cotton plants infested with 60 aphids/plant; a3. Bt cotton without aphid (Bt cotton control); a4. non-Bt cotton plants infested with 30 aphids/plant; a5. non-Bt cotton plants infested with 60 aphids/plant; and a6. non-Bt cotton without aphid (Bt cotton control).

3.2.2 Dispersal pattern of *A. gossypii* in Bt and non-Bt cotton plants

Bioassays were performed to study aphid behaviour and associate it with data obtained from electrical signalling bioassays. A randomized block design, distributed in 10 blocks = 10 plants/treatment, with four treatments was used: a1. Bt cotton plants infested with 30 aphids/plant; a2. Bt cotton plants infested with 60 aphids/plant; a3. non-Bt cotton plants infested with 30 aphids/plant and a4. non-Bt cotton plants infested with 60 aphids/plant. Bt and non-Bt cotton varieties were planted in plastic pots containing soil conditioning substrate (Forth1) and kept under the same climate conditions mentioned before.

Aphid infestations were performed on Bt and non-Bt cotton that reached the six-leaf stage. We used a paintbrush to infestation the plants with the aphids. The plants were divided into the following three equidistant regions: bottom, middle and top. The number of aphids was recorded in each plant region at 0 (immediately during infestation) and 24, 48 and 72 h after infestation. To evaluate aphid dispersal behaviour as a function of varieties (Bt and non-Bt cotton) and aphid densities, the negative binomial distribution parameter k was used.

There are three basic spatial pattern distributions: random distribution, regular or uniform distribution, and aggregate or contagious distribution. This parameter k is an indicator of uniform distribution, where when k tends to zero, the distribution is highly aggregated, k ranging from 2 to 8 indicates moderate aggregation, and values greater than 8 ($k > 8$) indicate that the distribution is random (39). The k values were estimated by the method of moments (a statistical method for constructing an estimator).

3.2.3 Data analyses

Characterization of electrical signalling potential of Bt and non-Bt cotton plants.

Descriptive analyses were conducted with boxplots aiming to characterize the quantiles, medians, maximum and minimum values, and outliers of the variables and time for the emission of VPs (variation potentials) after infestations with aphids on Bt and non-Bt cotton plants and the amplitude of VPs.

Correlation analyses were conducted between the VP amplitude and signal emission time after infestation plants within each cotton variety. The degree of correlation between the variables in each condition was studied using Spearman's rank correlation coefficient ($P < 0.05$) using the cor.test function of the R program.

Data on the number of signals per time interval after infestation of Bt and non-Bt cotton plants with aphids were subjected to deviance analysis with the purpose of studying the interaction involving cotton variety, aphid/plant density and time interval. A generalized linear model with a quasi-Poisson distribution was used. The goodness of fit of the model was evaluated with a simulated normal envelope using the hnp package in the R program [17].

Deviance analysis was applied to study the interaction involving cotton variety, aphid/plant density and period (photophase/scotophase) in the number of VPs. Data were divided into four sections, three of which corresponded to the data recorded during three days of observation, and the last section corresponded to the accumulated data recorded during the three days of evaluation. Negative binomial generalized linear models were used for approximately the 1st and 2nd evaluation days, while quasi-Poisson models were adopted for data recorded on the 3rd day and total accumulated over the three days of evaluations. We used a half-normal plot with a simulated envelope with the hnp package [17] to assess the goodness of fit of the models.

Aphis gossypii **dispersal pattern on Bt and non-Bt cotton plants**

The parameter k in each cotton variety and density was compared by confidence intervals. Confidence intervals were generated from the values of k for each block. We used the nonparametric bootstrap technique, with 10,000 pseudoreplications, and for the resampled parameter in each treatment, we used the R program boot package [18].

Multinomial analysis. The probability of aphids occurring within each region of Bt and non-Bt cotton plants in each treatment (variety and aphid density) was estimated and compared with a multinomial linear model. The analyses to estimate the probabilities and their comparisons were conducted with nnet [19] and emmeans [20] packages from R.

3.3 Results

3.3.1 Characterization of the electrical signalling potential of Bt and non-Bt cotton plants

In descriptive analysis, it was possible to visualize that Bt cotton plants infested with *A. gossypii* emitted the first VPs ("minimum value" in the boxplot) between time intervals of 0.31 h (60 aphids/plant) and 0.64 h (30 aphids/plant) (Fig 2). In the absence of aphids, only two Bt cotton plants emitted these electrophysiological signals. Non-Bt cotton plants emitted the first VPs ("minimum value" in boxplot) after 0.80 h when kept at 30 aphids/plant and after 1.60 h at a density of 60 aphids/plant (Fig 2).

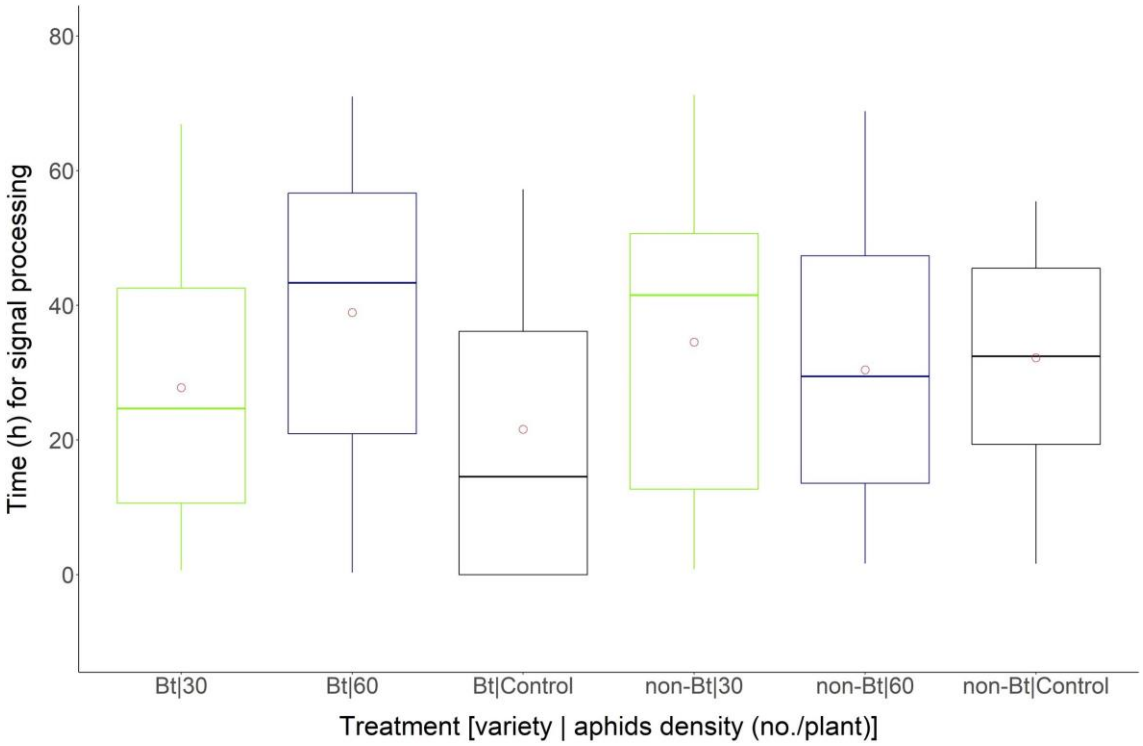

Fig 2. Boxplot of time (h) of exposure of Bt and non-Bt cotton plants to different densities of *A. gossypii* emitting potentials of variation (VPs) (mV). Bt and non-Bt cotton plants were infested at densities of 30 and 60 aphids/plant and in the absence of aphids (Control). The circle within the boxplot corresponds to the mean time for each treatment. The published version is available at: https://doi.org/10.1371/journal.pone.0249699.g002

The Bt and non-Bt cotton plants infested with aphids emitted signals after 60 h of aphid infestation (Fig 2), while in cotton plants used as a control, the maximum signal emission values were observed at 55 and 57 h in non-Bt and Bt cotton plants, respectively (Fig 2).

The maximum amplitude (mV) ("maximum value" in the boxplot) of VP found in the control cotton plants was near -28 mV. In general, the mean amplitude (mV) of VP (points within the boxplots) was near all treatments, ranging from -17 mV (Bt–control cotton plants) to -11.22 mV (non-Bt cotton plants infested with 30 aphids/plant). Outliers (points out of

boxplots) occurred for 30 aphid/plant (-129 mV)-infested non-Bt cotton plants and 60 aphid/plant (-116.60 mV)-infested Bt cotton plants (Fig 3).

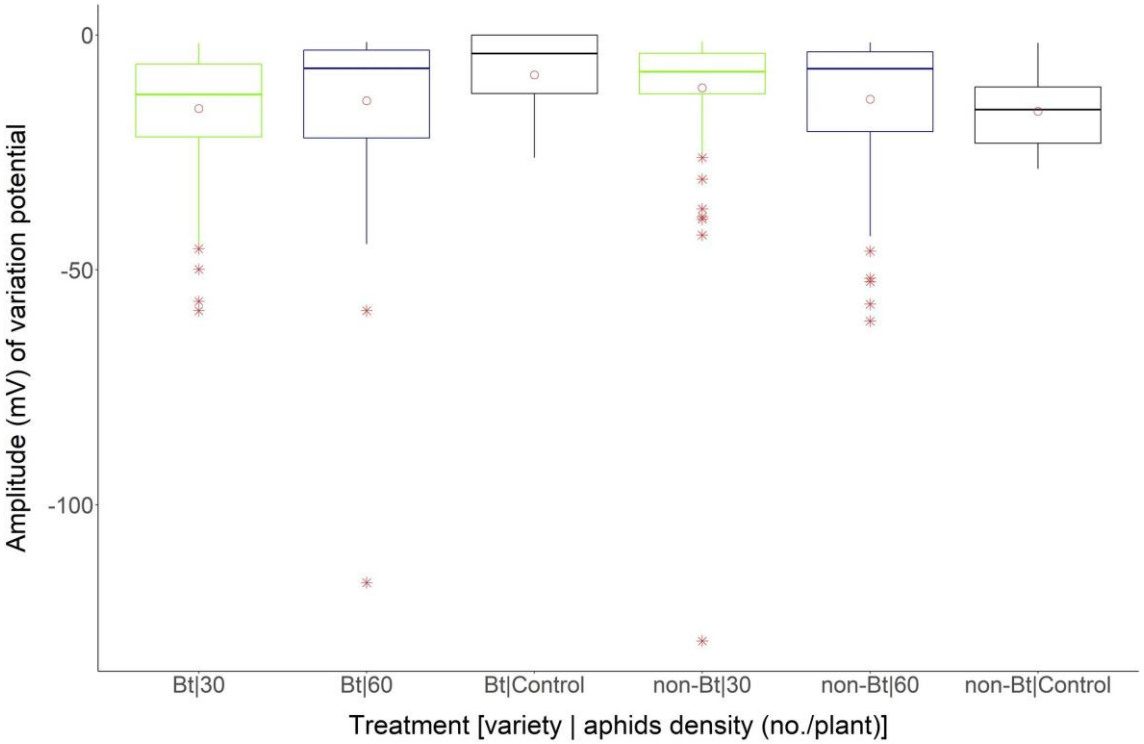

Fig 3. Boxplot of amplitude (mV) of variation potentials in Bt and non-Bt cotton plants exposed to different densities of *A. gossypii* Bt and non-Bt cotton plants exposed to densities of 30 and 60 aphids/plant and in the absence of aphids (Control). The circle within the boxplot corresponds to the mean time for each treatment. Asterisks outside of the boxplot correspond to outliers for each treatment. The published version is available at: https://doi.org/10.1371/journal.pone.0249699.g003

Spearman rank analysis revealed that there was no correlation between the amplitude (mV) of VP of VP and time (h) to emission of signals by cotton plants after aphid infestation at all densities studied within each variety (Bt and not Bt), except at the densities of 30 aphids / non- Bt cotton plants (ρ = -0.2659; P = 0.0060) and 60 aphids/Bt cotton plants (ρ = - 0.3528; P = 0.00254). Analyzing the number of VPs emitted by the cotton plants, we observed that infestation-free plants emitted few signals, with an average accumulation of 0.75 (control Bt cotton) and 2.50 signals (control non-Bt cotton) over 72 h. Only two Bt cotton plants emitted electrical signals in the absence of aphid stress (Table 1).

