BIOCHEMICAL AND MOLECULAR BASIS OF *Tuta absoluta* (MEYRICK) (LEPIDOPTERA: GELECHIIDAE) RESISTANCE TO PYRETHROIDS AND SPINOSYNS

by

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(Under direction of Professor Herbert Álvaro Abreu de Siqueira – UFRPE)

ABSTRACT

The *Tuta absoluta* is a key pest of tomato crops and the use of insecticides is still the main method of control. However, overuse of them has contributed to the development of resistant populations. Although the pyrethroid resistance mechanism in this species has been described, there is no information on their status in Brazilian populations. Regarding the spinosyns, despite the reports of resistance to these molecules, the underlying mechanisms are unknown. The objective of this research was to characterize the resistance to pyrethroids and spinosad in Brazilian populations of *T. absoluta* by conventional methods (bioassays), enzymatic and molecular assays. All populations tested were resistant to pyrethroids. The GSTs activity and cytochrome P450-mediated N-demethylation was significantly correlated with the level of resistance to deltamethrin and permethrin suggesting that these enzymes can play a role in resistance. TaqMan assays demonstrated that the mutation in L1014F sodium channel is fixed in all populations and is associated with other mutations T929I and M918T. Spinosad synergism (synergistic with PBO, DEF, and DEM) indicated that the metabolic enzymes are not involved in resistance. Comparison of the nucleotide sequence of the subunit of the nicotinic receptor α6 *T. absoluta* susceptible and resistant to the point spinosad allowed identification nucleotide change resulting in the substitution of a glycine (G) in the susceptible insects by glutamic acid (E)

resistant insects (G275E). The TaqMan assays revealed that the frequency of the resistant allele in the population is low. The diagnostic dose bioassays correlated with the resistant allele frequency. The *T. absoluta* Brazilian populations are resistant to pyrethroid insecticides and the main mechanism of resistance is the L1014F mutation, while for spinosad insecticide, most people are sensitive and the G275E mutation can lead to the loss of efficiency of this product for control of this plague.

KEY WORDS: Tomato leafminer, mutation, sodium channel, nicotinic acetylcholine receptor.

BASES BIOQUÍMICAS E MOLECULARES DA RESISTÊNCIA DE *Tuta absoluta* (MEYRICK) (LEPIDOPTERA: GELECHIIDAE) A PIRETROIDES E ESPINOSINAS

por

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(Sob Orientação do Professor Herbert Álvaro Abreu de Siqueira– UFRPE)

RESUMO

A *Tuta absoluta* é uma praga importante para a cultura do tomateiro e o uso de inseticidas ainda é principal método de controle. No entanto, a utilização indiscriminada destes tem contribuído para seleção de populações resistentes. Embora o mecanismo de resistência à piretroides nessa espécie tenha sido descrito, não há informações sobre seu *status* em populações brasileiras. Com relação a espinosinas, apesar dos relatos de resistência a estas moléculas, os mecanismos envolvidos são desconhecidos. O objetivo desta pesquisa foi caracterizar a resistência a piretroides e espinosade em populações brasileiras de *T. absoluta* por métodos convencionais (bioensaios), ensaios enzimáticos e moleculares. Todas as populações testadas foram resistentes a piretroides. A Atividade de GSTs e citocromo P450 mediado por *N*-desmetilação correlacionou-se significativamente com o nível de resistência a deltametrina e permetrina sugerindo que estas enzimas podem desempenhar um papel na resistência. Ensaios TaqMan demonstraram que a mutação L1014F no canal de sódio está fixa em todas as populações e foi associada com outras mutações M918T e T929I. Sinergismo de espinosade com PBO, DEF, DEM indicou que as enzimas metabólicas não estão envolvidas na resistência. A comparação da sequência de nucleotídeos da subunidade α6 do receptor nicotínico de *T. absoluta* suscetíveis e resistentes a espinosade possibilitou a identificação de alteração pontual de nucleotídeo,

resultando na substituição de uma glicina (G) nos insetos suscetíveis por um ácido glutâmico (E) em insetos resistentes (G275E). Os ensaios de TaqMan revelaram que a frequência do alelo resistente está baixa nas populações. Os bioensaios de dose diagnóstica correlacionaram com frequência do alelo resistente. As populações brasileiras de *T. absoluta* são resistentes aos inseticidas piretroides e o principal mecanismo de resistência é a mutação L1014F, enquanto para o inseticida espinosade, a maioria das populações são sensíveis e a mutação G275E pode acarretar a perda da eficiência deste produto.

PALAVRAS-CHAVE: Traça-do-tomateiro, mutação, canal de sódio, receptor nicotínico.

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A Thesis submitted to the "Programa de Pós-Graduação em Entomologia Agrícola" of the "Universidade Federal Rural de Pernambuco" in partial fulfillment of the requirements for the degree "Doutor em Entomologia Agrícola"

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DEDICATION

I offer this thesis the most beautiful rose in my garden, my beloved mother. This woman was born with the strength of a lioness, always ready to be with me and my sisters in the most difficult moments of my life.

I dedicate this thesis to my God for making possible the greatest miracle of my life that is the existence, allowing me to obtain all victories in this life.

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CHAPTER 1

INTRODUCTION

The tomato plant (*Solanum lycopersicum* L.) is one of the most widespread vegetable plants that are cultivated at the most different geographical latitudes on the planet. In 2012, Brazil produced 3.8 million tons of tomatoes (IBGE 2013). Such a large area expansion of tomato cultivation has favored the development of several pest species that considerably affect production each year. A remarkable pest is the tomato leafminer, *Tuta absoluta* (Meyrick, 1917); (Lepidoptera: Gelechiidae). This invasive pest was first detected in Brazil in 1980 (Morais & Normanha Filho 1982). Nowadays, it has become a worldwide tomato threatening pest after its detection in the North of La Plana de Castellón province, eastern Spain in late 2006 (Urbaneja *et al.* 2007). Since then it has spread to several places of the Mediterranean basin, causing serious losses to tomato crops (Urbaneja *et al.* 2009). In 2009, its presence was recorded in Italy, France, Malta, United Kingdom, Greece, Switzerland, Portugal, Morocco, Algeria, Tunisia, Libya and Albania (EPPO 2009).

The leaf mining larvae of *T. absoluta* injure a large part of the tomato superior canopy (Coelho & França 1987). Fruit boring depreciates the value of the tomatoes for market, and shoot injuring, sometimes decapitate plants causing eventual death (Michereff Filho & Vilela 2001). *T. absoluta* outbreaks usually lead to the complete destruction of crops. In these conditions, damaged fruits are usually lost for market and can also facilitate pathogen outbreaks (fungi, bacteria) (Coelho & França 1987).

Adults of *T. absoluta* are microlepidoteran of silver-grey color, with wingspan ranging between 9 and 11 mm (Coelho & França 1987, Michereff Filho & Vilela 2001). The eggs are

elliptical and initially bright white or light bright yellow, becoming brownish or reddish close to larval hatching. The eggs are laid isolated or in a cluster on the upper or lower foliole surfaces (Coelho & França 1987).

The main method of control used to reduce *T. absoluta* infestations is through insecticide spraying. This is the most simple and fast response that has a high cost-efficiency (Miranda *et al.* 1998). Overuse of insecticides in tomato cropping has often been associated with the absence of precise Economic Injury Levels (EIL) for chemical tactics, which leads to 36 applications of all sorts of insecticides per season in some regions (Picanço *et al.* 1995). The indiscriminate use of insecticides has resulted in the rapid development of resistant populations of *T. absoluta* to the various classes of insecticides (Guedes *et al.* 1994, Lietti *et al.* 2005, Salazar & Araya, 2001; Silva *et al.* 2011, Siqueira *et al.* 2001, 2000).

Mechanisms of Insecticide Resistance

The reports of insect resistance intensified in the 1940s after introduction of synthetic insecticides and acaricides. There are more than 7,740 reports of resistance cases to 331 compounds, involving more than 540 species of insects and mites (Whalon *et al.* 2008). Resistance is defined as the inherited ability of an organism to survive doses of a xenobiotic, which would be lethal to the majority of individuals of a species (Croft *et al.* 1988). In the evolutionary context, according to Dobzhansky (1951), resistance to insecticides is characterized as being a pre-adaptive, genetic and heritable trait. In the agricultural context, resistance has been identified as one of the most serious threats to maintenance and development of integrated pest management (IPM) practices (Metcalf 1980, Labbe *et al.* 2005).

There are basically three kinds of resistance mechanisms in insects: through metabolism, target site insensitivity, and reduced penetration (physiological), although a fourth one, behavior may be included. Resistance through reduced insecticide penetration appears to be a common

phenomenon. Resistance may be associated with lipid reservoirs in the cuticle that can retain insecticides, and an enzyme that can metabolize them or, simply, a thicker or less permeable integument (Plapp 1976). Biochemical studies have shown that the cuticle of the *Heliothis virescens* (Fabrícius) is resistant to DDT and had more proteins and lipids than a susceptible strain of the insect (Vinson & Law, 1971). Resistance to pyrethroids in the Pakistani populations *Helicoverpa armigera* (Hübner) was also attributed to a reduction in insecticide penetration. The intake time for deltamethrin was one hour for the susceptible colony and six hours for the resistant (Ahmad *et al.* 2006). This kind of resistance was also reported in *Helicoverpa zea* (Boddie), *Musca domestica* (Linnaeus.), *Spodoptera exigua* (Hubner), *Plutella xylostella* (Li) and *Boophilus microplus* (Can) (Gunning *et al.* 1991, Little, *et al.* 1989, Abd-Elghafar *et al.* 1994; Scott *et al.* 1986, Delorme *et al.* 1988,Schnitzerling *et al.* 1983).

The metabolic mechanism of resistance is related with a variety of enzymes that may be induced after contact with an insecticide (Sogorb & Vilanova 2002, Srinivas *et al.* 2006). There is then increased expression of genes that encode the three main groups of enzymes involved in the metabolism: cytochrome P450 dependent monooxygenases, glutathione-*S*-transferases (GSTs) and esterase (Zhu *et al.* 2000, Kostaropoulos *et al.* 2001, Baffi *et al.* 2008). The excess of these detoxification enzymes are then able to metabolize the insecticides.

The esterases are hydrolases present in insects that breakdown the ester bonds of insecticides by adding a water molecule and generating acid and alcohol parts (Yu 2008**)**. Resistance to organophosphates, pyrethroids, spinosad, metaflumizone and indoxacarb has been linked to increased esterase activity (Zhu *et al.* 2004, Ono *et al.* 1999, Ashraf *et al.* 2010, Herron *et al.* 2014, Nehare *et al.* 2010). The gene expression and duplication of esterases is a phenomenon commonly associated with insecticide resistance (Li *et al.* 2007). Cytochrome P450-dependent monooxygenases are a superfamily of enzymes that occur in all cellular organisms.

When highly expressed, these enzymes are important in the metabolism of xenobiotics and the resistance to insecticides (Scott 1999). The P450s are hemeproteins that are induced by numerous nonpolar substances and they have Nicotinamide Adenine Dinucleotide Phosphate (NADPH) as a cofactor for their catalytic activity (Hodgson 1985).

Glutathione *S*-transferases (GSTs) are members of a large family of intracellular enzymes involved in the endogenous detoxification of insecticides (Mannervik *et al*. 1988, Pickett & Lu, 1989, Yang *et al*. 2001). Increased expression of GSTs has been also associated to resistance development in many insect species. One or more GSTs were related to resistance to organophosphates in the fly *Musca domestica* (Linnaeus) (Wei *et al*. 2001), organochlorines in the fly *Drosophila melanogaster* (Meigen) (Tang & Tu, 1994) and pyrethroids in the *Nilaparvata lugens* (Stal) (Vontas *et al*. 2001, 2002).

The nervous system is the major site of insecticide action in insects (Narahashi 1996; Bloomquist 1996). Insecticide target site alteration is another type of resistance caused by mutations on genes that encode the site of insecticide action. Gene mutations can prevent and hinder the insecticide connection with its target molecule. These alterations may partially or fully impair the insecticidal activity in question. The main targets of insecticides are the *para* voltage-gated sodium channels (*vsgc*) (Narahashi 1992), the ionotropic receptors that mediate acetylcholine action (Gundelfinger 1992), γ –aminobutiric acid (GABA) (Sattelle 1990; Rauh *et al*. 1990) and L-glutamate (Cully *et al*., 1996) and the acetylcholinesterase enzyme (AChE) that is responsible for the acetylcholine breakup (Massoulie *et al*. 1993).

Sodium channels as Target for Insecticides

Electrical signals known as action potentials propagate rapidly along the neuron axons and through synapses between neurons. Voltage-gated sodium channels (vsgc) are responsible for the initiation and propagation of action potentials along the axon. Each sodium channel comprises an

alpha subunit (~ 260 kDa) consisting of four homologous domains (I to IV), which in turn contain six transmembrane segments (S1-S6) (Fig.1) (Soderlund & Knipple 2003).