In the accumulated emission of VPs over 72 h, it was verified that Bt cotton plants exposed to 60 aphids/plant density emitted fewer signals compared to the other conditions (P < 0.05) under aphid stress. However, by assessing the emission within the intervals, the deviance analysis revealed that the signal emission pattern in each variety was influenced by the time

interval and aphid density, as there was a significant interaction between these three factors (P = 0.0488) (Table 1).

Table 1. Number of variation potentials emitted (mean ± SE) by Bt and non-Bt cotton plants when subjected to different exposure times and densities of *A*. gossypii/plant.

Time interval (h)	Bt cotton			non- Bt cotton		
	0 (control)	30	60	0 (control)	30	60
0-12	0.00 ± 0.00^{nia}	6.0 ± 2.17 A a	3.50 ± .89 A b	0.50 ± 0.50^{nia}	6.00 ± 2.08 A a	6.25 ± 2.39 A a
12-24	0.00 ± 0.00^{nia}	4.50 ± 1.04 B a	1.75 ± 0.47 C b	0.00 ± 0.00^{nia}	4.75 ± 1.75 AB a	4.50 ± 1.25 BC a
24-36	0.50 ± 0.25^{nia}	2.75 ± 0.62 C b	1.00 ± 1.00 D d	0.75 ± 0.47^{nia}	1.75 ± 0.75 C c	5.25 ± 1.88 AB a
36-48	0.00 ± 0.00^{nia}	4.75 ± 1.25 B a	3.50 ± 1.04 A b	0.50 ± 0.28^{nia}	3.75 ± 0.94 B ab	3.50 ± 0.86 C b
48-60	0.25 ± 0.25^{nia}	1.25 ± 0.62 D c	2.50 ± 0.50 B b	0.75 ± 0.75^{nia}	5.75 ± 0.85 A a	5.00 ± 1.00 AB a
60-72	0.00 ± 0.00^{nia}	2.25 ± 1.65 C b	4.00 ± 0.08 A a	0.00 ± 0.00^{nia}	4.25 ± 1.54 AB a	1.50 ± 0.64 D c
∑*accumulated* (total)	0.75 ± 0.25^{nia}	22.00 ± 0.17 a	16.25 ± 0.13 b	2.50 ± 0.50^{nia}	26.25 ± 0.21 a	26.00 ± 0.20 a

Capital letters compare averages within each column, and lowercase letters compare averages within each row. Means followed by the same letters do not differ from each other by overlapping confidence intervals generated by the quasi-Poisson model (P <0.05). nia = not incorporated in the analysis because of the absence of variability. The published version is available at: https://doi.org/10.1371/journal.pone.0249699.t001

The highest number of signals emitted by Bt cotton plants when exposed to aphids occurred in the time interval after infestation of 0–12 h (30 aphids/plant) and 0–12, 36–48 and 60–72 h (60 aphids/plant) (Table 1, Fig 4).

When we compared the signal emission pattern between combined treatments involving aphid densities and cotton varieties within each time interval, it was possible to verify a delay in terms of the production pattern of signalling on Bt cotton plants under stress with 60 insects/plant because until the time interval of 36 h after infestation, there was a lower signal emission by Bt cotton plants when exposed to 60 aphids/plant in relation to the other condtions of aphid density/Bt or non-Bt cotton variety (Table 1, Fig 4).

In the time interval of 36-48 h, the emission of signals by Bt cotton plants was lower only in relation to Bt cotton with 30 aphids/plant. Additionally, in the time interval of 60-72 h, the signal production by cotton plants was higher when the Bt and non-Bt cotton plants were exposed to densities of 60 and 30 aphids, respectively, in relation to other conditions (Table 1, Fig 4).

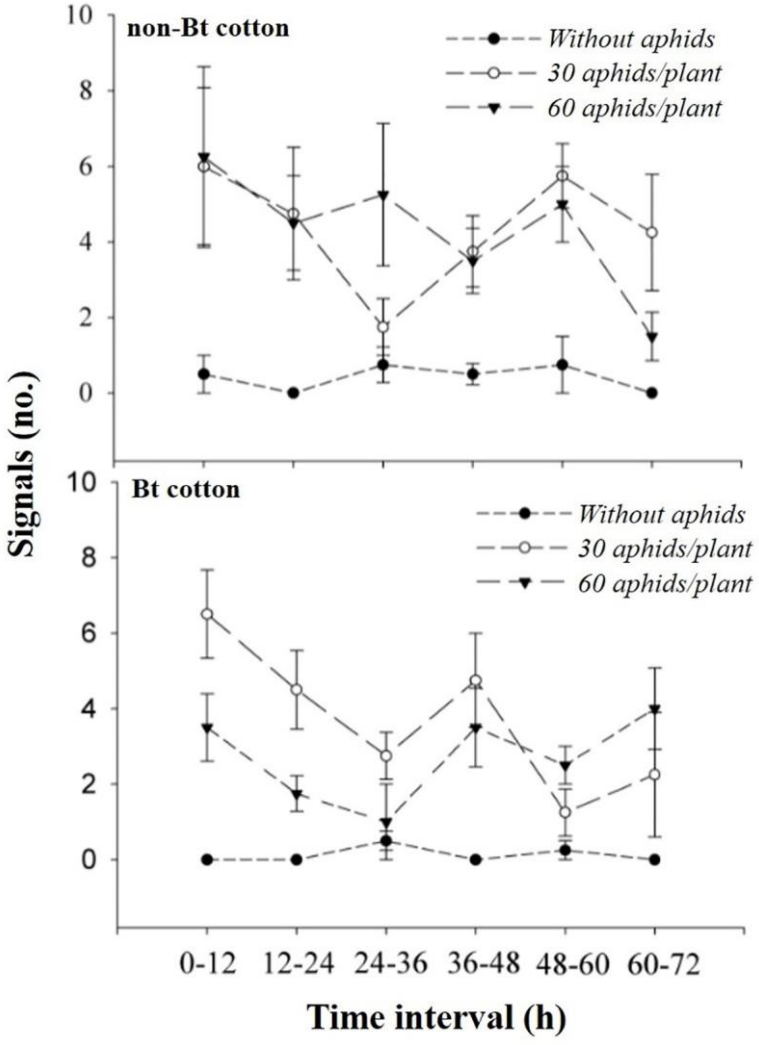

Fig 4. Variation potentials emitted (no.) (MEAN ± SE) by Bt and non-Bt cotton plants at different time intervals (h) after infestation of 30 and 60 aphids/plant and control (absence of aphid). Mean data (points) and error bars (SE) predicted by the generalized linear quasi-Poisson model, except for the plotted values for the control used in both cotton varieties (Bt or not Bt). The published version is available at: https://doi.org/10.1371/journal.pone.0249699.g004

The deviance analysis on the interaction of the factors aphid density versus cotton variety versus light period within each studied day (1st, 2nd or 3rd day) and accumulated over these three days influencing the number of VPs emitted by plants shows that there was no interaction (P> 0.05) among the studied factors for the 1st and 2nd day and the accumulated days of exposure of Bt and non-Bt cotton plants to aphids. The factor density [$F_{density}$ = 2.29, $P_{density}$ = 0.1294 (1st day); $F_{density}$ = 0.0070, $P_{density}$ = 0.95 (2nd day); $F_{density}$ = 0.1734, $P_{density}$ = 0.6813 (cumulative total)], variety [$F_{variety}$ = 0.003, $P_{variety}$ = 0.95 (1st day); $F_{variety}$ = 3.0896, $P_{variety}$ = 0.07 (2nd day); $F_{variety}$ = 2.2726, $P_{variety}$ = 0.1466 (cumulative total)] and period [F_{period} = 1.3882, P_{period} = 0.2387 (1st day); F_{period} = 1.0805, P_{period} = 0.3679 (2nd day); F_{period} = 0.2966, P_{period} = 0.5918 (cumulative total)] did not affect the number of VPs emitted by cotton plants.

There was an interaction between the aphid density versus cotton variety versus light/dark phase (F = 7.7295, P = 0.04150) for the number of VPs observed during the 3rd day of exposure of Bt cotton plants and non-Bt to aphids. On the third day, VP production was higher in non-Bt cotton plants exposed to 30 aphids/plant density than in Bt cotton plants exposed to the same density during the photophase (Table 2). In addition, VP production by Bt and non-Bt cotton plants exposed to 60 and 30 aphids/plant, respectively, was higher in the light phase than in the dark phase (Table 2).

Table 2. Number of variation potentials emitted (mean ± SE) by Bt and non-Bt cotton plants exposed to different aphid densities/cotton plant and photophase and scotophase on the 3rd assessment day.

Variety	Density (aphid/plant)	Photophase	Scotophase
non Bt Cotton	30	6.75 ± 1.43 A a	2.50 ± 1.50 A b
Bt cotton		1.50 ± 0.86 B a	1.75 ± 1.03 A a
non Bt Cotton	60	3.25 ± 1.18 AB a	4.50 ± 1.18 A a
Bt cotton		5.00 ± 0.70 AB a	2.00 ± 1.08 A b
non Bt Cotton	0 (control)	0.25 ± 0.25 *nic*	0.25 ± 0.25 *nic*
Bt cotton		0.00 ± 0.00 *nic*	0.25 ± 0.25 *nic*

Uppercase letters compare averages within each column. and lowercase letters compare averages within each row. Means followed by the same letters do not differ from each other by overlapping confidence intervals generated by the quasi-Poisson model (P <0.05). *nia* = not incorporated in the analysis because of the absence of variability. The published version is available at: https://doi.org/10.1371/journal.pone.0249699.t002

3.3.2 Dispersal pattern of *A. gossypii* on Bt and non-Bt cotton plants

The behaviour of *A. gossypii*, independent of the factors exposure time of plants to aphids, aphid density or cotton variety, followed a highly within-plant aggregated distribution pattern ($k < 2$) (Table 3, Fig 5A–5D).

Comparisons of the k index, based on confidence interval values, revealed that the highest k index of aphid aggregation with 30 aphids/non-Bt cotton plants was found at 48 h and 72 h after infestation of cotton plants with *A. gossypii* (Table 3). However, non-Bt cotton plants exposed to that density had a lower aphid k aggregation index at 72 h of infestation in relation to Bt cotton with 60 aphids/plant (Table 3).

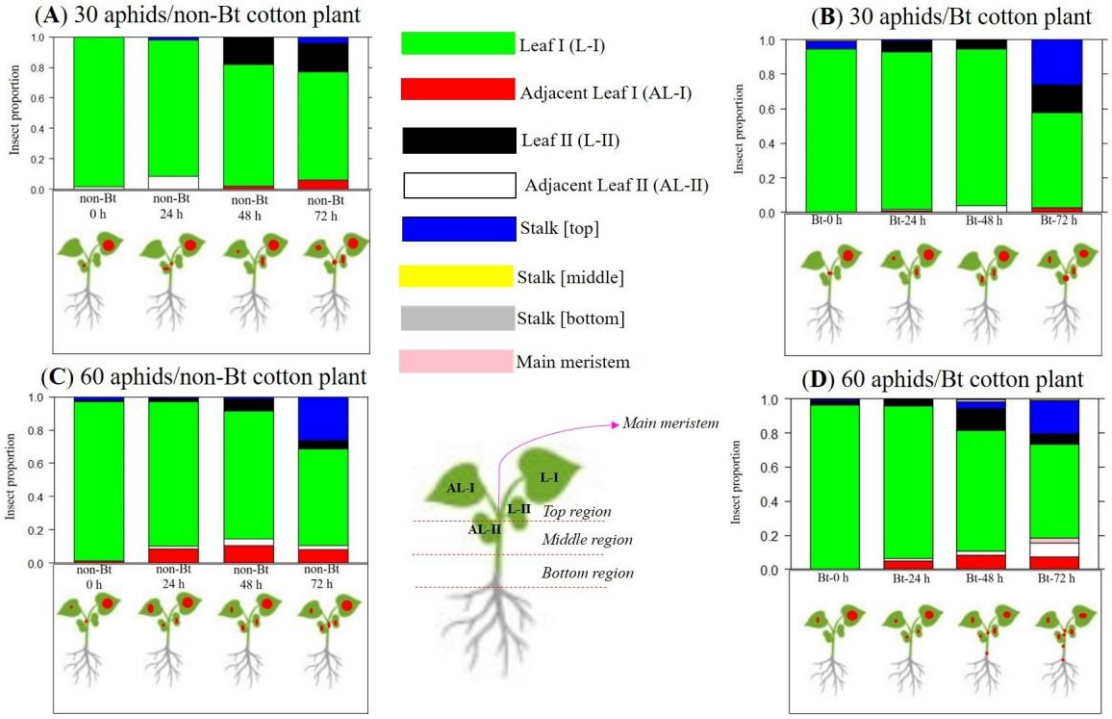

Fig 5. Multinomial distribution with occurrence rate of *A. gossypii* in the regions of non-Bt and Bt cotton plants at infestation times of 0 h, 24, 48 and 72 h with densities of 30 and 60 aphid/cotton plants. The red circle diameter represents the intensity of aphid infestation on cotton plants. The published version is available at: https://doi.org/10.1371/journal.pone.0249699.g005

Table 3. Confidence intervals associated with the *A. gossypii* aggregation index (95% CI) in Bt and non-Bt cotton plants submitted to densities of 30 and 60 aphids/cotton plants quantified at 0. 24. 48 and 72 h after the infestation of cotton plants with aphids.

Density/Variety	Time (h)			
	0	24	48	72
30 aphids/non-Bt cotton/	0.42–0.52 A b	0.47–0.74 A ab	0.55–0.85 A a	0.58–0.77 B a
60 aphids/non-Bt cotton	0.45–0.73 A b	0.55–1.67 A ab	0.78–3.03 A a	0.67–1.48 AB ab
30 aphids/Bt cotton	0.45–1.16 A a	0.45–0.83 A a	0.57–0.87 A a	0.77–1.55 AB a
60 aphids/Bt cotton	0.42–0.90 A b	0.63–0.80 A b	0.70–1.05 A b	1.12–1.46 A a

Uppercase letters compare averages within each column. and lowercase letters compare averages within each row. Means followed by the same letters do not differ from each other by overlapping confidence intervals generated by Boostrap (P <0.05). The published version is available at: https://doi.org/10.1371/journal.pone.0249699.t003

The dispersal rate of *A. gossypii* was higher on Bt cotton with 60 aphids/plant than on non-Bt cotton plants with 30 aphids/plant at 72 h (Table 3). In fact, according to the multinomial distribution in the within-plant distribution of *A. gossypii*, it was confirmed that with 30 aphids/non-Bt cotton plants at 72 h, the highest proportions of aphids were on the adaxial (0.18) and abaxial (0.49) regions of the leaf (leaf I); however, there was increased insect dispersal to other positions, such as leaf II, adjacent leaf I and main meristem (Fig 5A). On the other hand, on Bt cotton plants infested with 60 aphids/plant, we observed the most dispersal pattern with 72 h of infestation, where there was clearly an increased insect dispersal, with 0.16 and 0.41 of aphids found in the adaxial and abaxial regions of leaf I, respectively, and 0.20 in the main meristem of the cotton plant.

No significant difference was observed among the treatments within the infestation times of 0 h, 24 h and 48 h in relation to the k index and multinomial distribution, except for the treatment with 60 aphids/Bt cotton plants within 48 h, which showed the most dispersal behaviour because it reached more regions of the cotton plants (Fig 5D).

In the comparisons of aggregation level among the time intervals within non-Bt cotton exposed to 60 aphids/plant, we perceived that the highest k aggregation index was during the infestation time of 48 h (Table 3). With Bt cotton plants at a density of 30 aphids/cotton plants, it was found that there were no significant alterations in the aphid dispersal pattern at all time intervals (Table 3).

3.4. Discussion

The results from our study showed that both cotton varieties (Bt and non-Bt), when attacked by *A. gossypii*, emitted electrical signals of the variation potential type. Abiotic and biotic wounds are perceived differently by plants, as has been shown by other studies on plant-herbivore interactions [6, 21, 22]. Insect damage in plants plays a vital role in recognizing the type of biotic stress to the plant [23, 24]. Plants differentiate herbivory from mechanical damage by recognizing compounds present in insect saliva because oral secretion of herbivores can induce ionic flux and promote depolarization of the plant membrane potential [25].

Here, in our research, it was possible to describe how Bt and non-Bt cotton plants react to *A. gossypii* stress by changing the transmembrane potential by recording extracellular electrical signals. Although plant responses to herbivorous attack are complex and involve a number of signals, it is important to note that different types of stimuli caused by insect action against plants trigger characteristic electrical signals evoked by plants with a specific influence on plant physiological processes [26]. The cascade of events involved in plant signaling as a function of stress perception begins at the plasma membrane of cells with changes in transmembrane potential or ion flow; these are the first responses of plants to biotic and abiotic stresses [27]. Attack on herbivorous plants is known to promote membrane potential changes that trigger an electrical signal that can travel to the entire plant or even trigger local plant defence mechanisms [28].

Our results indicate the presence of VP on Bt and non-Bt cotton plants at all assessed interval times. An aphid continuously inserts its buccal apparatus into the phloem vessels, altering the hydrostatic pressure in these vessels. VP is a signal whose propagation properties vary with the intensity and distance of the stimulus site and is probably a local electrical response, which is induced by a hydraulic signal, chemical signal or the combined action of these signals [29]. The hydraulic signal is a wave that results from increased hydraulic pressure in the plant, which propagates through the xylem and initiates the generation of a VP by triggering mechanosensitive ion channels present in the cells adjacent to the plant xylem vessels [30].

Therefore, harmful stimuli such as local damage, burning and mechanical injuries can evoke VPs [31, 32]. These kinds of electrical signals emitted by plants under stress are

especially important for hazard perception and response; thus, the plant can become able to mount an appropriate defence response [33].

Distinct response patterns were attributed to the perception and response to *A. gossypii* by each cotton variety and aphid density used in the research. Although we reported the first emission of signals on Bt cotton plants, there was a delay in terms of the propagated signal amount on Bt cotton plants with 60 aphids of infestation with *A. gossypii*, which produced the smallest numbers of signals between 0 and 36 h. Another important result was the greater dispersal behaviour related to this same treatment, mainly during and after 48 h of infestation.

We suggest that the results could be supported by two hypotheses and explained independently or combined. The first hypothesis is based on the possibility of a trade-off in terms of the defence of the Bt plant; a high dispersal could mean a larger exploitation of food resources by aphids and ease penetration of mouth apparatuses by aphids on Bt cotton plants, which may explain why Bt cotton plants emitted faster electrical signals than non-Bt cotton plants in the first moment, showing that Bt cotton plants may be more susceptible to aphid stress. Inducibility of a plant stress response is the ability to respond to stress only on demand. This is a strategy that is considered cost saving [34]. Therefore, this inducibility of plant defence may indicate a delay in the operation of defensive mechanisms; on the other hand, it may also mean a strategy to save energy and prevent self-poisoning [35] because it is possible that the Bt cotton plants may save energy with less production of signal until 36 h and producing them later. Since induction of a stress response implies that the plant starts activating resistance mechanisms upon encounter with the stressor, this strategy may lead to delay in mounting an effective response [36, 37], a first stress experience may prime the organism for an improved response to a subsequent stress.

With an electrical penetration graph (EPG) used for monitoring the penetration of the mouth apparatus by aphids on Bt and non-Bt plants and recording the waveforms that reflect different aphid feeding activities, a lower percentage of waveform np (nonpenetration) was observed when the aphid was walking or grabbing the food with the rostrum on Bt cotton plants [38]. This suggests that aphids spend less time finding suitable places for penetration of their mouthparts on Bt cotton plants, probably due to the suitability of the tissue structure of Bt cotton plants to feed these aphids [38].

The second hypothesis is that the higher aphid dispersal on Bt cotton plants may indicate that the first signals emitted by the Bt cotton plants, even in smaller numbers than the non-Bt cotton plants, were enough to activate the Bt cotton plants' defence, which prevented or hindered aphid feeding, such as occlusion of phloem sieve elements (SEs), which are the main conductive cells in the phloem, by clogging the sieved pores [39]. This is presumed to prevent sap loss [40, 41], and this process is seen as a primary plant defence response [39]. At the same time, the saliva constituents of sucking insects affect cellular processes [42] and therefore are perceived by cells, leading to the activation of signaling mechanisms, supporting the supposition that local damage induces the propagation of a specific injury substance through the xylem, which induces the electrical response [43]. The main candidates for signaling molecules are H_2O_2 [44], systemin [45], jasmonic acid, abscisic acid, glutamate, among others. Both H_2O_2 and glutamate may activate calcium permeable channels, increasing intracellular calcium concentrations in plants [46] and being an important trigger for the generation and propagation of VP in plants [47].

The observed delay in the quantitative signalling pattern of Bt cotton plants when exposed to 60 aphids/plant could be attributed to self-preservation under stress and may be supported by the inclusion of resource reallocation for the production of metabolites and defensive structures (first and second hypotheses simultaneously). In the supplementary material, we show that almost twice the amount of Cry1F protein was observed on Bt cotton plants in the presence of aphids than in the absence of the insect. This result may be related to the fact that Bt cotton plants were faster to propagate VP signals. Well-known anti-herbivorous defence proteins include proteinase inhibitors (PIs) and polyphenol oxidases (PPOs), both of which are considered to interfere with digestive processes in herbivore intestines [35]. Therefore, simultaneous resource reallocation can serve not only to save resources; thus, the plant can subsequently use them for growth and reproduction but also to deprive the herbivore of food and consequently increase aphid dispersal, as observed in our results on Bt cotton with 60 aphids/plant, to search for the available food source. These abovementioned hypotheses generate a basis for further studies that seek to highlight what possible defence mechanisms are involved and how effective they may be as a function of Bt and non-Bt cotton varieties.

In conclusion, the stress caused by the aphid *A. gossypii* was sufficient to trigger specific responses in Bt and non-Bt plants. Bt cotton plants propagated VP signals faster; however, they produced signals in a smaller quantity with the highest aphid density, also promoting the

greatest within-plant aphid dispersal. Our results may guide future studies that aim to elucidate the factors involved in resistance to stress and plant defence processes and thus assist in the development of successful strategies in integrated pest management.