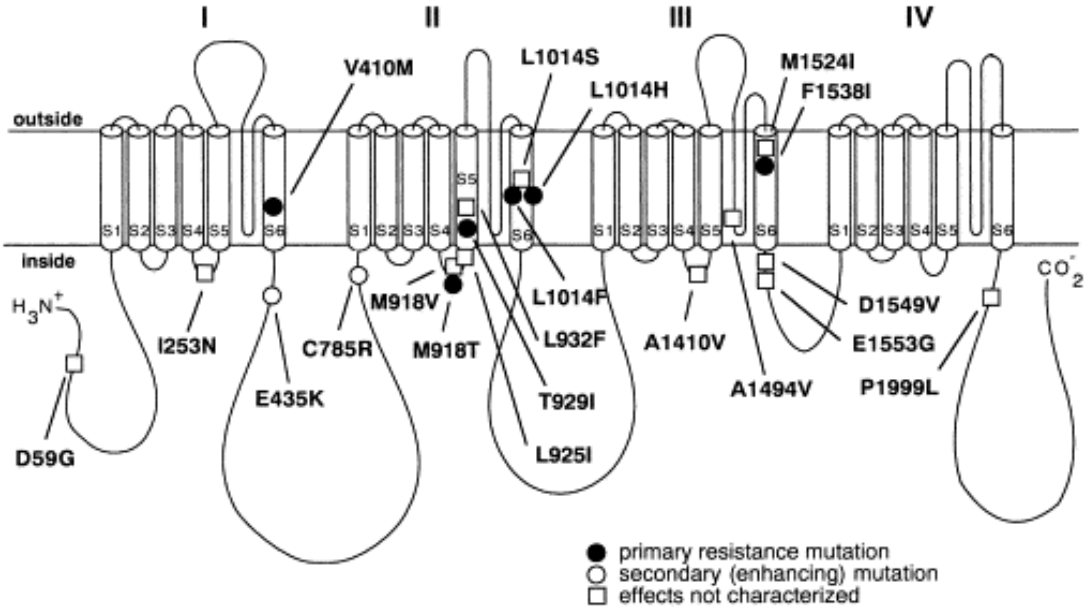

Figure 1. Diagram of the extended transmembrane structure of voltage-sensitive sodium channel *α* subunits showing the four internally homologous domains (labeled I–IV), each having six transmembrane helices (labeled S1–S6 in each homology domain), and the identities and locations of mutations associated with knockdown resistance. The symbols used to identify mutations indicate their functional impact as determined in expression assays with *Xenopus oocytes*. (Soderlund, & Knippl 2002).

The opening and closing of the sodium channel is regulated by two processes: activation and inactivation (Zlotkin 1999, Catterall 2000, Dong *et al.* 2014). When a neuron is at rest, sodium channels are closed. When the membrane of a neuron is depolarized, sodium channels are enabled. Influx of sodium ions through sodium channels further depolarizes the membrane and is responsible for increasing the action potential. Within a few milliseconds, sequential openings of sodium channels occur. The inactivation process is partly responsible for the potential of the falling phase of action, due to the departure of sodium ions (Goldin 2003, Dong *et al.* 2014). This change in the electrical potential of the membrane propagates along the neuron/axon,

characterizing the nerve impulse or action potential (Bloomquist 1996, Zhao *et al.* 2003, Nicholson 2007 Silver *et al.* 2010).

Pyrethroids prevent the normal closing of sodium channels, resulting in the continuous transmission of nerve impulses, causing tremors, paralysis and death of the insect (Narahashi 1996, Bloomquist 1996). These insecticides are synthetic derivatives of botanical pyrethrins present in the pyrethrum extract from the chrysanthemum species (Elliott 1977). Dried chrysanthemum flowers were used as insecticides in ancient China and Europe during the Middle Ages. The flowers were sold as "Persian dust" by Armenian merchants (Davies 2007). Commercial production of pyrethrins on a large scale began in the mid-nineteenth century. However, its general use in agriculture was limited by its low photostability and the cost of production. The discovery in the mid-1970s of permethrin and deltamethrin, two new pyrethroids with unprecedented activity combined with photostability made them suitable for agricultural use (Davies, 2007). These insecticides are grouped into two categories (Type I, Type II) based on their symptoms of poisoning, effects on the nervous system and their chemical structure (Du *et al.* 2009). Type I pyrethroids do not have an α-cyano moiety and cause repetitive discharges in response to a single stimulus. Conversely, Type II pyrethroids have the α-cyano group and cause a depolarization of the membrane accompanied by the suppression of the action potential (Dong 2007).

The main mechanisms of resistance to pyrethroids are caused by mutations in the Na^+ channel gene. These mutations were identified by comparing the sequences of sodium channel genes from resistant and susceptible individuals in several arthropod species (Rinkevich, *et al*., 2013, Dong *et al.* 2014). These mutations cause reduced neuronal sensitivity, known as knockdown resistance (*KdR*) (Soderlund, & Knippl 2002). This type of *KdR* resistance was first documented in the *M. domestica*. DNA nucleotide sequence analysis of the Na^+ channel gene

domain IIS6 demonstrated a single nucleotide polymorphism that resulted in the substitution of leucine for phenylalanine at position 1014 of the Na^+ channel (L1014F) (Williamson *et al.* 1993, Miyazaki *et al.* 1996). A second mutation, that resulted in the substitution of methionine to threonine at position 918 (M918T) was characterized together with the L1014F mutation (M918T + L1014F) and named super-*KdR* (Ingles *et al.* 1996). This genotype leads to higher levels of resistance to pyrethroids in the insect (Williamson *et al.* 1996). The M918T mutation alone caused a decrease of 5 to 10 times in sensitivity of the Na^+ channel to deltamethrin, the two mutations together can reduce 100 times the channel sensitivity to this insecticide (Dong *et al*. 2010, Lee *et al*.1999).

More than 30 mutations associated with resistance to pyrethroids were detected in arthropods (Soderlund, & Knippl 2003). In the mosquitoes, *Culex pipiens* (Li) and *Anopheles gambiae* (Giles) a mutation at position 1014 was identified, with the substitution of leucine for serine (L1014S) (Torres-Martinez *et al.* 1999, Ranson *et al.* 2000). A valine to methionine mutation in position 410 (V410M) located in the domain IS6 and the alteration of leucine to histidine at position 1014 (L1014H) in the domain III was found in different insecticide resistant populations of the moths *Heliothis virescens*, *Myzus persicae* (Sulzer) and *Triatoma infestans* (Klug) (Park & Taylor 1997, Martinez-Torres *et al.* 1999;. Fabro *et al.* 2012.). A threonine to isoleucine mutation (T929I) in the domain IIS5 was related to resistance to pyrethroids in the *Plutella xylostella* (Li), *Pediculus capitis* (De Geer), *Frankliniella occidentalis* (Pergande) and *Leptinotarsa decemlineata* (Say) (Lee et *al*., 2000, Kwon *et al*., 2004, Rinkevich *et al*. 2012, Forcioli *et al.* 2002). Recently, the L1014F, M918T and T929I mutations were reported in *T. absoluta* populations resistant to pyrethroids located in Colombia, Ecuador, Argentina, Canary Islands, Spain, Italy, Algeria and Israel (Haddi *et al*. 2013).

Nicotinic Receptors as Target for Insecticides

The nerve impulse that reaches the pre-synaptic membrane causes the release of acetylcholine as a neurotransmitter. This temporarily binds to the nicotinic receptor postsynaptic membrane, promoting the opening of the Na^+ channels in adjacent cells, and then the passage of nerve impulses from one cell to another. The activity of acetylcholine is terminated by the acetylcholinesterase catalysis, which rapidly hydrolyzes the neurotransmitter (Nation 2001).

Nicotinic acetylcholine receptors (nAChR) are members of the Cys-loop family of ligand-gated ion channels (Lester *et al*. 2004) and are important in excitatory neurotransmission in arthropods and in vertebrate nervous systems (Jones & Sattelle 2010). These receptors are selective to cation channels, such as Na^+, Ca^{2+}, K^+ (Laster *et al.* 2004). The acetylcholine, bind to the N- terminal extracellular domain of the subunits at a site formed by six loops (Loop A-F) located at subunit interfaces (Corringer *et al*. 2000). The N-terminal extracellular domain also contains a Cys-loop, characteristic of cys-Loop, which consists of two disulphide-bond forming cysteines separated by 13 residues (Corringer *et al*. 2000). Each subunit also has four transmembrane regions (TM1–TM4) (Green 1997). The subunits with two vicinal Cys at loop C which are involved in acetylcholine binding are classified into α type, whereas other subunits lacking the two Cys residues are regarded as non-α type (Fig. 2) (Kao *et.al.* 1986). Spinosyn insecticides mimic the action of acetylcholine binding to nAChR receptors, causing a change in its conformation, which opens ion channels and thus the conduction of the nerve stimulation, causing tremors, paralysis and death of the insect (Salgado *et al.* 1998, Thompson *et al.* 2000, Cisneros *et al.* 2002). This insecticide was isolated from a mixture of secondary metabolites during fermentation of the soil actinomycete, *Saccharopolyspora spinosa* (Mertz & Yao). It consists basically of two macrocyclic lactones, spinosyn A and spinosyn D (Sparks *et al.* 1998).

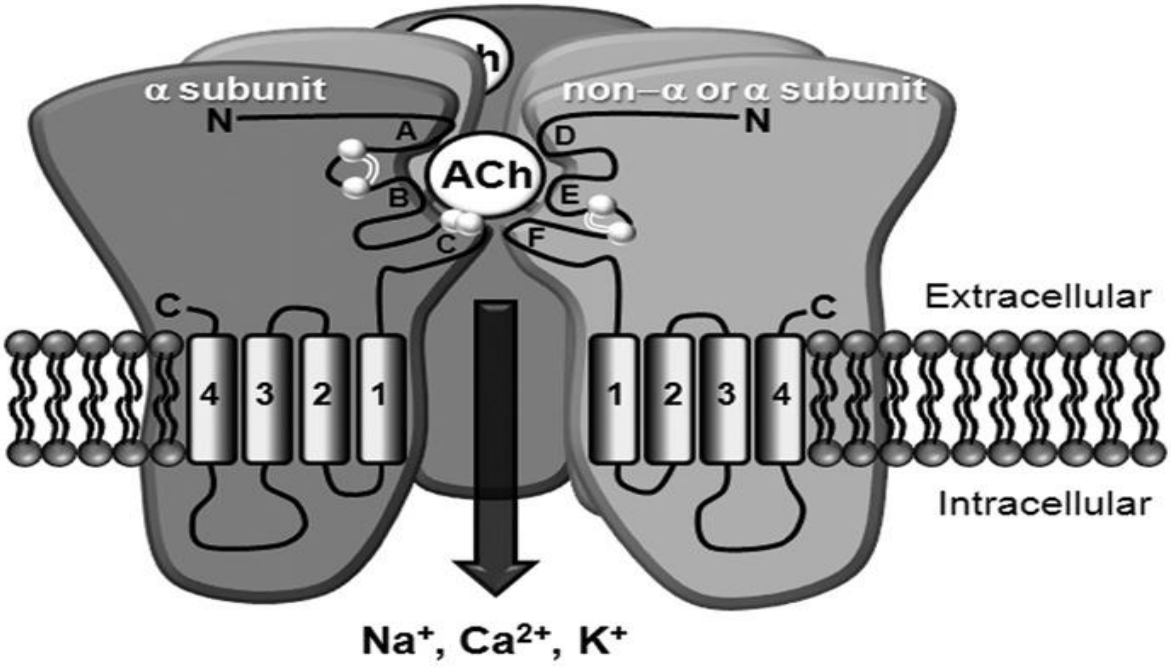

Figure 2. Structure of the nicotinic acetylcholine receptor. Schematic representation of a heteromeric receptor consisting of two α (dark grey) and three non‑α subunits (light grey). The polypeptide layout of two subunits are shown highlighting the Cys‑loop (two white circles connected by a white double line), the two vicinal cysteines in loop C defining α subunits and four transmembrane domains (TM1- 4) with a large intracellular loop between TM3 and TM4. The six binding loops (A-F) that contribute to ligand binding are shown and two acetylcholine (ACh) molecules are bound to this particular nAChR (Jones & Sattelle 2007).

High levels of resistance to spinosad were reported in field populations of several insects, including the moths *S. exigua* (> 345 fold; Wang *et al.* 2006), *P. xylostella* (> 20,600 fold; Sayyed *et al.* 2004, Zhao *et al*. 2002), *H. virescens* (> 669 fold; Wyss *et al.* 2003) and *M. domestica* (> 150 fold; Shono & Scott 2003). Resistance to spinosad in the *L. decemlineata*, *M. domestica* and *H. virescens* was a result of increased metabolic activity (Mota-Sanchez *et al.*2006, Shono & Scott 2003, Young e*t al.*2003). Whereas, in the fly *D. melanogaster* resistance was caused by a mutation that causes loss of function in the alpha subunit gene (Dα6) of the receptor AChR (Perry *et al.*2007). Alternatively, resistance in the *P. xylostella* was due to a mis-splicing of the α6 subunit of the gene that produced a truncated protein (Baxter *et al.* 2010) and by point mutations

that generated premature stop codons (Rinkevich *et al.* 2010). In the thrips, *F. occidentalis* (Pergande) and *Thrips palmi* (Karny) resistance was associated with a single nucleotide change in Dα6 subunit of the receptor gene, resulting in the substitution of a glycine (G) in susceptible insects and glutamic acid (E) in resistant insects (Puinean *et al*. 2013, Bao *et al.* 2014).