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4. Imidacloprid-mediated stress on non-Bt and Bt cotton, aphid and ladybug interaction: Approaches based on insect behaviour, fluorescence, dark respiration and plant electrophysiology[3]

Abstract

Plants and insects are parts of a complex system that involves interactions among many trophic levels, and it is important to understand the nature of such interactions. In the complex of interactions involving aphids and transgenic cotton expressing Bacillus thuringiensis, both the spraying of neonicotinoids and the occurrence of predatory coccinellids are common. However, there are gaps regarding the possible impacts of neonicotinoids on physiological variables of the host plant and behavioural traits of the aphid (*Aphis gossypii*) and predator (*Cycloneda sanguinea*). Therefore, this study aimed to highlight the photosynthetic and electrical responses of plants to stress caused by aphid attack combined with the stress generated by the use of imidacloprid in Bt and non-Bt cotton (*Gossypium hirsutum* L.) cultivars and to evaluate how this stress can influence the behavioural ecology of the predator and prey. Chlorophyll a fluorescence tests, dark respiration and electrophysiology on non-Bt and Bt cotton were carried out, and the behaviour of the prey and predator was also evaluated with a video capture system. Our research is a study model that generates insights about possible impacts when using imidacloprid without the occurrence of the pest on the plant because the exposure of non-Bt and Bt cotton plants and the predator to imidacloprid unnecessarily may result in stress on the physiology of the cotton plants and on the behaviour of the predator.

Keywords: Neonicotinoid, Impact, Plant physiology, Predator behaviour.

Highlights

- Cotton plants, aphids and coccinellid predators are parts of a complex system that involves important interactions.
- The understanding of such interactions must consider insecticide use.
- Imidacloprid exposure on Bt and non-Bt cotton plants and *C. sanguinea* in the absence of aphids result in stress on plant physiology and predator mobility.
- Our results demonstrate impacts when using imidacloprid in cases of non-occurrence of the aphid.

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4.1 Introduction

The growth and development of genetically modified cotton (*Gossypium hirsutum* L.) that expresses *Bacillus thuringiensis* Berlinier (Bt), can be affected by the constant attack of aphids. *Aphis gossypii* Glover (Hemiptera: Aphididae) is one of the most frequent aphid species and is of great importance due to the direct damage caused by sap suction, which results in the removal of nutrients from plants by modifying the flow phloem mass and causing losses in productivity (Dixon, 1998; Pitino et al., 2011), or indirect damage, such as transmission of viruses (Fernandes et al., 2001; Ma et al., 2006), both of which occur in practically all the phenological phases of cotton cultivars (Furtado et al., 2009).

Intense insecticide spray negatively affects the growth and development of plants (Shahzad et al., 2018; Sharma et al., 2015, 2016a). It can also interfere with the photosynthetic mechanism at various points in the electron transport chain; therefore, it is likely that it can influence the generation and propagation of electrical signals in plants (Ksenzhek and Volkov, 1998). In contrast, electrical signals are likely to integrate other signaling pathways (Sukhov, 2016; Sukhova et al., 2017) and directly influence photosynthesis (Sukhova et al., 2018) and respiration (Surova et al., 2016; Barbosa da Silva et al., 2020). In this way, electrical signaling plays an important role in inducing plant physiological reactions under stress conditions (Sukhov et al., 2019).

Sensitive or stressed plants may be more vulnerable to reduced photosynthesis, which is one of the physiological reactions observed to compensate for insect attack, and in most cases, the plant defence responses are associated with a reduced photosynthetic rate (Bilgin et al., 2010). Damage to *A. gossypii* in photosynthetic tissue can indirectly interfere with gas exchange by interrupting the transport of nutrients and water (Aldea et al., 2005; Nyka€nen and Koricheva, 2004).

Neonicotinoids are insecticides recommended for the control of *A. gossypii* both in the form of spray and during seed treatment (Ávila and Gómez, 2002), expressing systemic action on the plant and acting on insects as agonists of nicotinic acetylcholine receptors (nAChRs) (Nauen et al., 2001). The use of these molecules has raised concerns about their potential effects at different trophic levels, with an emphasis on the host plant (Alves et al., 2013) and nontarget insects such as predators (Malaquias et al., 2014), parasitoids (Kang et al., 2018) and pollinators (Jacob et al., 2019).

The excessive use of insecticides often causes an imbalance in the food web, which allows pest population outbreaks, changing important interactions in the population regulation of these individuals (Sujii et al., 2007). The compatibility of genetically modified plants with biological control has been highlighted by some studies (Kennedy, 2008; Malaquias et al., 2015) owing to the reduction in insecticide spraying to control defoliating lepidopteran species, promoting a more favourable environment for biocontrol. However, the use of neonicotinoids to control suckers may have sublethal effects on predators, with effects on the neurophysiology of these arthropods and on behavioural parameters, such as mobility, visual and chemical orientation and foraging capacity (Desneux et al., 2007).

Biological control of aphids with the use of coccinellids has proven to be a valuable and rational method within the concept of sustainable pest management (Pachú et al., 2018). Among the aphidophagous species that occur in cotton, *Cycloneda sanguinea* (Linnaeus) (Coleoptera: Coccinellidae) is one of the most abundant (Malaquias et al., 2017a). The growing importance that biological control has assumed in pest management programmes has been undeniable, especially when discussing integrated production to wards sustainable agriculture (Parra et al., 2014). Plants and insects are parts of a complex system that involves interactions between many trophic levels, and it is vitally important to understand the nature of such interactions. In the complex of interactions involving cotton and aphids, both the spraying of neonicotinoids and the occurrence of predatory coccinellids are common in Brazil. However, there are gaps regarding the possible impacts of neonicotinoids on the bioecological behavioural activities of nontarget coccinellids. Given this context, the present study investigated the photosynthetic and electrical responses of plants to the stress caused by the attack of the aphid *A. gossypii* combined with the stress mediated by the use of insecticides in Bt and non-Bt cotton cultivars (i) and assessed how the stress caused by the insecticide can influence the behavioural ecology of the predator *C. sanguinea* and its prey *A. gossypii* (ii).

4.2 Material and methods

4.2.1 Chlorophyll a fluorescence of Bt and non-Bt cotton plants

An experiment was carried out to evaluate leaf respiration and fluorescence of chlorophyll from Bt and non-Bt cotton plants. The bioassays were conducted at the Laboratory for the Study of Plants under Stress (LEPSE) at ESALQ-USP, Piracicaba, São Paulo.

Cotton plants expressing the genes for the Bt proteins Cry1Ab/Cry2Ae [variety FM 940 GLT (TwinLink®)] and Cry1Ac/Cry1F [variety FM 975 (WideStrike)]® and its non-Bt isoline [variety FM 993] were planted in plastic pots (one plant per pot) 25 cm in diameter and 40 cm in height containing soil conditioning substrate (Forth®) and kept in a phytotron chamber at 26 ± 1 °C with a relative humidity of 60 ± 10% and photophase of 12 h. The following treatments were adopted: T1: control (water); T2: insecticide; T3: insecticide aphid and T4: insect. The experimental unit consisted of a Bt or non-Bt cotton plant that reached the six-leaf stage with plant sizes ranging from 26 to 30 cm in height. We chose the variety WideStrike® due its importance to control lepidopteran species in Brazil (Malaquias et al., 2017b; Malaquias, 2020).

The plants were infested with 60 aphids in treatments T3 and T4. The aphid density was chosen in a preliminary bioassay and based on the potential to affect cotton plant physiology. In treatments T2 and T3, the insecticide used was imidacloprid at a dilution of 1.5 mL/L of distilled water; it is the commercial concentration recommended of the commercial product Provado® 200 SC (Imidacloprid) Bayer Crop Science for the control of aphids in cotton crops in Brazil. The spray was carried out with a hand operated pump (without pressure control) in a period of 12 h before the infestation of the aphids. Plants were sprayed until runoff point.

The chlorophyll a images and parameters (Table 1) were obtained using an Imaging PAM fluorometer (Maxi version, Heinz Walz GmbH, Effeltrich, Germany) and analyzed using maging Win software (Heinz Walz). To obtain the images (640 x 480 pixels), the plants were placed individually at a distance of 18.5 cm from the camera (CCD e charge-coupled device) coupled to the fluorescence device.

For the evaluations, the leaves were initially adapted to the dark so that the reaction centres were fully open (primary acceptors all oxidized) with minimal heat loss (Genty et al., 1989). The measurements were carried out in only one completely expanded leaf in the middle third of the plant. Under these conditions, the leaf tissue

was exposed to low intensity light (0.03 mmol $m^{-2}s^{-1}$) followed by a pulse of saturating light (>6000 mmol m^{-2} s^{-1}) for 0.8 s to determine fluorescence initial (F_0) and maximum (F_m). From these initial measurements, the maximum photochemical efficiency of photosystem II (PSII) was calculated using the relationship F_v/F_m (F_meF_0)/F_m (Genty et al., 1989).

The leaf tissue was then exposed to actinic light to obtain steady-state fluorescence (F). Subsequently, a saturating pulse was applied to obtain maximum fluorescence in the dark-adapted state (F_m). From these values, it was possible to calculate the effective quantum yield of photosystem II (PSII), (Y(II) ¼ (F_meF)/F_m)) and the quantum yield of unregulated energy dissipation (Y(NO) (F/F_m)) according to Genty et al. (1989) and Hendrickson et al. (2004). YII was used to calculate the electron transport rate, ETR Y(II)* PAR*LeafABS*0.5 (Bilger et al., 1995), where PAR is the flow of photons (mmol $m^{-2}s^{-1}$) in the leaves, LeafABS is the fraction of incident light that is absorbed by the leaves, and 0.5 is the fraction of the excitation energy directed to photosystem II (Laisk and Loreto, 1996). The photochemical dissipation (qP ¼ (F_meF)/(F_meF_0)) and the fraction of open PSII reaction centres (qL) were determined according to Bilger and Bjorkman (1990) and Kramer et al. (2004).

Table 1 – Abbreviation and description of the effect of variables related to the fluorescence of chlorophyll a.

Abbreviations	Description
PSII	Photosystem II
F	Fluorescence yield measured briefly before application of a saturation pulse.
F_o	Minimal fluorescence yield of dark-adapted sample with all PS II centers open.
F_m	Maximal fluorescence yield of dark-adapted sample with all PS II centers closed.
F_v	Variable fluorescence of dark-adapted sample; given by: $F_m - F_o$.
F_v/F_m	Maximum quantum efficiency of PSII photochemistry; given by: (F_m-F_o)/F_m.
qP	Relates PSII maximum efficiency to operating efficiency. Non-linearly related to proportion of PSII centres that are open; given by: (F_m–F)/(F_m–F_o).
qL	Estimates the fraction of open PSII centres; given by: (F_q/F_v)/(F_o/F)
Y(II)	Quantum yield of photochemical energy conversion in PS II; given by: F_m-F/F_m
Y(NO)	Quantum yield of non-regulated non-photochemical energy loss in PS II; given by F/F_m

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4.2.2 Chlorophyll a fluorescence, respiration in the dark and electrophysiology of Bt and non-Bt cotton plants over time

Taking into account that the presence of the aphid did not provide a significant difference for the variables already addressed in relation to all treatments studied, only the following treatments were used: control, insecticide and insecticide aphid. Evaluations of this trial were carried out immediately after the infestation of the insects (Time 0) and at each interval of 24 h over three days. The method for spraying the insecticide was the same as that adopted in the previous test.

In addition, we also evaluated the respiratory rate (CO_2 mmol $m^{-2}s^{-1}$) of Bt and non-Bt cotton plants using the values obtained from A (CO_2 assimilation rate) at 0 mmol $m^{-2}s^{-1}$ of photosynthetically active radiation, with plants previously adapted to the dark. The measurements were performed with an infrared gas analyzer (IRGA, model LI-6400xt, Liquor, Lincoln, Nebraska, USA).