Justifications

The characterization of resistance mechanisms occurs only after the implementation of measures for insect control. Therefore, it is extremely important to design tools to be able to detect early survivors and monitor the frequency of genes in insect populations under selection pressure. The resistance of *T. absoluta* to pyrethroids is well characterized in other populations in the world (Haddi *et al.* 2013). However, there are no reports of the mechanisms that control resistance to this insecticide in Brazilian populations. Field populations of *T. absoluta* in Brazil showed up to 93.3 fold resistance to spinosad and cross-resistance to other spinosyns (spinetoram) (Campos *et al.* 2014). After selection pressures on populations due to insecticide application, a resistant strain showed high levels of resistance (180,000 times). Metabolic activity of esterase and cytochrome P450-dependent monooxygenases decreased through population generations, however, suggesting that these enzymes are not involved in resistance to spinosad (Campos *et al.* 2014). The authors also showed that resistance was recessively inherited and monofactorial, characteristics usually associated with high levels of resistance and target site alterations. Therefore, the most plausible hypothesis is that the resistance is related with the alteration of the nACh receptor. Thus, this study aims to characterize and monitor resistance to pyrethroid and spinosad in *T. absoluta* populations from Brazil by conventional bioassays, biochemical, and molecular methods. This study should therefore generate useful information that can be applied in the management decision-making for controlling this invasive pest.

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CHAPTER 2

STATUS OF PYRETHROID RESISTANCE AND MECHANISMS IN BRAZILIAN POPULATIONS OF *Tuta absoluta* (MEYRICK) (LEPIDOPTERA: GELECHIIDAE)

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ABSTRACT – This study surveyed the resistance of *T. absoluta* populations from four regions in Brazil to pyrethroid insecticides, the frequencies of L1014F, T929I and M918T sodium channel mutations, and the role of detoxification metabolism in the resistance. Resistance ratios varied from 1- to 11-times among populations and insecticides, but control failure likelihood assays showed that all pyrethroids assessed exhibited no efficacy at all (and thus, 98-100% control failure likelihood) against all *T. absoluta* populations. The activity of glutathione S-transferase and cytochrome P450-mediated N-demethylation in biochemical assays was significantly correlated with the level of resistance to deltamethrin and permethrin suggesting that these enzymes may play a role in resistance. TaqMan assays were used to screen for the presence of knockdown resistance (*kdr)* mutations and revealed that the L1014F *kdr* mutation was fixed in all populations and associated with two super-kdr mutations, M918T and particularly T929I, at high frequency. Altogether, results suggest that control failures are because of mutations in the domain II of the sodium channel, as a prevailing mechanism of resistance to pyrethroids in populations of *T. absoluta* in Brazil. But, enhanced cytochrome P450-dependent monooxygenases and GST activities also play an important role in the resistance of some populations, which reinforce that pyrethroids must not be used overall to control *T. absoluta.*

KEY WORDS: Insecticide resistance, sodium channel, mutations, metabolism

RESUMO – Este estudo teve como objetivo caracterizar a resistência de populações *T. absoluta* de quatro regiões do Brasil a inseticidas piretroides, frequências das mutações L1014F, T929I e M918T no canal de sódio, e o papel do metabolismo desintoxificativo na resistência. Os índices de resistência aos inseticidas variou de 1 a 11 vezes entre as populações, mas ensaios de falha de controle mostraram que todos os piretroides avaliados exibiram nenhuma eficácia (portanto, 98-100% de falha de controle de probabilidade) em todos as populações *T. absoluta*. Em ensaios bioquímicos atividade da glutationa-S-transferase e citocromo P450 mediado por N-desmetilação foi correlacionada de forma significativa com o nível de resistência a deltametrina e permetrina sugerindo que estas enzimas podem desempenhar um papel na resistência. Ensaios TaqMan foram usadas para rastrear a presença de resistência knockdown (KDR), a mutação L1014F kdr foi fixada em todas as populações e foi associada com mutações duas super-KDR M918T e particularmente T929I, em alta frequência. No seu conjunto, os resultados sugerem que as deficiências de controlo são causa de mutações no domínio II do canal de sódio, como um mecanismo predominante de resistência aos piretroides em populações de *T. absoluta* no Brasil. Mas, o reforço do citocromo P450 monooxigenases-dependente e atividades de GST também desempenham um papel importante na resistência de algumas populações, o que reforça que os piretroides não deve ser utilizado em geral para controlar *T. absoluta*.

PALAVRAS CHAVE: Resistência a insecticidas, canais de sódio, mutações, metabolismo

Introduction

The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is one of the most economically important insect pests of tomato worldwide (Guedes & Picanço 2012, Desneux *et al.* 2010). The larval stages of *T. absoluta* feed on tomato leaves but also damage the flowers, fruits and stems (Picanço *et al.* 1997, Desneux *et al.* 2010), resulting in yield losses between 40-100% (Giustolin *et al*. 2001, Oliveira *et al*. 2009). This species is native to South America with its presence confirmed in southern Brazil, in the early 1980s (Morais & Normanha Filho 1982). Since then it has spread to all major tomato-producing regions in Brazil often seriously compromising productivity due to its destructive capacity (Coelho & França 1987). This pest has also become a major concern for tomato cultivation in Europe, Africa and the Middle East (Urbaneja *et al.* 2007, Desneux *et al.* 2011). The control of *T. absoluta* in most settings has relied heavily on the use of chemical insecticides; unfortunately, this has resulted in the development of resistance populations now described with resistance to a wide range of compounds (Lietti *et al*. 2005, Roditakis *et al*. 2013). Although, only recently, characterisation of resistance mechanisms have had attention, which may be used as tools to survey populations (Haddi *et al.* 2012, Reyes *et al.* 2012)

Pyrethroids are an important class of synthetic insecticide widely used to control many arthropod pests, including *T. absoluta*, as a result of their rapid action, high insecticidal activity and low mammalian toxicity (Rinkevich *et al.* 2013). Pyrethroids interact with the voltage-gated sodium channel and modify its kinetic function, leading to nervous system exhaustion and death (Narahashi 1996, Goldin 2003, Soderlund & Knipple 2003). One of the main mechanisms of pyrethroid resistance is reduction of neuronal sensitivity, known as knockdown resistance (*kdr*) (Soderlund & Knipple 2003, Dong 2007, Soderlund 2012). This type of resistance was first documented in the housefly (*Musca domestica* - Linnaeus) and was subsequently shown to be

caused by two amino acid substitutions in domain II of the channel, a leucine to phenylalanine (L1014F) replacement in transmembrane segment IIS6, termed '*kdr*' and a threonine to methionine substitution upstream of segment IIS5, with the latter associated with an enhanced form of resistance termed super-*kdr* (Williamson *et al.* 1996). Subsequently, additional mutations associated with pyrethroid resistance, primarily in domains II or III of the channel, have been identified in a range of arthropod species (Davies *et al*. 2007).

Pyrethroid resistance in *T. absoluta* has recently been reported to be associated with the presence of the mutations L1014F, M918T and T929I in the sodium channel (Haddi *et al*. 2012). These authors found all three mutations at high frequency in field strains collected from Europe and South America, although no field populations from Brazil have been screened to date for the presence of those mutations. Furthermore, the role of metabolic detoxification in pyrethroid resistance in this species has not yet been investigated. In other insects metabolic resistance to pyrethroids has been associated with elevated levels of cytochrome P450 monooxygenases (P450s), carboxylesterases (CEs), and glutathione *S*-transferases (GSTs) (Ranson *et al.* 2002, Li *et al.* 2007, Low *et al.* 2007).

Metabolism has been in particular assessed only in Chilean populations (Reyes *et. al* 2012) and it is well known that such mechanism can confer broad resistance to insecticides, which impacts more the agriculture of developing countries. Full characterisation of Brazilian populations of *T. absoluta* regarding to resistance has long been a necessity for improving the chemical management of this pest. Here, we provide a survey of resistance of *T. absoluta* populations from different geographical regions of Brazil to three representative pyrethroids using biochemical and molecular approaches as well as toxicological measures.

Material and Methods

Insecticides. Concentration-response curves were estimated for each population of *T. absoluta* through bioassays of larvae mortality using the following insecticides: Deltamethrin (Decis 25 CE, Bayer CorpScience S.A, recommended label rate, 7.5 mg a.i/L of water), alpha-cypermethrin (Fastac 100 SC, BASF S.A., recommended label rate, 10 a.i/L of water) and permethrin (Valon 384 CE, Dow Agrosciences Industrial Ltda, recommended label rate, 49 mg a.i/L of water). The efficacy of each insecticide to control *T. absoluta* using the recommend label rate was also assessed.

Insects. Eight different populations of tomato leaf miner from commercial tomato crops in the Northeast, Midwest, Southeast and South of Brazil were collected in the period between 2010 and 2011 (Table 1). Individual larvae were obtained from various parts of plants, including stems, leaves and fruits. The populations were established and reared individually on leaves of tomato variety "Santa Clara" under ambient conditions as described in Campos *et al.* (2014).

Bioassays. A toxicological bioassay was conducted using a completely randomized design with two replications, and the whole bioassay was repeated twice. For control failure likelihood (Gotijo *et al.* 2013), bioassays were conducted with the label rates stated for each insecticide: Deltamethrin (7.5 mg a.i/L of water), alpha-cypermethrin (10 mg a.i/L of water), and permethrin (49 mg a.i/L of water). Full dose-response bioassays were carried out using 7-8 concentrations of each insecticide that resulted in mortality of between 0 and 100%. Distilled water plus Triton X-100 at 0.01 was used as the control treatment. Leaflets of tomato cultivar "Santa Clara" were cleaned using a solution based on sodium hypochlorite 5%. After cleaning the leaflets in tap water, they were immersed horizontally for a minute in insecticide or control solution. The leaflets were kept on paper towels at room temperature until completely dry and then transferred to Petri dishes (80 x 15 mm) containing filter paper misted with distilled water. Each replicate

comprised 10 second instar (L2) larvae of *T. absoluta* placed on a treated leaflet in a petri-dish. Petri dishes were sealed and maintained in a climate chamber (BOD) set at an average temperature of 25 ± 1°C, 65 ± 5 % relative humidity and photoperiod of 12 h. Mortality was evaluated after 48 hours with the aid of a light source and magnifying glass (Olympus SZ61, Olympus ®, Center Valley, PA, USA). The larvae were considered dead if they could not move at least the extent of their length after touching (Tabashnik *et al.* 1990). Mortality values were corrected for control mortality, using Abbott's formula (Abbott 1925).

Sample extractions for Enzyme Assays. For enzyme assays, 10 L3 larvae of each population were transferred to a microfuge tube with three replicates for each assay. For esterase and glutathione *S*–transferase assays, each sample was homogenized in 200 µl of sodium phosphate buffer (0.02 M, pH 7.2) or sodium phosphate buffer (0.1 M, pH 7.5), respectively using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 15,000 *g* and 4°C for 15 min and supernatants harvested and stored at –20°C. For cytochrome P450 assays, samples were homogenized in 500 µL sodium phosphate buffer (0.1M, pH 7.5) + glycerol at 20% and microsomes were prepared in the same buffer. Homogenates were centrifuged at 15,000 *g* and 4°C for 15 min and the supernatant was ultra-centrifuged at 100,000 g for 60 min in an Optima^{TM} L-80 XP ultracentrifuge (Beckman Coulter, Palo Alto, CA) with the resulting microsomal pellet resuspended in homogenization buffer containing 20% glycerol. Quantitation of protein was determined by the bicinchoninic acid method using bovine serum albumin (BSA) as standard (Smith *et al.* 1985).

Esterase Assays. Esterase activity was measured with a method adapted from Van Asperen (1962). Stock solutions (250mM) of α-naphthyl acetate and β-naphthyl acetate substrates were prepared in acetone. For each reaction, 2 µL α-naphthyl acetate at 25mM, 10 µL of sample

diluted to 1:100 and 188 µL of sodium phosphate buffer (0.02M, pH 7.2) were used. The same procedure was carried out for esterase analysis using β-naphthyl acetate as substrate, however the samples were diluted to 1:10. Reactions were incubated at 30ºC for 15 min then stopped using 33.2 µL of 0.3% FAST Blue B. Absorbance was read at 595 ηm on a microtiter plate reader (ELx800, BioTek®, Winooski, VT, USA). Each sample was analysed in triplicate. A standard curve was prepared with α-naphtol and β-naphtol. Esterase activity was expressed as ηMol naftol x min^{-1} x mg of $protein^{-1}$.

Glutathione S-transferase Assays. Conjugation activity of reduced glutathione was determine using CDNB (1-chloro-2,4-dinitrobenzene) as substrate in the presence of glutathione *S*–transferase forming 2,4-dinitrophenyl-*S*-glutathione (Habig *et. al.* 1974). CDNB solution (150 mM) was prepared in ethanol and reduced glutathione (10 mM) was dissolved in sodium phosphate buffer (0.1M, pH 7.5). For each reaction, 138 µL of sodium phosphate buffer (0.1 M, pH 7.5), 10 µL of sample containing 1 µg of protein, and 150 µL of reduced glutathione (10 mM) were mixed and incubated in a water bath at 30ºC for 5 min then 2 µL of CDNB (150 mM) added to the reaction. The formation of 2,4-dinitrophenyl-*S*-glutathione was immediately measured at 340 ηm using a biophotometer (Eppendorf, Hamburg, Germany) with the reaction analysed for 5 min using read intervals of 30 sec. Each sample was analysed in triplicate. Absorbance data were analysed as a function of reaction time after addition of CDNB. The slope of the line (absorbance/min) was transformed using the extinction coefficient of CDNB (9.6 $mM^{-1}.cm^{-1}$).

Cytochrome P450 Monooxygenase (*O-demethylase*) Assays. Cytochrome P_{450} activity was determined by assessing the *O*-demethylation of the substrate *p*-nitroanisole ($O_2N–C_6H_4–O–CH_3$) to nitrophenol. Reactions were carried out by mixing 178.8 µL of sodium phosphate resuspension buffer (0.1M, pH 7.5), 56.2 µL of sample, 2.5 µL *p*-nitroanisole (150 mM in ethanol) and 12.5 µL of reduced NADPH (9.6 mM) to each well of a microtiter plate in order. The mix was incubated

for 15 min at 37°C with HCl (1M) added to stop the reaction. The reaction mix was then centrifuged at 14,000g for 10 min, and 200 µL of the supernatant read at 405 ηm on a microtiter plate reader. Each sample was analysed in triplicate. Activity of cytochrome P_{450} per sample was determined based on a standard curve of *p*-nitrophenol and expressed as ηMol *p*-nitrophenol x min^{-1} x mg of $protein^{-1}$.

Cytochrome P450 Monooxygenase (*N-demethylation*) Assays. Assays were performed according to Scharf *et al*. (2000).The substrate 4-chloro-N-methylaniline was used to determine N-demethylation activity. Reactions comprised 50 µL of sodium phosphate buffer with 2% Tween-20 (0.1 M, pH 7.5), 25 µL of sample, 25 µL of 4-chloro-*N*-methylaniline N-(4-CNMA) 7.5 mM diluted in 20% v/v ethanol, and 25 µL of reduced NADPH (9.6 mM). The reaction was processed for 16 min at 37°C then stopped by the addition of 187.5 µL *p*-dimethylaminobenzaldehyde to 233.33 mM diluted in 3.0 N sulphuric acid. Samples were then centrifuged for 15 min at 10,000 g at 4°C and 200 µL of the supernatant read at 450 ηm on a microtiter plate reader. Activity of cytochrome P450-dependent monooxygenases per sample was determined based on a standard curve of 4-chloroaniline and expressed as ηMol 4-chloroaniline x min^{-1} x mg $protein^{-1}$. Assays were replicated three times using three different protein preparations.

TaqMan Diagnostic Assays. Three TaqMan assays developed previously were used to genotype samples for the *kdr*/super-*kdr* mutations L1014F, M918T and T929I (Haddi *et al*. 2012). DNA was extracted from individual insects by grinding larvae in a microfuge tube using a micropestle and liquid nitrogen followed by extraction using DNAzol reagent (Life Technologies) following the manufacturer's protocol but reducing reagent volumes by 1/5th. PCR reactions (10 µl) contained 2 µL of genomic DNA, 7.5 µL of SensiMix DNA kit (Quantace, London, UK), 800 nM of each primer and 200 nM of each probe. Samples were run on a Rotor-Gene 6000 (Corbett Research, UK) using temperature cycling conditions of: 10 min at 95°C followed by 40 cycles of

9 °C for 10 s and 60 °C for 45 sec. The increase in fluorescence of the two probes was monitored in real time by acquiring each cycle on the yellow (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 nm emission) of the Rotor-Gene respectively. Reference template controls (a wild-type homozygous, a mutant homozygous and a heterozygous sample) were included in each run to aid genotype scoring.

Data Analysis. Bioassay data were corrected for mortality observed in the control Abbott (1925) and subjected to Probit analysis (Finney 1971) using the POLO-Plus program (LeOra-Software 2005) using the POLO-Plus program to estimate concentration-response curves for each population and insecticide. Confidence intervals at 95% probability were calculated for both LC_{50} and LC_{80} values. The resistance ratios (RR) were calculated using the LC_{50} value of the most susceptible population to each insecticide as the reference with the 95% confidence limits for each RR calculated using the method of Robertson & Preisler (1992). Differences in mortality of *T. absoluta* populations to the recommended label rate (control failure likelihood) for each insecticide were estimated according to Gontijo *et al.* (2013) by comparing (using Student's paired t-test at $P<0.05$) the corrected observed mortality with the minimum expected efficacy (80% mortality) required for insecticide registration by the Brazilian Ministry of Agriculture, Livestock and Supplement. Also, the frequency of populations in control failure was estimated as in Gontijo *et al*. (2013). The mean values of esterase, glutathione *S*-transferase and cytochrome P450-dependent monooxygenase activity were subjected to analysis of variance (PROC ANOVA) and Tukey's test (HSD) $P < 0.05$ to identify significant differences using the SAS program (SAS Institute 2001). *Pearson* correlation analysis between reduced susceptibility to insecticides and enzymatic activity of third-instar larvae as well as with the genotype frequencies of both *sKdR* mutations were estimated using (SAS Institute 2001).

Results

Bioassays. All of the pyrethroids assessed through the recommended field rate exhibited no efficacy at all (and thus, 98-100% control failure likelihood) against all of *T. absoluta* populations (Table 2). In full dose response bioassays the Tianguá-CE population had the lowest LC_{50} value for deltamethrin, while the Iraquara-BA population had the lowest LC_{50} values for the insecticides alpha-cypermethrin and permethrin (Table 3). Anápolis-GO was the most resistant population to deltamethrin (LC_{50} 561 mg/L), whereas Venda Nova-ES (LC_{50} 2595 mg/L) and Pelotas-RS (LC_{50} 1417 mg/L) were the most resistant populations to the insecticides alpha-cypermethrin and permethrin respectively (Table 3). The resistance ratios to deltamethrin, alpha- cypermethrin and permethrin ranged from 1.2 to 5.1; from 3 to 11 and 1.3 to 5.3 times respectively (Table 3) using the most susceptible population as a reference. The LC_{80} values for deltamethrin ranged from 273 (Tianguá - CE) to 1078 mg AI/l (Anápolis - GO), for alpha-cypermethrin 733 (Iraquara -BA) to 6756 mg AI/l (Venda Nova-ES), and for permethrin from 755 (Iraquara-BA) to 3335 mg AI/l (Pelotas-RS) (Table 3).

Enzyme Assays. Biochemical assays of esterase activity differed significantly among populations of *T. absoluta* using the substrate α-naphthyl acetate but not when using β-naphthyl acetate. The α-esterase activity ranged from 1.35 ± 0.09 mmol/min/mg (Anápolis-GO) to 2.09 ± 0.31 mmol/min/mg (Venda Nova-ES), while the β-esterase activity varied from 1.02 ± 0.06 µmol/min/mg (Anápolis-GO) to 1.30 ± 0.06 mmol/min/mg (Tianguá-CE). Assays of glutathione *S*-transferase (GST) activity showed significant differences between *T. absoluta* populations with variation of up to 3.12-fold observed among populations (Table 4). The Pelotas-RS population had the greatest GST activity (77.7 ± 1.97 µmol/min/mg) while the Tianguá -CE population had the lowest activity (24.83 ± 1.31 µmol/min/mg). The activity of cytochrome $P450_s$ using the

model substrate 4-chloro-N-methylaniline differed significantly between the populations tested with variation of up to 5.7-fold with N-demethylation activity ranging from 1.03 ± 0.13 ηmol/min/mg for the Paulínia-SP population to 5.86 ± 0.17 ηmol/min/mg for Anápolis-GO (Table 4). *O*-demethylation activity varied by up to 3.5-fold among the *T. absoluta* populations with activity of 4.23 ± 0.72 ηmol/min/mg for the Paulinia-SP population to 14.75 ± 0.77 ηmol/min/mg for Anápolis-GO (Table 4).

TaqMan Diagnostic Assays. TaqMan assays revealed that the L1014F, M918Tand T929I *kdr* mutations were at high overall frequency in the eight *T. absoluta* populations (Table 5). The frequency of the L1014F mutation was fixed at 100% in all eight populations (Table 5). The overall frequency of T929I was higher (0.65) than M918T (0.37) and this was also the case for each individual population except for Pelotas-RS where M918T was at higher frequency (0.80) than T929I (0.25) (Table 5). All individuals tested carried either M918T or T929I in combination with L1014F apart from a single individual of the Iraquara-BA population which only had the L1014F mutation. As reported previously (Haddi *et al.* 2012) the M918T and T929I mutations were only found in combination in individual larvae when both mutations were in the heterozygous form. Overall the most common genotype observed was F1014 (homozygous) + M918 (homozygous) + I929 (homozygous) followed by the genotype F1014 (homozygous) + M918T (heterozygous) + T929I (heterozygous).

Correlations. To investigate whether there is a relationship between the level of resistance to pyrethroids, and the enzymatic activities of different *T. absoluta* populations, enzyme activity was correlated with the LC_{50s} obtained in bioassays (Table 6). Esterase biochemical assays were negatively correlated with resistance using the substrate α-naphthyl acetate for deltamethrin (r = - 0.45) and permethrin (r = - 0.61). Activity for the substrate β-naphthyl acetate had a significant negative correlation with LC_{50} values for deltamethrin (r = - 0.33), for permethrin the correlation

was not significant (r = - 0.02) and for alpha-cypermethrin there was a low, albeit, significant positive correlation (r = 0.23). A significant positive correlation was observed between the LC_{50s} of deltamethrin and permethrin and the enzymatic activity of GSTs (*r* =0.50); but there was no correlation between GST_s activity and the insecticide alpha-cypermethrin. P450 enzyme activity using the substrate 4-chloro-N-methylaniline correlated significantly with the LC_{50s} of deltamethrin (r = 0.56) and permethrin (r = 0.36) but not with alpha-cypermethrin whereas P450 activity using 4-nitroanisole as substrate showed a modest significant correlation with the LC_{50s} to alpha-cypermethrin. The LC_{50s} of deltamethrin and permethrin were significantly correlated (r = 0.50), however, the insecticide alpha-cypermethrin showed only a modest negative correlation with deltamethrin (*r* = -0.28) (Table 6). Genotype frequencies correlated only between T929I and alpha-cypermethrin (r = 0.778, P= 0.023, N=8) variables. Also, negative significant correlation was observed between T929I and M918T (r = -0.73, P= 0.040, N=8) variables. No correlation was observed for the other pairwise variables, either using genotype or allelic frequencies.

Discussion

Pyrethroids were first registered for control of *T. absoluta* in Brazil in 1980 (Morais & Normanha) and resistance to the pyrethroid permethrin was first reported in Brazilian populations in 2000 (Siqueira *et al.* 2000). Since then resistance to bifenthrin and deltamethrin in populations in Brazil has also been described (Silva *et al.* 2011). To date, recommended label rate of permethrin, deltamethrin and alpha-cypermethrin exhibit total control failure of *T. absoluta* populations collected from eight different regions of Brazil, scenario previously showed in Brazil to pyrethroids (Gontijo *et al.* 2013, Silva *et al.* 2011). Resistance ratios calculated from full dose response bioassays varied only from ~1- to 11-times (compared with the most susceptible population), underestimated because of lacking of a fully pyrethroid susceptible strain for use as a

reference. A similar problem has recently been described by others and likely results from widespread resistance to this chemical class worldwide (Siqueira *et al.* 2000, Haddi *et al.* 2012, Roditakis *et al.* 2013). The development of insecticide resistance in Brazilian populations of *T. absoluta* is perhaps unsurprising given the high selection pressure from insecticide use in agricultural regions where tomato growers may apply insecticides up to three times a week during the growing season (Guedes *et al.* 2012). For many years, permethrin and deltamethrin along with the nereistoxin-derived cartap were the few insecticides registered for use against *T. absoluta* leading to widespread use (Souza *et al.* 1986). Although pyrethroids are no longer widely used to control *T. absoluta* in Brazil, small and unassisted growers still make use of them because of their cheaper prices. Such practice, not only provides inefficacy, but also worsens the resistance scenario to pyrethroids against other pests and other insecticides against *T. absoluta.*

Three approaches were used in this survey to determine the status of *T. absoluta* resistance to pyrethroids in Brazil: toxicological assessment, biochemical and molecular tools. Biochemical investigation of the major enzymes, most frequently implicated in metabolic resistance (P450s, GSTs and CEs), suggested P450s and GSTs may play a role in resistance to certain pyrethroids, but there was less evidence that CEs contribute to resistance in these strains. The N-demethylation activity of microsomal preparations of the different *T. absoluta* populations varied by 5.7-fold and significantly correlated with the level of resistance to deltamethrin and permethrin suggesting enhanced P450 monooxygenases activity may contribute to resistance to these insecticides. In contrast O-demethylation activity using the model substrate 4-nitroanisole showed no significant correlation with resistance to any of the pyrethroids. A plausible explanation for the latter result is related to the structure of the pyrethroids in question, which do not have methoxy or alkoxy groups. In other insect pests P450s dependent monooxygenases have been shown to metabolize deltamethrin and permethrin to less toxic secondary metabolites such as 4-hydroxy-deltamethrin

and 4-hydroxy-permethrin (Martin *et al.* 2003, Yang *et al.* 2004, Zimmer *et al.* 2011, Stevernson *et al.* 2011). Further analysis of the metabolic fate of these insecticides in *T. absoluta* is required to confirm if a similar route of P450-mediated detoxification occurs in resistant strains of this species.