The measurement of electrical signals was performed in a separate experiment, and the electrical signalling profile was contrasted between Bt and non-Bt cotton plants, with two treatments: control (water) (T1) and insecticide (T2). A technique was used to detect differences in electrical signalling potential for long periods. At the time of measuring the electrical signal, the cotton plants were placed in a Faraday cage to ensure electromagnetic isolation from the environment at 26 ± 1 °C, with a relative humidity of 60 ± 10% and photophase of 12 h. Measurements were made in completely expanded leaves (middle third of the plant) using electrodes consisting of a silver wire with 0.25 or 0.50 mm in diameter chlorinated in 3 M KCl solution. Five electrodes were used, four of which were inserted in different arrangements along the stem of cotton plants. The fifth electrode was the reference electrode and was inserted at the base of the plant. The electrodes are connected to a four-channel data acquisition system with a built-in amplifier (World-Precision Instruments Lab-Trax-4/24 T model) connected to a computer with LabScribe software version 3.0 that decodes the signal (adapted from Zawadzki et al., 1995).

The recordings of the electrical activity in the cotton plants were carried

out continuously for three days. The following variables were obtained: amplitude and duration of the signal, number of signals generated, and time of signal generation by cotton plants.

4.2.3 Behavioural evaluation of Aphis gossypii and Cycloneda sanguinea in Bt and non-Bt cotton plants in the presence and absence of an insecticidal molecule

The tests were conducted at the Laboratory of Ecology and Forest Entomology of the Luiz de Queiroz College of Agriculture e ESALQ of the University of São Paulo e USP.

The behaviour of the predator and pest was evaluated; thus, the ladybugs *C. sanguinea* and *A. gossypii* were used as model insects. Adults of both species were collected in the vicinity of the Department of Entomology and Acarology, ESALQ e Piracicaba and taken to the laboratory. Then, they were maintained in the insect rearing cage following the methodologies described in Castro (2010) and Farhadi et al. (2010) for ladybugs and Oliveira et al. (2010) for aphids. The insects were kept in 500 mL plastic containers in air-conditioned chambers (BODs) at a temperature of 25 ± 1 °C, relative humidity of 70 ± 10% and photophase of 12 h.

Bt and non-Bt cotton plants in the 8-leaf stage after emergence were sprayed until the runoff point with two treatments used: control (water) (T1) and imidacloprid insecticide (T2), at a dilution of 1.5 mL/L of water. Adult males and females of the ladybugs were individualized and maintained in one of the treatments, whereas in the test for aphids, only apterous adults were used. The insects (prey and predator) were monitored for 10 min with Ethovision® software (Noldus et al., 2002), a computational platform aimed at automating behavioural experiments. The variables extracted from the Ethovision® equipment were average, maximum and total distance covered by the insects in the arenas.

4.2.4 Data analysis

The experiment was conducted with a randomized block design using 10 blocks, with one plant of each treatment per block totaling 10 plants per treatment in the bioassays with plants (items 2.1 and 2.2) and 4 blocks for the insect (prey or predator) bioassays, with 10 insects of each species per block and 40 insects per treatment in total (item 2.3). All statistical analyses were performed using the R Core Team (2019) program.

4.2.4.1. Chlorophyll a fluorescence of non-Bt and Bt cotton plants

A generalized linear model with gamma distribution was used for the variables qP, Y(II) and qL. The goodness of fit of the statistical model was tested with a half-normal plot with simulated envelopes using the hnp package. The other variables were not addressed in this first trial due to the lack of adjustment of this and other models.

4.2.4.2. Chlorophyll a fluorescence, respiration in the dark and electrophysiology of Bt and non-Bt cotton plants over time

A theoretical stepwise multiple regression model was built to describe the hypothesis of relationships between variables related to fluorescence and the respiratory rate to obtain consistent statements about the magnitude or direction of these relationships. For this analysis, the olssr package was used.

With the nlme package, a mixed linear model with repeated measures, using the REML method, was adopted to analyze the effect of time versus treatment versus cotton cultivar interaction on respiratory and fluorescence responses. Confidence intervals for the model parameters were obtained using the lsmeans package.

For the analysis of the effect of the insecticide on the electrical signalling of Bt and non-Bt cotton plants, a generalized linear model with a quasi-Poisson distribution was used. The goodness of fit of the model was assessed with the hnp package.

4.2.4.3. Behavioural evaluation of Aphis gossypii and Cycloneda sanguinea in Bt and non-Bt cotton plants in the presence and absence of an insecticidal molecule

Multiple nonparametric comparisons were performed with the Kruskal-Wallis test (0.05) to test the hypothesis of differences between treatments; for this purpose, the agricolae package was used.

Supplementary material provides the *C. sanguinea* survival curves, which were estimated using the Kaplan-Meier product limit method using the survival package.

4.3. Results

4.3.1. Chlorophyll a fluorescence of Bt and non-Bt cotton plants

In our experiment, the most significant reductions in the effective quantum yield of photosystem II (YII) occurred under the following conditions: aphid insecticide in Bt and non-Bt cotton and only in the treatment with the aphid in non-Bt cotton (Table 2). Such reductions had a direct effect on the activation of thermal dissipation mechanisms, as observed by the increase in Y(NO) (variable considered in the subsequent bioassay).

Treatments with insecticide sprays in the presence of aphids on Bt and non-Bt cotton and insects only on non-Bt cotton reduced photochemical dissipation (qP) (Table 2).

Table 2- Comparative analysis of chlorophyll a on Bt and non-Bt cotton plants under stress induced by the insecticide imidacloprid and/or with *Aphis gossypii* infestation for 24 h

Cultivar	Treatment	Y(II)	qP	qL
Bt	Control	0.3340 ± 0.01 a	0.4502 ± 0.01 a	0.1761 ± 0.01 a
	Insecticide	0.3411 ± 0.06 a	0.4608 ± 0.09 ab	0.2090 ± 0.06 a
	Insecticide +Aphid	0.2294± 0.01 bc	0.2996 ± 0.02 bc	0.0920 ± 0.01 c
	Aphid	0.2840 ± 0.01 ab	0.3811 ± 0.02 ab	0.1381 ± 0.70 c
non-Bt	Control	0.3394 ± 0.01 a	0.4370 ± 0.01 a	0.1485 ± 0.01 b
	Insecticide	0.3217 ± 0.04 a	0.4247 ± 0.06 ab	0.1056 ± 0.04 b
	Insecticide + Aphid	0.2022 ± 0.01 c	0.2581 ± 0.01 c	0.0704 ± 0.01 c
	Aphid	0.2100 ± 0.01 bc	0.2775 ± 0.01 c	0.0850 ± 0.70 c

Means within the same treatment (column) with the same letters are not significantly different when the confidence interval overlaps (95% CI). The 95% CIs were estimated with a quasi-Poisson generalized linear model. **Y(II)**: Quantum yield of photochemical energy conversion in Photosystem II (PS II). **qP**: Relates PSII maximum efficiency to operating efficiency; it is nonlinearly related to the proportion of PSII centers that are open, also known as Fq′/Fv′. **qL**: Estimates the fraction of open PSII centres. The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#tbl2

The spraying of insecticide in the absence of aphids in Bt cotton did not provide a significant difference between all treatments for either the variable Y(II) or qP (Fig. 1).

The occurrence of only the aphid or in combination with the insecticide significantly reduced the proportion of open reaction centres (qL) for both varieties, which reflects directly on the values of Y(II) and qP (Table 2).

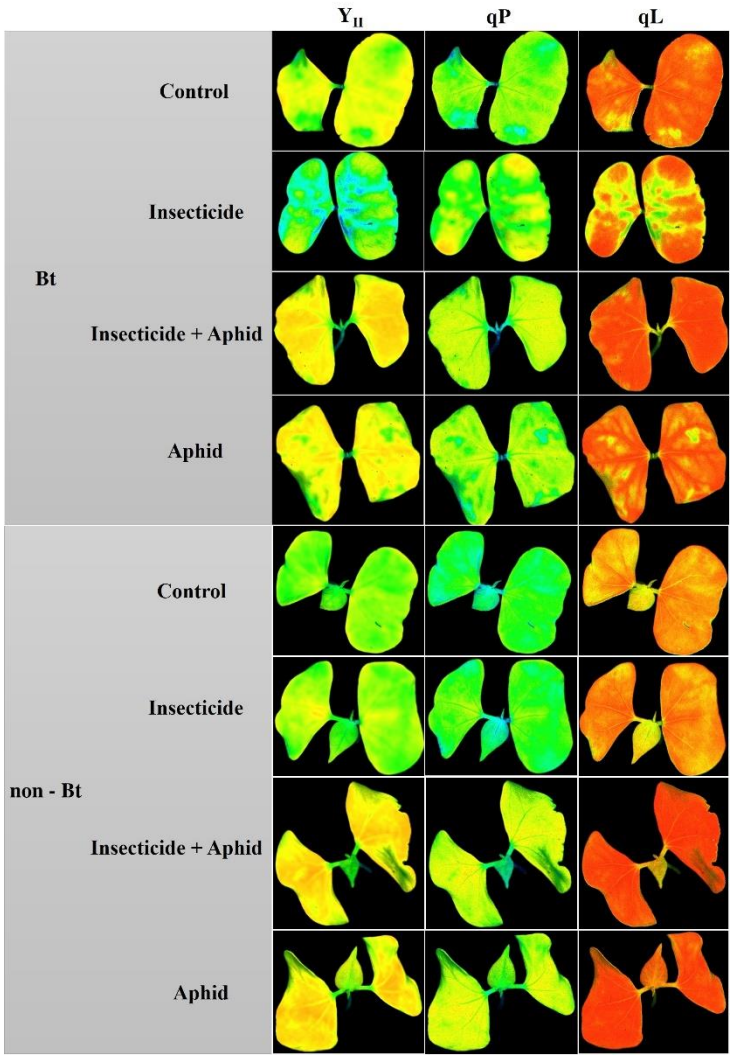

Figure 1 – Image representation of Bt and non-Bt cotton plants under stress mediated by the isolated insecticide effect or combined with the occurrence of *Aphis gossypii*. The assessed variables are **Y(II)**: Quantum yield of photochemical energy conversion in Photosystem II (PS II); **qP**: Relates PSII maximum efficiency to operating efficiency, it is non-linearly related to proportion of PSII centres that are open, also known as Fq′/Fv′, and **qL**: Estimates the fraction of open PSII centres. The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#fig1

4.3.2. Chlorophyll a fluorescence, respiration in the dark and electrophysiology of Bt and non-Bt cotton plants over time

Regarding the respiratory rate in the dark, when we compared the cultivars within each time interval, we noticed that in the insecticide aphid condition at all time intervals, the respiratory rate was lower in Bt cotton than in non-Bt cotton. For treatment with insecticide only, there was no significant difference between cultivars in 24 h, and in the period of 48 h,

the respiratory rate in Bt cotton was lower. In the 72 h period, it was possible to observe an inversion of this rate, that is, it was higher in Bt cotton than in non-Bt cotton (Table 3).