Variation among the *T. absoluta* populations in this study was also observed for GST activity with enzyme activity significantly correlating with the level of resistance (LC_{50} values) to permethrin and deltamethrin in dose-response bioassays. These finding suggest a role for this enzyme system in resistance to these two pyrethroids. In other insect species pyrethroids have not been shown to be directly metabolised by GSTs, rather studies have suggested they may sequester pyrethroids until they are metabolised by other detoxification enzymes or protect against lipid peroxidation products and oxidative stress induced by pyrethroid exposure (Lin *et al*. 2013, Zhu *et al.* 2010). Further work is required to investigate these two possibilities in *T. absoluta.*

In other insects enhanced expression of esterases have been shown to confer modest levels of resistance to pyrethroid insecticides (Li *et al.* 2007), however, there was little indication of a role for this enzyme class in the resistance of *T. absoluta* populations from Brazil using two substrates for this enzyme family, with only moderate levels of positive correlation herein seen between LC_{50} values of alpha cypermethrin and activity to the substrate β-naphthyl acetate.

One of the primary mechanisms of pyrethroid resistance in many insect species is *kdr*-type mutation of the voltage-gated sodium channel (Dong 2007). Indeed three such mutations have been previously identified at known resistance 'hot spots' in pyrethroid resistant field populations of *T. absoluta* from 12 countries (Haddi *et al.*2012), although this did not include populations from Brazil (see introduction). In the current study TaqMan diagnostic assays demonstrated that the same three mutations, L1014F, M918T and T929I, are fixed or at high frequency in *T. absoluta* populations in Brazil. The L1014F mutation was fixed in all populations analysed. In

other insects this mutation confers moderate resistance factors of 10 to 30 times to pyrethroids with cross-resistance observed across all pyrethroids (Roditakis *et al*. 2013, Zimmer *et al.*2014, Kostaropoulo *et al.* 2001). The 100% frequency of this mutation in Brazilian populations is consistent with results observed in populations of *T. absoluta* from Europe and other South American countries where the L1014F mutation has also undergone fixation (Haddi *et al.*2012). Indeed, to date, only populations from Colombia have been observed that do not carry L1014F at 100% frequency (Haddi *et al.* 2012).

Almost all individuals tested in the current study carried L1014F in combination with either M918T or T929I, and, as reported previously (Haddi *et al.* 2012), the M918T and T929I mutations were only found in combination in individual larvae when both mutations were in the heterozygous form. Both these mutations, when found in combination with L1014F, are known to give high levels of resistance to a range of different pyrethroids (Soderlund & Knipple 2003). The overall frequency of T929I (0.65) was higher than M918T (0.37), consistent with the previous study (Haddi *et al.* 2012), and suggesting that the T929I mutation may provide a slight selective advantage over M918T. This appears to be the case in *T. absoluta*, because high positive correlation was observed between alpha cypermethrin and the frequency of the T929I mutation. No significant correlation was observed between the genotype or allelic frequency of the M918T mutation and variability in the resistance of the eight populations to permethrin and deltamethrin. Indeed, when mutation frequency and resistance phenotype is compared it is apparent that certain populations with similar mutation frequencies (i.e. Paulínia-SP and Sumaré-SP) display different levels of resistance to certain pyrethroids providing further support to the hypothesis that additional metabolic mechanisms contribute to resistance as discussed above.

In summary the current study shows that target site resistance is widespread in *T. absoluta* populations in Brazil and may be further enhanced by metabolic detoxification. The *kdr* mutation

is uniformly fixed and the two *skdr* mutations are at relatively high frequency across Brazilian *T. absoluta* populations as they were in 12 other countries (Haddi *et al.* 2012).The presence of these mechanisms confers strong resistance to three representative pyrethroids rendering the use of these insecticides ineffective for control of *T. absoluta* in Brazil. Continued use of pyrethroids by small growers may impose a scenario of resistance for other pests, and thus they must not be used overall to control *T. absoluta.*

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Table 1. Sites of *Tuta absoluta* populations collected in Brazil.

Populations/Locale/State	Geographic Position	Collection date	History[1]
Anápolis – GO	16° 29' 46" S, 49° 25' 35" W	Dec/2011	Pyr, IGR, OPs, Av
Guaraciaba do Norte – CE	4° 10' 01" S, 40° 44' 51" W	Feb/2010	Pyr, OPs, Cartap
Iraquara – BA	12° 14' 55" S, 41° 37' 10" W	Nov/2011	Pyr, IGR, OPs, Cartap, Sp
Paulínia – SP	22° 45' 40" S, 47° 09' 15" W	Aug/2010	Pyr, IGR, OPs
Pelotas – RS	31° 46' 19" S, 52° 20' 33" W	Nov/2011	Pyr, IGR, OP, Cartap
Sumaré – SP	22° 49' 19" S, 47° 16' 01" W	Sept/2011	Pyr, IGR, OPs,
Tianguá – CE	3° 43' 56" S, 40° 59' 30" W	Feb/2010	Pyr, IGR, OP, Cartap
Venda Nova – ES	20° 20' 23" S, 41° 08' 05" W	Aug/2011	Pyr, IGR, OP, Cartap, Bt

[1]Pyr – pyrethroids, IGR – insect growth regulator, OPs – organophosphates, Bt – *Bacillus thuringiensis*. Av – Avermectins, Sp – Spinosyns.

Table 2. Corrected Mortality (%) (± SE) of *Tuta absoluta* populations exposed to label rate of pyrethroids.

Region	Population	Deltamethrin (7.5 mg a.i/L*)	α-Cypermethrin (10 mg a.i/L*))	Permethrin (49 mg a.i/L[1])
	Guaraciaba- CE	0.0 ± 0.0*	0.5 ± 0.5*	1.0 ± 0.6*
Northeast	Iraquara - BA	0.0 ± 0.0*	1.0 ± 0.6*	0.5 ± 0.5*
	Tianguá - CE	1.5 ± 0.8*	0.0 ± 0.0*	0.0 ± 0.0*
	Paulínia - SP	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Southeast	Sumaré - SP	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
	Venda Nova-ES	1.0 ± 0.6*	0.0 ± 0.0*	0.0 ± 0.0*
Central-West	Anápolis - GO	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
South	Pelotas - RS	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*

[1]Mortality significantly lower than 80% since label rate dose does not lay within the confidence interval of the LC_{80} for a given insecticide and population.

Table 3. Relative toxicity of pyrethroids to L2 larvae of *Tuta absoluta*.

Insecticides	Population	n[1]	DF[2]	Slope ± SE[3]	LC_{50} (CI95%) mg a.i/L*)	(LC_{80} (95%/CI) (mg a.i/L*)	χ^2	RR_{50} (95%CI)[4]
α-Cypermethrin	Iraquara – BA	277	6	1.65 ± 0.22	234 (154 - 320)	733 (507 – 1183)	4.77	
	Guaraciaba – CE	308	6	1.96 ± 0.24	298 (224 - 378)	801 (626 – 1101).	2.66	1.2 (0.8 – 1.9)
	Paulínia – SP	277	5	2.37 ± 0.35	382 (236 - 520)	864 (638 – 1324)	5.30	1.6 (1.0 – 2.5)
	Pelotas – RS	292	6	1.72 ± 0.18	409 (552 - 1735)	1257 (895 – 1994)	6.28	1.7 (1.1 – 2.7)
	Anápolis – GO	272	5	3.20 ± 0.49	588 (495 – 699)	1093 (903–1402)	3.34	2.5 (1.7 – 3.7)
	Tianguá – CE	261	5	1.96 ± 0.34	656 (397 - 858)	1732 (1203- 2946)	6.60	2.8 (1.8 – 4.3)
	Sumaré – SP	271	5	2.39 ± 0.24	1493 (1224 - 1823)	3157 (2534- 4178)	1.60	6.1 (4.0 – 9.2)
	Venda Nova – ES	266	5	2.03 ± 0.21	2595 (1774 - 3873)	6756 (4440-13200)	8.30	10.8 (7.1 – 16.2)
Deltamethrin	Tianguá – CE	292	5	2.12 ± 0.21	110 (88 - 135)	273 (217- 366)	2.90	
	Venda Nova – ES	250	5	1.14 ± 0.15	130 (68 - 213)	705 (400 - 1948)	6.08	1.2 (0.5 - 2.6)
	Iraquara – BA	248	5	1.41 ± 0.17	136 (82 - 212)	552 (350 - 1144)	3.04	1.3 (0.6 – 3.0.)
	Paulínia – SP	278	5	1.75 ± 0.18	181 (143 - 229)	547 (414 -790)	1.01	1.6 (0.8 - 3.4)
	Sumaré – SP	267	5	2.03 ± 0.26	196 (135 -281)	517 (351- 943)	6.78	2.1 (1.0 - 4.6)
	Pelotas – RS	258	5	2.00 ± 0.22	269 (195- 374)	612 (432-1019)	6.40	2.4 (1.8 - 3.3)
	Guaraciaba – CE	272	5	2.83 ± 0.34	282 (231 - 341)	560 (455- 737)	4.18	2.5 (1.2 - 5.2)
	Anápolis - GO	279	5	2.96 ± 0.33	561 (415-780)	1078 (776-1893)	8.20	5.1 (2.5 - 10.5)

[1]Total number of insects bioassayed. [2]Degree of Freedom. [3]Standard Error. [4]Resistance ratio: ratio of LC_{50} estimative between resistance and susceptible populations calculated through Robertson and Preisler (1992) method with confidence interval at 95%. Resistance ration significant if confidence interval does not encompass the value 1.0.

Table 3. Relative toxicity of pyrethroids to L2 larvae of *Tuta absoluta* (continued).

Insecticide	Population	n[1]	DF[2]	Slope ± SE[3]	LC_{50} (95%/CI) (mg a.i/L*)	LC_{80} (95% CI) (mg a.i/L*)	χ^2	RR_{50} (95%CI)[4]
Permethrin	Iraquara – BA	281	6	1.87 ± 0.21	269 (205 - 342)	755 (582 - 1052)	5.49	
	Paulínia – SP	261	5	1.90 ± 0.29	338 (215 - 460)	783 (490 -1330)	0.90	1.2 (0.8 – 2.0)
	Tianguá – CE	270	5	1.46 ± 0.20	455 (293 - 627)	1716 (1270 - 2510)	1.65	1.7 (1.0 – 2.6)
	Guaraciaba - CE	280	5	2.05 ± 0.26	508 (293 - 737)	1310 (904 - 2254)	7.54	1.8 (1.3 – 2.7)
	Venda Nova – ES	271	5	2.82 ± 0.32	659 (534 - 796)	1180 (851 - 1803)	1.78	2.4 (1.7 – 3.3)
	Anápolis – GO	244	4	2.06 ± 0.24	801 (633 – 991)	2048 (1611 - 2820)	3.12	3.0 (2.2 - 4.1)
	Sumaré – SP	271	5	2.28 ± 0.24	1074 (869 - 1315)	2509 (1999 - 3358)	0.50	4.0 (2.8 – 5.5)
	Pelotas – RS	275	5	2.27 ± 0.23	1417 (1059 – 1871)	3335 (2470 - 5066)	5.48	5.2 (3.8 – 7.3)

[1]Total number of insects bioassayed. [2]Degree of Freedom. [3]Standard Error. [4]Resistance ratio: ratio of LC_{50} estimative between resistance and susceptible populations calculated through Robertson and Preisler (1992) method with confidence interval at 95%. Resistance ration significant if confidence interval does not encompass the value 1.0.

Table 4. Mean activity of detoxificative enzymes from *Tuta absoluta* populations.

Population	α-esterase mmol/min/mg	β-esterase mmol/min/mg	GST µmoles/min/mg	CypO µmoles/min/mg	CypN µmoles/min/mg
Anápolis - GO	1.35 ± 0.09 cd[1]	1.02 ± 0.06 a	76.56 ± 1.10 a	14.75 ± 0.77 ba	5.86 ± 0.17 a
Guaraciaba - CE	2.07 ± 0.07 ab	1.14 ± 0.02 a	71.29 ± 1.81 b	5.57 ± 0.49 dc	4.43 ± 0.30 bc
Iraquara-BA	2.32 ± 0.10 a	1.07 ± 0.06 a	65.00 ± 1.54 c	18.13 ± 1.31 a	3.15 ± 0.29 d
Paulínia - SP	1.88 ± 0.05 abc	1.26 ± 0.04 a	54.97 ± 0.20 e	4.23 ± 0.72 d	1.03 ± 0.13 e
Pelotas-RS	1.88 ± 0.10 d	1.15 ± 0.10 a	77.67 ± 1.97 b	14.39 ± 1.48 ab	4.99 ± 0.39 ab
Sumaré -SP	1.62 ± 0.10 bcd	1.27 ± 0.08 a	68.61 ± 2.24 d	14.56 ± 2.19 ab	2.28 ± 0.23 d
Tianguá - CE	2.10 ± 0.09 ab	1.30 ± 0.06 a	24.83 ± 1.31 c	10.58 ± 1.50 bc	3.30 ± 0.12 cd
Venda Nova-ES	2.09 ± 0.31 ab	1.27 ± 0.07 a	71.25 ± 2.09 b	14.59 ± 1.50 ab	4.48 ± 0.30 b

[1]Means followed by the same letter are not statistically different by Tukey's test at 5% probability.

Table 5**.** *Kdr* genotyping frequencies (%) (L1014F, M918T, T929I) in *Tuta absoluta* populations from Brazil determined by TaqMan assays.

Population	L1014F				M918T				T929I			
	RR	RS	SS	R-Allele Frequency	RR	RS	SS	R-Allele Frequency	RR	RS	SS	R-Allele Frequency
Anápolis - GO	100	00	00	100	11	33	56	28	60	30	10	75
Guaraciaba - CE	100	00	00	100	10	50	40	35	20	50	30	45
Iraquara - BA	100	00	00	100	30	20	50	40	40	20	40	50
Paulínia - SP	100	00	00	100	10	40	50	30	60	40	0	80
Pelotas - RS	100	00	00	100	70	20	10	80	10	30	60	25
Sumaré - SP	100	00	00	100	00	30	70	15	70	30	00	85
Tianguá - CE	100	00	00	100	10	50	40	35	40	50	10	65
Venda Nova - ES	100	00	00	100	0	10	90	5	90	10	00	95

[1]RR: resistant, RS: heterozygous, SS: susceptible

Table 6. Pearson correlation coefficients between reduced susceptibility to insecticides and enzymatic activity of third-instar larvae in field populations of *Tuta absoluta*.

		Deltamethrin	α-Cypermethrin	Permethrin
Insecticides	Deltamethrin	---		
	α-Cypermethrin	*r*= -0.26*	---	
	Permethrin	*r*= 0.50****	*r*= 0.18 ^{ns}	---
Substrate	α-Naphthyl Acetate	*r*= -0.45****	*r*= 0.07 ^{ns}	*r*= -0.61***
	β-Naphthyl Acetate	*r*= -0.33***	*r*= 0.23*	*r*= -0.02 ^{ns}
	CDNB	*r*= 0.50****	*r*= 0.13 ^{ns}	*r*= 0.47****
	4-Chloro-*N*-Methylaniline	*r*= 0.56**	*r*= 0.05 ^{ns}	*r*= 0.36****
	4-Nitroanisole	*r*= -0.04 ^{ns}	*r*= 0.22 ^{ns}	*r*= 0.26 *

^{ns}Not significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ $^{****}P < 0.0001$.

CHAPTER 3

THE G275E MUTATION IN THE NICOTINIC ACETYLCHOLINE RECEPTOR (nAChR) α6 SUBUNIT OF *Tuta absoluta* (MEYRICK) (LEPIDOPTERA: GELECHIIDAE) CONFERS HIGH LEVELS OF RESISTANCE TO SPINOSYNS[1]

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[2]Silva, W.M., The G275E mutation in the nACh receptor α6 subunit of *Tuta absoluta* confers high levels of resistance to spinosyns. To be submitted to Insect Biochemistry and Molecular Biology.

ABSTRACT– *Tuta absoluta*, is a major pest of tomato crops worldwide. Recently, high levels of resistance to spinosad were observed in populations from Brazil. Further selection led to very high level of resistance that conferred cross-resistance to spinetoram, but not to other nAChr-interfering insecticides. Selection with spinosad also led to reduced metabolism, excluding it from involvement in the resistance. These studies strongly support the hypothesis of target-site alteration in the nAChr of *T. absoluta*. Synergism of spinosad as well as the metabolic activity was assessed to rule them out from resistance. We have cloned the nicotinic acetylcholine receptor (nAChR) α6 subunit from *T. absoluta* (Taα6) and compared the nucleotide sequence of Taα6 from susceptible and spinosad-resistant insect populations. A single nucleotide change has been identified in Taα6, resulting in the replacement of a glycine (G) residue in susceptible insects with a glutamic acid (E) in resistant insects. Altogether, results show that G275E mutation in the exon 9 of the nAChR receptor α6 subunit is the primary mechanism of resistance to spinosyns in populations of *T. absoluta* in Brazil.

KEY WORDS**:** Insecticide resistance, nACh receptor, mutations, metabolism

RESUMO –*Tuta absoluta* é a principal praga das culturas de tomateiro no mundo. Recentemente, altos níveis de resistência a espinosade foram observadas em populações do Brasil. Além disso, seleção com inseticida espinosade conduziram há niveis elevados de resistência que confere resistência cruzada com a espinetoram, mas não para outros insecticidas que interferem no nAChr. Seleção com espinosad também levou a um metabolismo reduzido, excluindo-o envolvimento na resistência. Estes estudos apoiam fortemente a hipótese de alteração do sítio no nAChr de *T absoluta*. O sinergismo de espinosade, bem como a atividade metabólica foi avaliada para o papel delas na resistência. Clonámos o receptor nicotínico de acetilcolina (nAChR) da subunidade α6 de *T. absoluta* (Taα6) e comparamos com a sequência de nucleótidos de Taα6 a partir de populações de insectos suscetíveis e resistentes a espinosad. Uma única alteração de nucleótido foi identificada em Taα6, resultando na substituição de uma glicina (G) nos insetos suscetíveis e ácido glutâmico (E) em insectos resistentes. No seu conjunto, os resultados indicam que a mutação no éxon 9 G275E do nAChR da α6 subunidade do receptor é o mecanismo principal de resistência à espinosinas em populações de *T. absoluta* no Brasil.

PALAVRAS-CHAVE: Resistência a insecticidas, receptores de nACh, mutações, metabolismo

Introduction

The tomato leafminer, *Tuta absoluta* is a global threat to tomato crop because of its high potential of damage, rapid propagation and rising production costs, due to the increasingly frequent use of insecticides (Coelho & Franca 1987, Guedes *et al.* 1994, Desneux *et al.* 2010). This insect has its origin in South America and was introduced in Brazil between 1979 - 1980 and quickly spreaded nationwide, and has caused serious damage to tomato crops since then (França & Castelo Branco 1992, Guedes & Picanço 2012). This pest was introduced in Europe, North Africa and the Middle East becoming a serious problem for tomato crop in several countries since 2006 (Desneux *et al.* 2010).

The main method of *T absolouta* control is through the application of insecticides, however, this method is not always effective due to the development of resistance to insecticides (Picanço *et al.* 1995). The intensive use of pyrethroids, benzoylureas, avermectin, indoxacarb and diamides caused high resistance levels in *T. absoluta* populations of Brazil, Chile, Argentina, Greece and Italy (Guedes *et al.* 1994, Siqueira *et al.* 2000a, Siqueira *et al.* 2000b, Salazar & Araya 2001, Siqueira *et al.* 2001, Lietti *et al.* 2005, Silva *et al.* 2011, Roditakis *et al.* 2015).

The spinosad insecticide was commercially introduced for pest control in 1997 (Sparks *et al.*, 2012). The active ingredient of spinosad is a mixture of two compounds, spinosyn A and spinosyn D, produced by the microorganism *Saccharopolyspora spinosa* (Mertz & Yao) during fermentation (Salgado & Sparks 2005). The spinosad mimic the action of acetylcholine binding to its receptor causing a change in the conformation, which leads to the opening of ion channels to the conduction of nerve stimulation, causing tremors, paralysis and death of the insect (Salgado *et al.* 1998, Thompson *et al.* 2000, Cisneros *et al.* 2002). The nicotinic acetylcholine receptor (nAChr) is a protein of five subunits, each containing four transmembrane (TM1-TM4) domains and extracellular N-terminal domains that include the acetylcholine binding site (Sine & Engel

2006). The acetylcholine binding site is located at the interface of two subunits and possibly comprises six loops (Grutter & Changeux 2001).

Neuronal insensitivity to spinosad was related to molecular alterations in the nACh receptor (Salgado & Sparks 2005). The resistance of *Drosophila melanogaster* (Meigen), was associated to a deletion in the alpha 6 subunit (Dα6) of nACh receptor (Perry *et al.* 2007). In *Plutella xylostella* (Li) and *Bactrocera dorsalis* (Hendel) resistance was linked to mis-spliced transcripts of the nAChr α6 subunit, producing a truncated subunit protein (Baxter *et al.* 2010, Hsu *et al.* 2012). Recently, *Frankliniella occidentalis* (Pergande) and *Thrips palmi* (Karry) resistance was associated to a single nucleotide change in the nACh receptor alpha 6 subunit, resulting in the replacement of a glycine (GGG) residue in susceptible insects with a glutamic acid (GAG) in resistant insects. (Puinean *et al.* 2013, Bao *et al.* 2014).

High levels of resistance to spinosad were observed in field population from Brazil (Campos *et al.* 2015a). Further selection led to very high level of resistance that conferred cross-resistance to spinetoram, but not to other nicotinic acetylcholine receptor (nAChR) interfering insecticides (Campos *et al.* 2015c). Campos *et al.* (2014) reported very high resistance of *T. absoluta* to spinosad after six generations of selection in laboratory, a strain named after as IRA-Sel. The spinosad resistance was autosomal, recessively inherited, monofactorial, and showed high cross-resistance with spinetoram (spinosoid) but not to cartap and neonicotinoid, suggesting it is very specific and related to target-site alteration. Therefore, the underlying mechanisms of resistance to spinosyns in *T. absoluta* still need to be unraveled. Herein, the *T. absoluta* nAChr alpha six subunit (*Taα6*) was PCR amplified, cloned and examined the deduced amino acid sequences of the leafminer strains that showed different levels of resistance to spinosad and developed diagnostic tools that allow sensitive detection of these mutations in individual *T. ab*soluta larvae and adults. Furthermore, the possible involvement of cytochrome P450-dependent

monooxygenases ($P450_s$), glutathione *S*-transferase (GST_s) and esterases in the spinosad resistance strain IRA-Sel was examined using synergists and enzymatic assays.

Material and Methods

Chemicals. The insecticide used in the experiments was spinosad (Tracer 480 g a.i/L concentrated suspension, Dow AgroSciences industrial Ltda, Franco da Rocha, SP, Brazil. synergists dimethyl maleate (DEM - 99%, Sigma, Milwaukee, WI, USA), S,S,S triphenyl phosphate (DEF - 93%, Sigma, Milwaukee, WI, USA) and piperonyl butoxide (PBO - 90%, Sigma, Milwaukee, WI, USA). The reagents and solvents used in enzyme assays were purchased from Sigma-Aldrich (Milwaukee, WI, USA), except for the protein assay kit which was purchased from Pierce Chemical Co. (Rockford, IL, USA).

Insects. The susceptible strain of *T. absoluta* (Pelota - RS, named here in as PLT-Sus) was collected and maintained subsequently in the laboratory without exposure to insecticide. The other population from Iraquara-BA, previously reported as resistant to spinosad (Campos *et al.* 2014a), was divided into two strains: one without exposure (named here as IRA-Unsel) and the other (named here as IRA-Sel) subjected to further selection with spinosad, which is currently maintained under selection with 500 mg a.i./mL under laboratory conditions (Campos *et al.* 2014c). For phenotyping and genotyping, 17 other populations were as well assessed to determine the resistance status.

Bioassays. Toxicological bioassays were conducted with PLT-Sus, IRA-Unsel, and IRA-Sel using a completely randomized design with two replications per treatment, and the whole bioassay was repeated twice. Concentration-response bioassays were carried out using 7-8 concentrations of spinosad that resulted in mortality between 0 and 100%. The concentration-mortality bioassays were performed as described previously (Campos *et al.* 2014b). The spinosad

solutions were diluted in the control solution (to record natural mortality), which comprised water containing 0.01% Triton X-100. Spinosad-treated tomato leaflets were placed in Petri dishes (80 mm diameter) with 10 lavae of 2nd instar larvae of *T. absoluta* and bioassays were maintained under controlled environmental conditions [25 ± 1 ºC, 65 ± 5% R.H and 12:12 (L:D) photoperiod]. Larval mortality was assessed 48 hours after exposure by prodding the insects with a fine paintbrush. Larvae were considered dead if they were unable to move the length of their body.

The phenotyping bioassays were performed using discriminatory doses established by Campos *et al.* (2014c). For the discriminating heterozygous and resistant homozygous individuals the doses 0.25 mg/L and 5 mg/L prepared as aforementioned were respectively used. Five replications each comprising 10 larvae of 2nd-instar larvae of *T. absoluta* + a control treatment were used. Mortality data were assessed 48 hours after exposure. The surviving insects were collected and stored in alcohol for further genotyping using molecular tools.

Synergism of Spinosad. The 2nd-instar larvae of spinosad susceptible (PLT-Sus), unselected (IRA-Unsel) and selected (IRA-Sel) colonies were exposed in concentration-mortality bioassays in the presence of spinosad + PBO + DEF and + DEM to determine whether metabolism is involved in the resistance. The bioassays were performed as those previously described for the concentration-mortality bioassays, but all larvae were topically treated (0.2 µL/larvae) with 1.0 mg/mL of either PBO, DEM or DEF before exposure to spinosad. The synergist concentrations caused no mortality for *T. absoluta.*

Sample Extractions for Enzyme Assays. For enzyme assays, 10 larvae of 2nd-instar larvae from each population were transferred to a microfuge tube with three replicates for each assay. For esterase and glutathione *S*–transferase assays, each sample was homogenized in 200 µL of sodium phosphate buffer at 0.02 M, pH 7.2 or sodium phosphate buffer (0.1 M, pH 7.5), using a Potter-

Elvehjem homogeniser. Homogenates were centrifuged at 15,000 g and 4°C for 15 min and supernatants harvested and stored at –20°C. For cytochrome P450-dependent monooxygenase assays, samples were homogenised in 500 µL sodium phosphate buffer (0.1M, pH 7.5) + glycerol at 20% and microsomes were prepared in the same buffer. Homogenates were centrifuged at 15,000 *g* and 4°C for 15 min and the supernatant was ultra-centrifuged at 100,000 *g* for 60 min in an OptimaTM L-80 XP ultracentrifuge (Beckman Coulter, Palo Alto, CA) with the resulting microsomal pellet resuspended in homogenization buffer containing 20% glycerol (Wright *et al.* 2000). Quantitation of protein was determined by the bicinchoninic acid method using bovine serum albumin (BSA) as standard (Smith *et al.* 1985).