Table 3 – Dark respiration (mmol CO_2 $m^{-2}s^{-1}$) of Bt and non-Bt cotton plants under stress mediated by the isolated insecticide effect or combined with the occurrence of *Aphis gossypii*

Time (h)	Treatment	Bt	non-Bt
24	Water	0.67 ± 0.11 Ca	0.59 ± 0.10 Da
	Insecticide	0.53 ± 0.09 Ca	0.53 ± 0.25 Da
	Insecticide + Aphid	0.45 ± 0.07 Cb	0.83 ± 0.11 Da
48	Water	2.15 ± 0.07 Ba	2.21 ± 0.05 Ca
	Insecticide	1.81 ± 0.09 Bb	3.46 ± 0.62 Ba
	Insecticide + Aphid	2.15 ± 0.24 Bb	3.46 ± 0.15 Ba
72	Water	3.86 ± 0.06 Aa	3.08 ± 0.16 Bb
	Insecticide	3.51 ± 0.28 Aa	2.23 ± 0.23 Cb
	Insecticide + Aphid	3.73 ± 0.08 Ab	4.19 ± 0.08 Aa

Means within the same plant variety (or column) with the same lowercase letter or means between varieties within of the same treatment (or row) with the same capital letters are not significantly different when the confidence interval overlaps (95% CI). The 95% CIs were estimated by a mixed linear model with repeated measures. The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#tbl3

When comparing the respiratory rate over time and within the non-Bt cultivar, it was observed within 24 h that there was no difference among treatments, whereas within 48 h, there was an increased respiratory rate in the insecticide and insecticide aphid treatments compared to the control (water). In 72 h, there was a reduction in the respiratory rate in the treatment with insecticide and an increase in this variable when the insecticide was adopted in the presence of the aphid in relation to the condition control (water) (Table 3).

In the comparisons involving treatments over time in the Bt cultivar, it is clear that although the respiratory rate increased over time, there was no difference between treatments within each time interval; therefore, the respiratory rate in Bt cotton plants followed the same pattern over time in the different treatments evaluated (Table 3).

In addition to the variables qL, qP and Y(II)I addressed in the previous study, the initial fluorescence (Fo), the maximum quantum yield of PSII (Fv/Fm), the unregulated quantum dissipation yield Y(NO) and the electron transport rate (ETR) were measured. Given the effect of treatments on respiratory rates, we used a multivariate multiple regression model to examine

the effect exerted by the variables Fo, Fv/Fm, Y(II), Y(NO), qP, qL and ETR on the dependent variable respiration. The result of this regression was an equation representing an additive model with the best prediction of the dependent variable respiratory rate from the independent variables selected using the backward and forward methods. The forward method is characterized when the equation starts empty and each predictor enters the equation one by one, while the backward method is characterized when all predictors are included in the equation at once and then removed.

The treatment with significant influence of the independent variables on the dependent variable respiratory rate was spraying of the insecticide only in the absence of the aphid in both cultivars. The variables that significantly interfered with the respiratory rate were Fv/Fm in non-Bt cotton and Y(II), Y(NO) and Fo in Bt cotton (Table 4), with the contribution of the variable Y(II) being considered low (adjusted R2 = 0.01) (Table 4).

Table 4 – Multivariate multiple regression of the effect of variables related to the fluorescence of chlorophyll a on the dark respiration rate

Cultivar	Treatment	Variable	$R^2_{adjusted}$	*AIC*	*RMSE*	*F*	*P*
non-Bt	Water	Y(NO)	0.00	31.93	1.15	0.31	0.59 ^{ns}
	Insecticide	F_v/F_m	0.43	30.39	1.06	7.17	0.03 *
	Insecticide + Aphid	ETR	0.00	43.24	1.45	0.96	0.35 ^{ns}
Bt	Water	F_v/F_m	0.08	44.32	1.30	2.05	0.18 ^{ns}
	Insecticide	Y(II)	0.01	33.37	1.06	10.80	0.01 *
		Y(NO)	0.65	23.66	0.63		
		F_o	0.76	20.14	0.51		
	Insecticide + Aphid	F_o	0.00	43.11	1.44	0.32	0.58 ^{ns}

$R^2_{adjusted}$ = Adjusted determination coefficient. *AIC*= Akaike information criterion. *MSE*= mean squared error. *F*= model *F-value*. *P* = value of the level of model significance. The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#tbl4

Curiously, the optimal values of the Fv/Fm ratio did not occur in the evaluation of treatment with only non-Bt cotton insecticide, expressing a value below that considered optimal immediately (see discussion) after exposure to the insecticide (Time 0), indicating a possible stress level (Fig. 2).

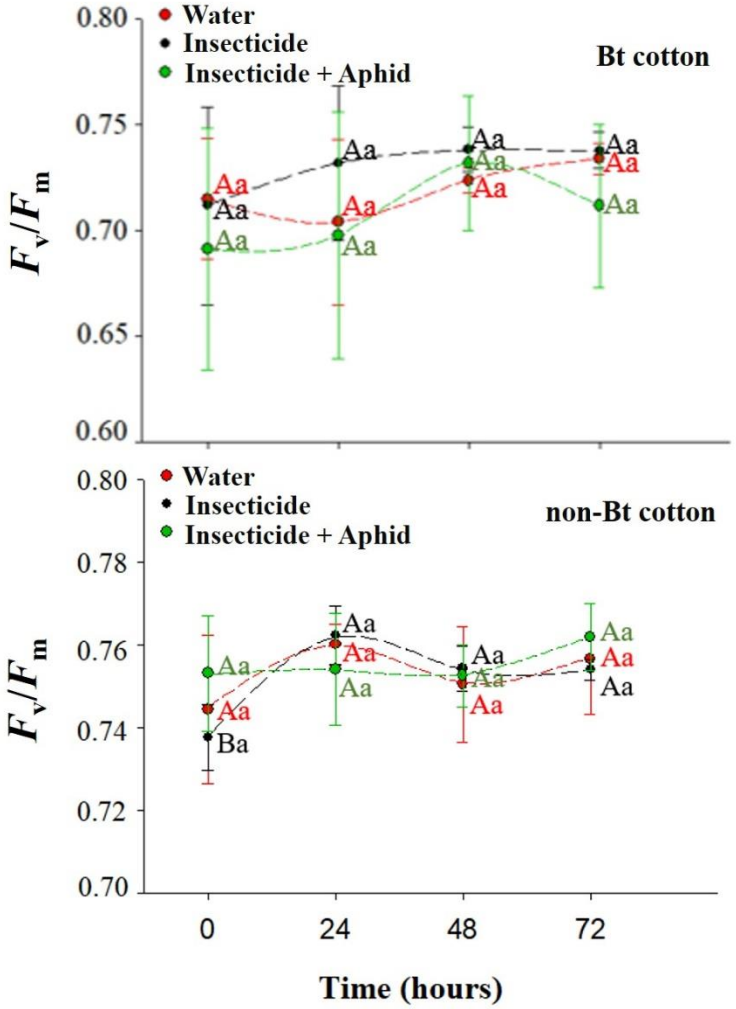

Figure 2. Maximum quantum efficiency of PSII photochemistry (F_v/F_m) in leaves of Bt and non-Bt cotton plants. Lowercase letters compare treatments (water, insecticide and insecticide + aphid) within time evaluated by overlapping confidence intervals (95% CI) estimated by a mixed linear model with repeated measures. The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#fig2

The Y(NO) variable is indicative of the mechanism used by plants to prevent damage or energy overload in photosynthetic machinery when exposed to stress. There was a significant difference for the Bt cotton cultivar after exposure to insecticide and insecticide aphid only in the period of 48 h. Regarding the non-Bt cultivar, the significant difference occurred only in the presence of the insecticide aphid in the 24 h period (Fig. 3).

The T2 treatment (only insecticide) promoted a greater F0 in relation to the T3 treatment (insecticide aphid) during the 0 h and 24 h periods in non-Bt cotton. But with no differences in the (F0) values for control. “In Bt cotton, there was a reduction in the initial fluorescence (F0)

compared to the control, with treatment with only insecticide during the evaluation period of 48 h (Fig. 4).

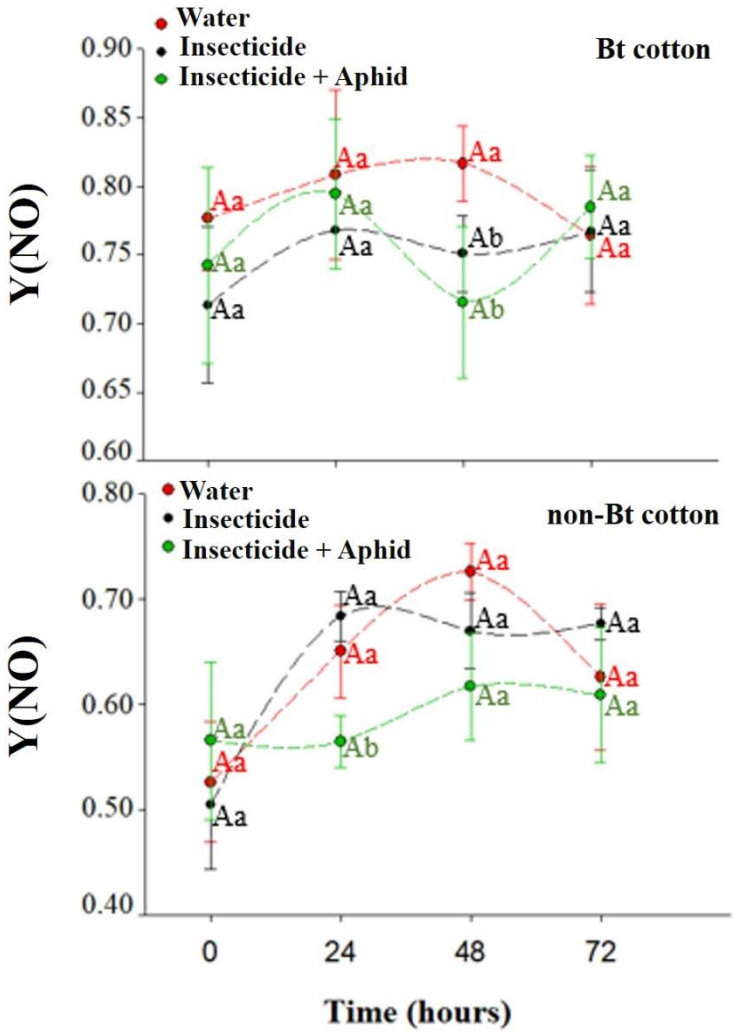

Figure 3- Quantum yield of nonregulated nonphotochemical energy loss in PS II (**Y(NO)**) in leaves of Bt and non-Bt cotton plants. Lowercase letters compare treatments (water, insecticide and insecticide + aphid) within time evaluated by overlapping confidence intervals (95% CI) estimated by a mixed linear model with repeated measures. The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#fig3

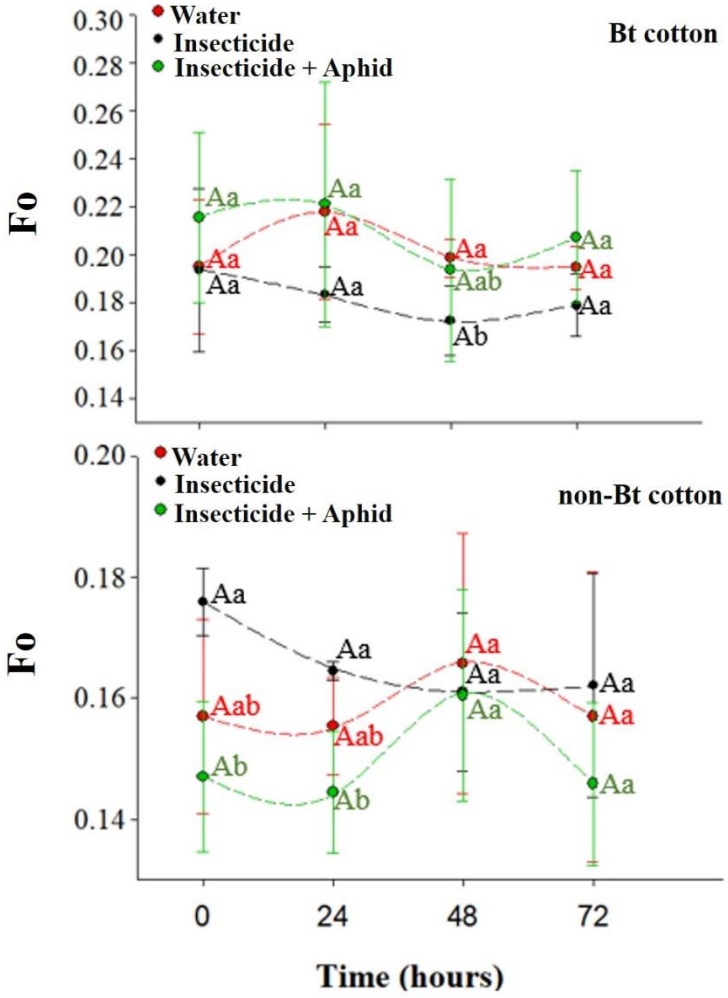

Figure 4- Initial fluorescence (***F*₀**) in leaves of Bt and non-Bt cotton plants. Lowercase letters compare treatments (water, insecticide and insecticide + aphid) within time evaluated by overlapping confidence intervals (95% CI) estimated by a mixed linear model with repeated measures. The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#fig4

For electrical signalling results, there was no record of electrical signals with application of water (control), both in Bt and non-Bt cotton. On the other hand, electrical signals of the type variation potentials (VPs) were registered in both cultivars when the plants were exposed to the insecticide. There was only a significant difference between treatments (insecticide and control), and there was no isolated effect of the cultivar, as well as the treatment versus cultivar interaction (Table 5).