Esterase Assays. Esterase activity was measured with a method adapted from van Asperen (1962). Stock solutions (250mM) of α-naphthyl acetate and β-naphthyl acetate substrates were prepared in acetone. For each reaction, 2 µL α-naphthyl acetate substrate, 10 µL of sample diluted to 1:100 and 188 µL of sodium phosphate buffer (0.02M, pH 7.2). The same procedure was done for esterase analysis using β–naphthyl acetate substrate, however the samples were diluted to 1:10. Samples were incubated in 30 ºC for 15 min. The reaction was stopped using 33.2 µL of 0.3% FAST Blue B. Absorbance was read at 595 ηm on a microtiter plate reader (Elx800, BioTek®, Winooski, VT, USA). Each sample was analysed in triplicate. A standard curve was prepared with α-naphtol and β-naphtol. Esterase activity was expressed as ηMol naftol x min^{-1} x mg de $protein^{-1}$.

Glutathione-S-transferase Assays. Conjugation activity of reduced glutathione was determine using CDNB (1-chloro-2,4- dinitrobenzene) substrate in the presence of glutathione *S*-transferase, forming 2,4-dinitrophenyl-*S*-glutathione (Habig *et al.* 1974). CDNB solution (150 mM) was prepared in ethanol and reduced glutathione (10 mM) was dissolved in sodium phosphate buffer

(0.1M, pH 7.5). For each reaction, 138 µL of sodium phosphate buffer (0.1 M, pH 7.5), 10 µL of sample containing 1 µg of protein and 150 µL of reduced glutathione (10 mM) were mixed. The mix was incubated in a water bath at 30 ºC for 5 min. Next, 2 µL of CDNB (150 mM) was added to the reaction. Immediately, formation of 2,4-dinitrophenyl-*S*-glutathione was measured using a biophotometer (Eppendorf) at 340 ηm. The reaction was analyzed for 5 min with read intervals of 30 sec. Each sample was analyzed in triplicate, and measures comprised a total of nine replicates. Absorbance data were analysed as function of reaction time after addition of CDNB. The slope of the line (absorbance/min) was transformed using the extinction coefficient of CDNB (9.6 $mM^{-1}.cm^{-1}$).

Cytochrome P_{450}–dependent Mono-oxygenase (*O*-demethylase) Assays. Activity of cytochrome P_{450} was determined through *O* – demethylation using the substrate *p*–nitroanisole ($O_2N–C_6H_4–O–CH_3$) to nitropheno (Rose & Brindley 1985). Samples were analysed in triplicate. Activity of cytochrome P_{450}–dependent monooxygenase was measured by mixing 178.8 µL of sodium phosphate resuspension buffer (0.1M, pH 7.5), 56.2 µL of sample, 2.5 µL *p*–nitroanisole (150 mM in ethanol) and 12.5 µL of reduced NADPH (9.6 mM) in each well and in this order. The mix was incubated for 15 min at 37°C. After, HCl (1M) was added to stop the reaction. Subsequently the mix was centrifuged at 14,000*g* for 10 min, and 200 µL of supernatant was transferred to microtiter plate wells to be read at 405 ηm on a microplate reader. Each sample was analysed in triplicate, and measures comprised a total of nine replicates. Activity of cytochrome P_{450}–dependent monooxygenases per sample was determined based on a standard curve of *p* – nitrophenol in ηMol *p*–nitrophenol x min^{-1} x mg of $protein^{-1}$.

RNA Extraction and cDNA Synthesis. Total RNA was isolated from of 40 larvae of susceptible and resistant populations to spinosad, using trizol reagent (Invitrogen® Life Technologies,USA) in quantities half of that recommended in the manufacturer's protocol. For cDNA synthesis was

used, SuperScript® III First Strand Synthesis Kit for RT-PCR system (Invitrogen® Life Technologies). In the reaction was used 200 ng of RNA, 1µL of random hexamers (50 ng/µL) and 1 µL dNTPs 10mM. The samples were incubated at 65Cº for 5 min and then added on ice for 1 min. Then the following reagents were added: 4 µL 10X RT buffer, 1 µL 25 mM $MgCl_2$, 1 µL DDT, 1 µL of RNase Out, 1 µL Superscript III (200 U/µL) and incubated for 25 Cº for 10 min/50 Cº/ 50 min the reaction was finished and 85 Cº/15 min, the RNA was removed by adding of 1 µL of RNA H to 37 Cº/20 min and stored the -20Cº until use.

Molecular Cloning of the *Tuta absoluta* nAChR α6 Subunit (Taα6) Nested PCR was used to amplify the α6 subunit of the nAChR receptor of susceptible populations, unselected and resistant. The specific primes were designed from the transcriptome of *T. absoluta* by Berger M, (unpublished). For the first reaction, Nested-PCR was performed with 1 µL of cDNA containing 10 pmol of each primer pair: Tuta_nachr_mid_F1 (GGA GGC GAT TTA TCA GAC T) and Tuta_nachr_mid_R1 (GAG TCT GGT GGC AGT GTA). In the second reaction, 1µL PCR was used, containing the primers Tuta_nachr_mid_F2 (TGG CGA ATG GTA TTT GAT AGG) and and Tuta_nachr_QPCR_R1 (AACACATGGCACGATCAGGT), 5 µL of 10X AccuPrime™ PCR Buffer II, 0,2 µL of AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen® Life Technologies) and 41,8 µL of nuclease-free H_20, to the PCR reaction mixture (50 µL). The amplification profile consisted of the following steps: initial denaturation at 94 ° C for 2 min followed by 35 cycles (94 °C / 30 s, 52°C / 1min and 72 °C / 2 min, followed by a final extension step at 72 ° C for 5 min. PCR products (~800 bp) were run on 1.5% agarose gels pre-stained with SYBR Safe DNA stain (Invitrogen® Life Technologies), and clean up System from Promega was used to recover DNA from gel slices according to the manufacturer's recommendations. Amplified fragments were cloned into pCR® 2.1-TOPO® TA vector (Invitrogen® Life Technologies) and sequenced in the automatic sequencer ABI 3500 (Applied Biosystems,

Cleveland, Ohio, USA). The assembly of sequence results was performed using Geneious R7.1 (Biomatters Ltd., New Zealand).

DNA Extraction. Insects were placed in liquid nitrogen and homogenized individually. The homogenates were centrifuged (Eppendorf 5810R) at 15,000 *g* and 4°C for 15 min after addition of 200 µl of DNAzol® (Invitrogen® Life Technologies). Following, samples were centrifuged at 15,000 g and 4°C for 20 min after adding 100µl of 100% ethanol to precipitate the DNA. The ethanol was removed and the pellet washed with ethanol 70%, and centrifuged for 5 min. The samples were dried for 5 min at room temperature to complete evaporation of the ethanol, and the DNA samples were then dissolved in 30 µl of nuclease-free H_20. The quality and quantity of DNA were spectrophotometrically assessed (Nanodrop Technologies).

Intron Amplification. Long PCR enzyme mix (Thermo Scientific, USA) was used to amplify the intron sequence before exon nine. The primers Ta_a6_ex8_761F (TCT CGC TGA CGG TGT TTT TGA ACC TG) and Ta_a6_ex9_934R (GCA TCT CAT GAA TGT CCG CCG TTC GAT) were designed for this amplification using the *T. absoluta* transcriptome (Berger M, unpublished). A master mix was made, containing 2.5 µl of 10X Long PCR buffer with 15 mM $MgCl_2$, 1 µl of dNTP mix (10mM), 18 µl of nuclease-free water, 1 µl of forward primer (10 µM), 1 c of reverse primer (10 µM) and 0.5 µl of Long PCR Enzyme Mix per reaction. Genomic DNA (50 ng) was added to each sample. A 16 hour programme (94°C 2 min, 35 cycles of: 94°C/10 sec 50°C/20 sec, 68°C/25 min, with a final extension of 68°C/20 min) was performed. PCR products (~4kb) were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR products were sequenced by Eurofins Genomics, Germany.

TaqMan Diagnostic Assays. Forward and reverse primers and two probes were designed using the Custom TaqMan Assay Design Tool (Applied Biosystems). The primer G275E_F ACA CTG

TAA GCA CAA TAC TGTTGATCTAAT and G275E_R- GCC ACC ATA AAC ATG ATG CAA TTGA, were used to amplify the region encompassing the G275E. For all assays the probe labelled with VIC-G275E-TGG CAG GGA CTTAC, was specific for the wild-type allele, while a second probe, labelled with FAM- G275E- TGG CAG AGA CTT AC was specific for the mutant allele. Each probe also carried a 3' non-fluorescent quencher. PCR reactions (15µL) contained 2 µL of genomic DNA extracted from individual insects using DNAzol reagent, 7.5 µL of SensiMix DNA kit (Quantace), 800 nM of each primer and 200 nM of each probe. Samples were run on a Rotor-Gene 6000 (Corbett Research) using the temperature cycling conditions of: 10 min at 95°C followed by 40 cycles of 95 °C for 10 sec and 65 °C for 45 ses. The increase in fluorescence of the two probes was monitored in real time by acquiring each cycle on the yellow (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 nm emission) of the Rotor-Gene respectively.

Data Analysis. Mortality data obtained from concentration–response bioassays were corrected with the mortality observed in the control treatment (Abbott 1925) and analysed by probit analysis at $P > 0.05$ (Finney 1971) using the program Polo-Plus® (LeOra-Software 2005). The resistance ratios were calculated through the "lethal ratio test" and were considered significant if 95 % confidence interval (CI) did not include the value 1.0 (Robertson *et al.* 2007)**.** Data gathered from activity of esterases, glutathione *S*-transferases, and cytochrome P450-dependent monooxygenases were analysed using SAS (SAS Institute 2001). The assumptions of normality and homoscedasticity were tested using PROC UNIVARIATE and PROC GLM (SAS Institute 2001). The activity data were subjected to an analysis of variance (ANOVA) using PROC ANOVA and the Tukey's test (HSD) at $P < 0.05$ for grouping the means (SAS Institute 2001). The R allele frequency from genotyping data was subjected to correlation analysis with the mortality data of the diagnosed populations, using PROC CORR (SAS Institute 2001).

Results

Bioassays. Diagnostic bioassays detected low levels of resistance in most of the 17 populations analyzed, except in Iraquara-BA. The mortality ranged from 6.7 ± 3.4 to 100 in the concentration of 0.25 mg/L and in the concentration of 5mg/L only 2 populations showed survivors (Table 1). In the dose-response bioassays the PLT-Sus population presented a LC_{50} of 0.020 mg/L and the IRA-Unsel population (Iraquara-BA) presented a LC_{50} of 5.8 mg/L, while the IRA-Sel population presented a LC_{50} of 1001 mg/L (Table 2). The IRA-Unsel population showed a resistance ratio of 284 times, while the population subjected to selection pressure showed a ratio of resistance of 48.900 times (Table 2).

Synergism Assays. The synergistic ratio of PBO, DEF and DEM towards spinosad was in that order of 1.1-, 3.5- and 1.6-fold in the PLT-Sus population, and of 2.4, 3.3- and 4.1-fold in the IRA-Unsel population (Table 3), being significant for all the three synergists in the unselected population. Significant synergism of spinosad was also observed in the PLT-sus population only by DEF. In the IRA-Sel population, the effect of inhibitors towards spinosad was respectively of 0.5-, 0.6- and 0.6-fold for PBO, DEF and DEM, clearly showing a decreasing in the toxicity of spinosad though not significant.

Enzyme Assays. Biochemical assays of esterase activity differed significantly among populations of *T. absoluta* using α-naphthyl acetate and β-naphthyl acetate as substrate. The α-esterase activity was 0.02 ± 0.004 mmol/min/µg, 0.05 ± 0.005 mmol/min/µg and 0.03 ± 0.008 mmol/min/µg for thes PLT-Sus, IRA-Unsel and IRA-Sel populations respectively. While the β-esterase activity varied from 0.050 ± 0.010 mmol/min/µg (IRA-Sel) to 0.09 ± 0.010 mmol/min/µg (PLT-Sus) (Table 4). Assays of glutathione S-transferase (GST) activity showed significant differences between *T. absoluta* populations with variation of 2.4-fold observed populations. The IRA-Sel population had the greatest GST_s activity (72.6 ± 1.1 µmol/min/µg) while the PLT-Sus population

had the lowest activity (30 ± 3.6 µmol/min/µg) (Table 4). The activity of cytochrome P450–dependent monooxygenases differed significantly between the populations tested with variation of up to 3.9-fold. Activity of cytochrome P450–dependent monooxygenases mediated by *O*-demethylase, ranged from 0.02 ± 0.004 mmol/min/µg for the susceptible population to 0.060 ± 0.002 µmol/min/µg for the selected population (Table 4).