Table 5 – Variation potential (VPs) recorded after insecticide application in Bt and non-Bt cotton

Cultivar	Treatment	
	Water	Insecticide
Bt	0.00 ± 0.00	3.20 ± 1.59
non-Bt	0.00 ± 0.00	1.00 ± 0.63
Average	0.00 ± 0.00 b	1.10 ± 0.88 a
df	1	
F	10.2907	
P	0.0013	

There was only a significant effect for the factor treatment. No difference was observed in relation to cultivar or significant interaction between cultivar versus treatment. The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#tbl5

4.3.3. Behavioural evaluation of Aphis gossypii and Cycloneda sanguinea in Bt and non-Bt cotton plants in the presence and absence of an insecticidal molecule

Insecticide affected the behaviour and insect survival. The *C. sanguinea* survival rate was estimated to be approximately 75% 5 days after insecticide exposure; however, there was a significant difference in the time mortality of *C. sanguinea* treated with imidacloprid (log-rank test < 0.05) (Supplementary Material II).

The comparison of aphid behavioural variables involving treatments with and without insecticide combined with Bt and non-Bt cultivars and of ladybugs of both sexes (males and females) kept only on non-Bt cotton under insecticide spray and control conditions was performed through the mean ranks, and the data were expressed in comparative boxplots (Fig. 5).

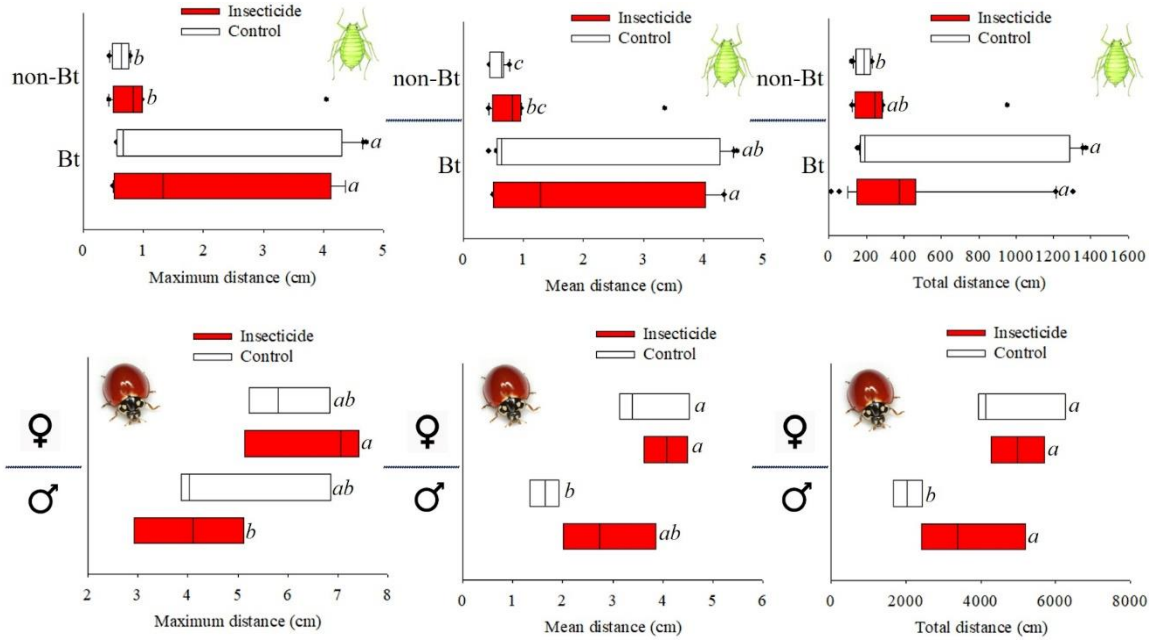

Figure 5 – Comparative boxplot of the distribution of *Aphis gossypii* movement data in Bt and non-Bt cotton and for males and females of *Cycloneda sanguinea* exposed to treatments with insecticide and the control (water). The height of the boxplot rectangle is defined by quartiles Q1 and Q3, while the line that cuts the rectangle represents the median value (Q2). The semistraight lines connecting quartiles Q1 and Q3 correspond to the minimum and maximum values of the data set, respectively. Asterisks correspond to outliers. Means followed by the same letters do not differ by the Kruskal-Wallis test ($\alpha = 0.05$). The published version is available at: https://www.sciencedirect.com/science/article/pii/S0045653520317562#fig5

There was no significant difference between the insecticide and control treatments within each cultivar for the variables maximum, mean and total distance travelled by the aphid. However, between the cultivars, the greatest maximum distance travelled by the aphid was observed in Bt cotton in relation to non-Bt cotton. The shortest mean and total distances were found in non-Bt cotton in the control treatment but did not differ from the same cultivar with insecticide (Fig. 5).

In the case of the behaviour of *C. sanguinea* when exposed to insecticide and without the presence of aphids, there was a significant difference for all evaluated variables, and when the aphids were exposed to the insecticide, the maximum distance travelled was higher for females than for males. The lowest mean distance found was observed in males without exposure to the insecticide (control) in relation to females in both conditions (insecticide or control); however, it did not differ when they were exposed to the insecticide (Fig. 5).

In relation to the total distance travelled by *C. sanguinea*, when we compared males between treatments, it was possible to show greater locomotion stimulus when they were exposed to insecticides in relation to the control group. In addition, the total locomotion distance

was significantly lower in males in the control group than in females in both groups (insecticide or control) (Fig. 5).

4.4 Discussion

An efficient way to monitor damage caused by herbivory stress has been the use of chlorophyll a fluorescence measurements associated with photosystem II (PSII) (Saglam et al., 2019). In the present study, it was found that insecticide spraying with the occurrence of aphids in both cultivars and only the presence of aphids in non-Bt cotton reduced the proportion of open reaction centres of PSII (qL), resulting in less transfer of light energy to PSII, as observed by the lower values of qP and Y(II). YII is indicative of the proportion of light absorbed by the leaf that is directly related to the photochemical phase of PSII and indicates the efficiency of the electron transport chain for photosynthesis (Jones, 2013).

The reduction in YII may induce less activity in the Calvin cycle due to the lower availability of ATP and NADPH (Jones, 2013). This effect demonstrates that plants suffer photochemical limitations in photosynthesis, which may reflect the lower activity of the Calvin cycle due to the lower availability of ATP and NADPH (Jones, 2013). It is possible that molecules of insecticides induce significant changes in the expression of genes involved in signal transduction activity, the electron transport chain, and photosynthesis (Cheng et al., 2012). On the other hand, aphids interfere with the interception of light and the photochemical phase by altering the chlorophyll content, leading to a limitation in electron flows triggered by light (Burd and Elliott, 1996). In addition, they can hinder the normal transport of phloem, which interferes with the delivery of nitrogen to cells and interrupts the flow of assimilates (Peterson and Higley, 1993). Similar results were observed after feeding aphids on soybean [*Glycine max* (L.) M.], wheat (*Triticuma estivum* L.) and cotton (*G. hirsutum*) (Macedo et al., 2003; Shannag et al., 1998).

Corroborating the changes in photosynthetic efficiency, the increase in the initial fluorescence (Fo) in non-Bt cotton exposed to insecticide spray in the absence of the insect between periods 0 h and 24 h is indicative of damage in the reaction centres or in the dissipation of energy via the antenna complex, resulting in less energy transfer to the PSII (Baker and Rosenqvst, 2004). This effect can be observed in the reduction in Fv/Fm 24 h with spraying of insecticide in the absence of the aphid, demonstrating that the damage of the light absorption complexes in PSII may also have contributed to the reduction of Y(II) and qP. Fv/Fm is a variable measured after the plant is adapted to the dark, and its values are commonly used to assess plant stress reflecting high PSII sensitivity to environmental stimuli, directly or

indirectly. Under normal conditions, the Fv/Fm ratio has optimal values of approximately 0.75 to 0.83 for most species (Baker, 2008; Maxwell and Johnson, 2000).

The activation of unregulated thermal energy dissipation mechanisms (Y(NO)) is among the mechanisms used by plants to prevent damage to photosynthetic machinery when exposed to stresses (Muller et al., 2001). The decrease in the efficiency of this process when the insecticide was sprayed with aphids in 24 h in non-Bt cotton requires the action of another protection mechanism against excess energy to avoid photooxidative damage. In the analysis of the interference of the chlorophyll a fluorescence variables and those related to the rate of respiration in the dark, the variables Fv/Fm in non-Bt cotton and Y(NO) and F0 in Bt cotton, when using the insecticide in the absence of aphid, were the only ones with significant influence on the respiration of these cultivars. Values below that considered normal in the Fv/Fm ratio were observed, which may indicate that oxidative stress occurred. Some studies have documented that the application of some pesticides causes this kind of stress in plants as a result of the generation of reactive oxygen species (ROS) (Sharma et al., 2018b), which results in the degradation of chlorophyll and protein and finally causes a reduction in the photosynthetic efficiency of plants (Xia et al., 2006; Sharma et al., 2015). The low photosynthetic activity can also be related to the reduction of the intercellular concentration of CO2 due to stomatal closure (Baker, 1993) but also to the increase in lipid peroxidation due to the deviation of the electron flow from the assimilation of CO2 to the reduction of O2 (Lemos Filho, 2000).

When stress is mediated by insecticide + aphid, the dark respiration rate in Bt cotton is lower than that in non-Bt cotton. Under stress with only insecticide, in the period of 48 h, the respiratory rate in Bt cotton was lower, while in the period of 72 h, the opposite result was obtained. High RD values may be attributed to low photosynthetic activity; thus, cultivars respond differently to the kind of stress and its intensity over time. Other studies have shown that the rates of stomatal conductance of transpiration and photosynthesis do not differ significantly between non-Bt and Bt cotton until 80 days after sowing (Hebbar et al., 2007).

In the analysis of the respiratory pattern over time between treatments, it is verified that for non-Bt cotton, in 48 h stress mediated by the insecticide in isolation or combined with the aphid promotes an increased respiratory rate in relation to the control (water); in 72 h, the insecticide in the absence of the aphid reduces the respiratory rate, while in the insecticide + aphid combination this variable increases in relation to the control (water) condition. For cultivar Bt, the respiratory rate increased over time, following the same pattern in the treatments

used. Therefore, some of these results reinforce that the effect of combined stress factors in plants is not always additive because the result is typically dictated by the nature of the interactions between stress factors (Prasch and Sonnewald, 2013; Atkinson et al., 2013; Pandey et al., 2015a, 2015b; Choudhary et al., 2016; Ramu et al., 2016). Our results may also indicate a combined stress in non-Bt cotton, as in addition to the effects caused by the insecticide, aphids cause direct injuries to the host plant by removing the phloem sap, which is richer in sugars than in amino acids. A large part of this sap is excreted as molasses by aphids, which can cover the leaf surface and, consequently, explain the reduction in photosynthesis efficiency (Shannag, 2007).

Another question of this research was in relation to the impact of the insecticide on the electrical signalling of cotton plants of the non-Bt and Bt cultivars. Our results showed that the application of insecticide to plants led to the generation of variation potentials (VPs). VP is a damage-induced long-distance electrical signal that can spread to undamaged areas (Stahlberg et al., 2006). The spread of VPs is associated with the transmission of hydraulic or chemical signals (Malone,1994; Stahlberg et al., 2006). There is evidence that several physiological processes are influenced by VPs, including gene expression, protein synthesis, respiration and photosynthesis (Sukhov et al., 2014). Surova et al. (2016a) demonstrated that after burning stress, VPs are generated and propagate to neighbouring leaves, resulting in the inactivation of photosynthesis. Approximately 15e20 min after VP, the authors verified increased nonphotochemical quenching (NPQ), which is an energy dissipation mechanism related to the protection of the photosynthetic apparatus. In addition, VP-induced respiration activation has been observed in several species, such as peas (Pisum sativum L) (Surova et al., 2016b), pumpkin (Curcubita pepo L.) (Sherstneva et al., 2015) and mimosa (Albizia julibrissin L.) (Lautner et al., 2014). However, studies evaluating the spraying of insecticides on the electrical activity of plants are scarce.

It is necessary to emphasize that there are several studies showing significant responses to the use of insecticides, including compounds that belong to the group of neonicotinoids on the physiology and biochemistry of plants, including cotton (Gonias et al., 2008). Nevertheless, these effects vary according to the product concentration, plant phenological age at the time of spraying and the plant cultivar. However, the results presented here point to the activation of stress tolerance routes, with small distinctions between Bt and non-Bt cotton. Additionally,

there is a vast body of literature that indicates that electrical signals are important stress signallers in higher plants (Sukhov et al., 2019).

The evaluation of sublethal effects of insecticides on the behaviour of target insects is also essential for the improvement of other control strategies, such as the use of genetically modified plants integrated with other control strategies such as conservative or applied biological control, as well as exploring behavioural control. *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) had locomotor behavioural activities affected, such walking distance, time and speed when exposed to *Cinnamomum zeylanicum* L. (Lauraceae) (Haddi et al., 2015). In our study, we observed that the insecticide does not change the behavioural pattern of *A. gossypii*, with behavioural differences occurring only between Bt and non-Bt cotton. When the aphids were kept on Bt cotton plants, they showed greater locomotion capacity. This can be explained by the fact that the sap of Bt cotton is less suitable for ingestion by these aphids, thus favouring higher dispersal of insects (Xue et al., 2008).

In the present study, we emphasized the sublethal behavioural effects of the insecticide on coccinellid predators. In the literature, both physiological and behavioural effects are described related to insect mobility, changes in the ability to search for prey or hosts and changes in feeding and oviposition behaviour (Desneux et al., 2007). It is well known that insecticides sprayed at certain concentrations can alter specific plant odours and cause rejection of feeding places. According to the effects observed in the present study by the application of imidacloprid on the physiological changes of cotton plants in both cotton cultivars, we decided to quantify the possible effects of this insecticide on behavioural variables of *C. sanguinea*, not taking into consideration the cultivar of cotton.

Males of *C. sanguinea* exposed to imidacloprid are stimulated to greater locomotion. It is known that neonicotinoid insecticides are neurotoxic and negatively affect the behaviour and performance of natural enemies, causing sublethal effects on their physiological and behavioural characteristics (Cabral et al., 2011; Fogel et al., 2013; He et al., 2012), can cause changes in foraging patterns, disturb sexual recognition and communication (Elzen, 1989), and influence the speed with which the insect walks. In other insects, the impact of neonicotinoids was evident, although the behavioural variables showed a different response degree according to the insecticide and nontarget species. In *Podisus nigrispinus* (Heteroptera: Pentatomidae), the impact of imidacloprid on its functional responses does not depend on the cotton cultivar (Bt or non-Bt) (Malaquias et al., 2015). In other nontarget insects, neonicotinoids promoted

changes in motor variables of stinging and stingless bees (Jacob et al., 2019a, 2019b). Imidacloprid and thiacloprid reduced the locomotion activity of *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae), acetamiprid did not cause changes in locomotion activity, and thiamethoxam induced high hyperactivity in this stingless bee. Acetamiprid, imidacloprid and thiacloprid affected the speed, distance travelled, duration and frequency of resting, and continuous mobility of *Apis mellifera* L. and *Scaptotrigona postica* (Latreille) (Hymenoptera: Apidae) (Jacob et al., 2019b).

We used an interdisciplinary approach based on plant physiological responses and prey and predator behaviour to understand imidacloprid-mediated stress on Bt and non-Bt cotton plants and on *A. gossyppi* and *C. sanguinea*. It is important to emphasize that our research does not represent totally field exposure conditions. It is only a model study that generates insights highlighting the exposure of Bt and non-Bt cotton plants and the predator *C. sanguinea* to insecticides without and with the occurrence of *A. gossypii* on the plant. Thus, this exposure can cause stress on the plant physiology and on the behaviour of the predator. Imidacloprid in Bt and non-Bt cotton plants without the presence of the aphid led to the generation of variation potentials (VPs), and these signals could have resulted in the inactivation or low efficiency of photosynthesis in some specific periods. Non-Bt cotton plants exposed to insecticide aphids resulted in low photosynthetic efficiency, indicating combined stress in this cultivar. The respiratory rate of cotton plants was also affected by the insecticide aphid. Bt cotton showed a low respiratory rate and low quantum yield, while non-Bt cotton showed higher respiration and lower quantum yield. No imidacloprid effects were observed in *A. gossypii* behaviour; however, in ladybugs, imidacloprid caused high locomotion of males, which could affect dispersal within and between mating and feeding patches, promoting possible negative impacts on the population structure of this species in cotton agroecosystems.

Our results can be used as a tool to implement integrative management programmes in the search for conservation and greater efficiency and sustainability to the agroecosystem and to demonstrate the possible impacts when using imidacloprid in cases of non-occurrence of the aphid *A. gossypii*.

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Supplementary Material I

Electrical signalling on Bt and non-Bt cotton plants under stress by *Aphis gossypii*[4]

Determination of Cry1F protein content - Methods

The Cry1F protein was quantified in Bt cotton plants in the presence of aphids (*A. gossypii* treatment) and in the absence of the insect (control). The plants were infested with the aphids with a paintbrush. We used the following 2 treatments: a1. Bt cotton plants infested with 60 aphids/plant, and a2. Bt cotton without aphids (Bt cotton control), distributed in 10 blocks = 10 plants/treatment.

The Cry1F protein concentrations in cotton leaf extracts were determined by immunological analysis (ELISA) using the Bt-Cry1F ELISA Kit (Quantitative DAS ELISA for the detection of the Bt-Cry1F transgenic protein, Catalog number: PSP 11700, Agdia®) according to the manufacturer's instructions. The leaf tissue of each subsample was individually homogenized using a clean mortar and pestle and 2 ml of phosphate buffered saline with Tween 20 Detergent (PBST) extraction buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween® 20 detergent, pH 7.4). The supernatant was collected after centrifugation at 11,180×g at 4°C for 20 min.

Enzyme conjugates (100 μl) were dispensed per well, and the same amount of each prepared sample, positive control, negative control and PBST buffer were dispensed in their respective wells. The standard Cry1F insecticidal protein, controls and samples were placed inside a humid box and incubated at 28°C for 60 minutes. After incubation, the plate was washed using 1X PBST. After washing, 100 μl of the TMB substrate solution was added to each well, and the plate was incubated in the dark for 20 min. The absorbance was recorded at 650 nm using Gen5 2.05 Software. Cry1Ac protein concentrations were calculated using the standard Cry1F insecticidal protein curve.

Results

[4] The original and published version is available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0249699#sec013

Our results showed differences in Cry1F concentration on Bt cotton leaves of *A. gossypii*-infested and control (noninfested) plants (**Table I**).

Table I – Cry1F concentration (ng/ml) on noninfested (control) WideStrike cotton plants infested with *Aphis gossypii*

Treatment	Cry1F concentration (ηg/ml)
Control (absence of aphids)	4.38±0.67 b
A. gossypii	7.85±0.28 a
$P_{adjusted}$	= 0.0163

There was a difference between the treatments ($P < 0.05$). The published version is available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0249699#sec013

Supplementary Material II

Imidacloprid-Mediated Stress on non-Bt and Bt Cotton, Aphid and Ladybug Interaction: Approaches Based on Fluorescence, Dark Respiration and Plant Electrophysiology and Insect Behaviour[5]

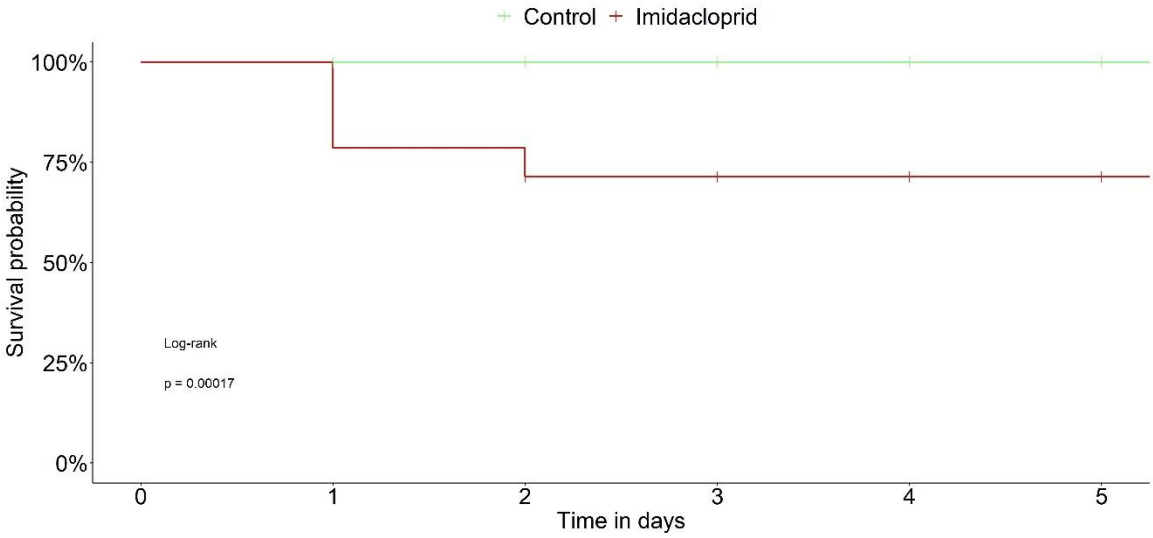

Figure I. Survival curves of adults of *Cycloneda sanguinea* **exposed to imidacloprid and the control (water).** Survival curve intervals grouped by the same fill color were not significantly different by the log-rank test (P = 0.05). The published version is available at (Download Word document): https://www.sciencedirect.com/science/article/pii/S0045653520317562#appsec1

[5] The original and published version is available at (Download Word document): https://www.sciencedirect.com/science/article/pii/S0045653520317562#appsec1