Cloning the nAChR Alpha Six Subunit of *Tuta absoluta* (Taα6). PCR fragments of the alpha subunit of PLT-Sus, IRA-Unsel and IRA-sel populations were amplified. The amplified DNA fragments (~800bp) were cloned and sequenced. Comparison of the sequence obtained with that from susceptible populations revealed the presence of a single point mutation in the unselected and resistant populations resulting in an amino acid substitution of glycine (GGG) to glutamic acid (GAG) at the position 275 in exon 9 of the alpha six subunit (Fig.1). The codon for the mutated amino acid was found to span exon 9 and exons 8a/8b, with the resistance-associated mutation being at the start of exon 9.

TaqMan Diagnostic Assays. For TaqMan assay were used 340 *T. absoluta* DNA samples from different regions in Brazil. The TaqMan assays use two probes, one specific for the resistant (mutant) allele labelled with FAM and the other specific for the susceptible (wild-type) allele labelled with VIC. A homozygous resistant individual will display a strong increase in FAM fluorescence, whilst a homozygous wild-type individual will show a strong increase in VIC fluorescence. Heterozygous individuals show an intermediate increase in both channels (Fig. 2). The TaqMan assay showed that the allelic frequency of the G275E mutation was low in populations of *T. absoluta* from Brazil (Tabela 1). The resistant allele was found only in populations of Anápolis, Brasília, Gameleira II, Iraquara, João Dourado I, João Dourado III - BA, Lagoa Grande, Paulínia e Sumaré. However, only the population of Iraquara was detected a high frequency of this allele (67,5%) in other frequency ranges of population 2,5% to12,5% (Table 1).

The frequency of sensitive allele was of 100% in the populations of América Dourada, Gameleira I, Guaraciaba, João Dourado II, Pelotas, Pesqueira, Tianguá e Venda Nova (Tabela 1). Mortality data strongly correlated with the R allele frequency (DD_1, r = -0.835, P < 0.0001, n=17) and (DD_2, r = - 0.958, P < 0.0001, n = 17), where increasing the R allele frequency, a decrease in mortality is observed.

Discussion

The indiscriminate use of insecticides has resulted in the rapid emergence of resistant populations of *T. absoluta* in Brazil (Siqueira *et al.* 2000b, Silva *et al.* 2011, Campos *et al.* 2014a). The loss of efficacy of traditional and cheaper products has caused an increase in the use of new molecules as spinosad. Recent studies have demonstrated high levels of spinosad resistance in some populations from Brazil (Campos *et al.* 2014a). Selection of *T. absoluta* population resistant to spinosad in laboratory led to a rapid increase in resistance to this insecticide (Campos *et al.* 2014c). The resistance to spinosad was also detected in *B. oleae*, *Liriomyza trifolii* (Burgess), *Helicoverpa armigera* (Hübner), *Spodoptera exigua* (Hübner) *e F. occidentalis* (Moulton *et al.* 2000, Ferguson 2004, Bielza *et al.* 2007, Kakani *et al.*,2010, Roe *et al.* 2010). The loss of spinosad efficiency was detected after two years of use to control *P. xylostella* populations in Hawaii (Zhao *et al.* 2002a). Herein, we provide the mechanism of resistance to spinosad in *T. absoluta*, which is also likely associated with the cross-resistance to spinetoram observed by Campos *et al.* (2014c).

The three enzymes system analyzed in the present study are the most commonly involved in resistance to several insecticides in different species (Feyereisen 1999, Hayes *et al.* 2005, Heidari *et al.* 2005, Hongchun *et al.* 2013). Increased monooxygenase (*O*-demethylase) activity was

associated with the resistance to spinosad in *S. exigua* and *H. armigera* (Wang *et al.* 2006, Wang *et al.* 2009). Reyes *et al.* (2012) showed that resistance to spinosad in Chilean populations of *T. absoluta* was associated with increased cytochrome P450-dependent monooxygenases. However, we did not find levels of that enzyme or synergism of spinosad in the IRA-Sel population, which would explain the very high level of resistance, suggesting metabolism may be associated with lower levels of resistance. Indeed, significant synergism of spinosad was observed in the IRA-Unsel founder population by all inhibitors. Campos *et al*., (2014c) observed a decreased activity of cytochrome P450-dependent monooxygenases and esterases following the selection course of IRA-Unsel colony. Other studies report that synergist DEF and PBO did not enhance the toxicity of spinosad in *P. xylostella, M. domestica, S. exigua* and *F. occidentalis* (Liu & Yue 2000, Zhao *et al.* 2002b, Bielza *et al.* 2007). Here, we showed that a mutation (G275E) previously described in two thrips species (Puinean *et al.*,2013, Bao *et al.* 2014) is also associated with resistance to spinosad in *T. absoluta*, confirming our hypothesis of alteration in the nicotinic acetylcholine receptor subunit alpha 6. Therefore, the mechanism based on metabolism seems to be overcome by target site alteration when resistance reaches higher levels.

The resistance-associated mutation (G275E) is predicted to lie at the top of the third α-helical transmembrane domain of Taα6 in *T. absoluta* from Brazil. Comparison of the nucleotide sequences of resistant populations and susceptible to spinosad in *F. occidentalis* also showed that a single nucleotide change, resulting in the substitution of a glycine in susceptible insects with a glutamic acid in resistant insects nAChR α6 subunit (Puinean *et al.* 2013). The resistance to spinosad in this insect was characterized through the expression of analogous mutation (A275E) in the human nAChR α7 subunit using *Xenopus laevis* (Wagler) oocytes and showed abolishment of the modulatory effects of insecticide (Puinean *et al.* 2013), effect that may occur likewise in *T. absoluta*. This same mutation was also associated with resistance to spinosad in *T. palmi* (Bao *et*

al., 2014). Conversely, resistance to spinosad in *P. xylostella* was associated with a truncated nAChR α6 subunit sequence in the mutant individuals and it might indicate that spinosad was interacting with the wild type TM3 and TM4, which is removed by this truncation (Baxter *et al.* 2010). Rinkevich *et al.* (2010) also reported that resistance to spinosad in this insect is associated with mutations that generate premature stop codons shortly after TM3. Truncated proteins of nAChR α6 subunit were also associated with resistance to spinosad in *B. dorsalis* and *D. melanogaster* (Watson *et al*,. 2010, Hsu *et al.*, 2012). Nevertheless, this is the first time that a point mutation is reported to a Lepidoptera and may not be linked to the receptor loss of function (Perry *et al*., 2007).

TaqMan assays have a high power to discriminate the allele frequency of resistance as demonstrated by other studies that have genotyped populations of *Aphis gossypii* (Glover), *Ctenocephalides felis* (Bouché) and *Anopheles gambiae* (Giles) (Daborn *et al.* 2004, Ramphul *et al.* 2009, Chen *et al.* 2014*)* This technique provided the R allele frequency of spinosad resistance caused by mutation G275E in nAChR in *T.absoluta* populations from Brazil. All three genotypes (rr, rs and ss) were identified in populations from different geographical regions in the country. This technique was already used for monitoring pyrethroid resistance in *T. absoluta* populations (Haddi *et al.* 2012, Silva *et al.* 2015).Therefore, the TaqMan assays can serve as an effective and rapid method for monitoring the resistance of spinosad in *T. absoluta*. The detection of the frequency of resistance alleles are one of the main factors for management of insecticide resistance (Tabashnik 1994)

The frequency of spinosad resistance was low or absent in some populations of *T. absoluta.* Thus, this insecticide was still effective to control this pest in some areas. However, an intense selection pressure with this insecticide in *T. absoluta* populations likely will lead populations to very high levels of resistance in a few generations (Campos *et al.* 2014c). The IRA-Unsel

population showed high frequency of resistant allele, although there were 44 generations without spinosad selection pressure when this assay was done. Such fact demonstrates that intensive use spinosad in this location will lead to field control failure by this insecticide or related molecules, since mutation in nAChR can confer cross-resistance to other spinosyns (Campos *et al.* 2014a). Therefore, rotation must be done with insecticides that do not present cross-resistance to spinosyns, such as chlorfenapyr, for instance (Silva 2013)

The use of diagnostic concentration technique can distinguish precisely between susceptible and resistant individuals (Roush & Miller 1986). Thus, allows early to determine the change in resistance frequency and change making initial decisions with different modes of insecticides action (Marçon *et al.* 2000). The point mutation G275E has been proposed to be the primary mechanism of spinosad resistance in *F. occidental* (Puinean *et al.* 2013). The frequency of the resistant allele in IRA-Unsel (67,5%), and it was the population with the lowest mortality. These results showed a significant correlation between the G275E mutation gene frequencies and mortality obtained in diagnostic assays doses. Populations that showed a high frequency of heterozygous individuals showed low mortality, indicating that these subjects have an important role in resistance to spinosad, Possibly this is due to the fact that the inheritance pattern for this insecticide is incompletely recessive (Campos *et al.* 2014c).

This work shows evidence that mutation G275E in nAChR can have a significant effect on the inefficiency of the insecticide spinosad. TaqMan assay detected the low frequency of mutation in populations of *T. absoluta* allowing knowledge of the geographical distribution of this mutation from Brazil. The dose of the diagnostic and TaqMan results indicated that the dose of 5mg/L can be effective in monitoring this pest in locations that showed low frequency of resistant allele. Therefore, this study provides important information for the status of resistance to spinosad in *T. absoluta*.

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Table 1. Mortality of larvae of *Tuta absoluta* exposed to spinosad diagnostic doses and R-allelic and genotype frequencies of larvae exposed to spinosad diagnostic doses.

Population	DD[a]$_1$ 0.25 mg/L	DD[b]$_2$ 5 mg/L	R-Allele Freq (%)	Gen Freq - SS(%)	Gen Freq - RS(%)	Gen Freq - RR(%)
América Dourada – BA	100.0 ± 0.0	100.0 ± 0.0	0	100	0	0
Anápolis – GO	96.6 ± 3.3	100.0 ± 0.0	2,5	95	5	0
Brasília – DF	83.3 ± 8.8	100.0 ± 0.0	2,5	95	5	0
Gameleira 2 – BA	83.3 ± 6.6	100.0 ± 0.0	0	100	0	0
Gameleira 1 – BA	61.3 ± 20.8	100.0 ± 0.0	10	80	20	0
Guaraciaba do Norte - CE	70.8 ± 14.4	100.0 ± 0.0	0	100	0	0
Iraquara – BA	6.7 ± 3.4	20.8 ± 4.1	67,5	10	45	45
João Dourado - BA I	54.1 ± 32.5	96.6 ± 3.3	10,5	78,9	21,1	0
João Dourado - BA II	68.5 ± 7.1	100.0 ± 0.0	0	100	0	0
João Dourado - BA III	93.3 ± 3.3	100.0 ± 0.0	12,5	75	25	0
Lagoa Grande – PE	96.6 ± 3.3	100.0 ± 0.0	5	90	10	0
Paulínia – SP	100.0 ± 0.0	100.0 ± 0.0	12,5	75	25	0
Pelotas – RS	100.0 ± 0.0	100.0 ± 0.0	0	100	0	0
Pesqueira – PE	100.0 ± 0.0	100.0 ± 0.0	0	100	0	0
Sumaré – SP	100.0 ± 0.0	100.0 ± 0.0	7,5	85	15	0
Tianguá – CE	100.0 ± 0.0	100.0 ± 0.0	0	100	0	0
Venda Nova – ES	83.3 ± 6.6	100.0 ± 0.0	0	100	0	0

[a] Diagnostic doses - 0,25 mg/L. [b] diagnostic doses-5 mg/L *: susceptible allele - S; resistant allele - R

Table 2. Resistance of larvae of *Tuta absoluta* populations to spinosad.

Population	n[1]	Slope ± SE[2]	LC_{50} (95% CI)[3]	LC_{80} (95% CI)[3]	χ^2 DF[4]	RR_{50} (95% CI)[5]	RR_{80} (95% CI)[5]
PLT-Sus	338	2.16 ± 0.20	0.020 (0.016 – 0.026)	0.05 (0.04 – 0.07)	7.6 (7)	1.0 (0.8 – 1.3)	1.0 (0.7 – 1.4)
IRA-Unsel	209	1.12 ± 0.21	5.87 (2.82 – 9.52)	29 (19 – 60)	3.7 (5)	284 (151 – 533)	672 (340 – 1328)
IRA-Sel	210	2.12 ± 0.30	1001 (729 – 1311)	2488 (1865 – 3706)	3.2 (6)	48900 (34500 – 69500)	49700 (32500 – 75900)

[1]Total number of larvae bioassayed. [2]Standard error. [3]Milligrams spinosad per liter water. [4]Chi-squared and Degree of Freedom. [5]Resistance ratio: ratio between LC_{50} resistant and LC_{50} susceptible, calculated through Robertson and Preisler (1992) method, and confidence of interval at 95%. Resistance ratio non-significant if the confidence interval brackets the value 1.0.

Table 3. Synergism of spinosad in susceptible and resistant larvae of *Tuta absoluta*. Temperature: 25 ± 1ºC; R.H.: 70 ± 5% and 12 h photophase.

Population	Insecticides	N[1]	Slope ± SE[2]	LC_{50} (95% CI)[3]	χ^2 DF[4]	SR_{50} (95% CI)[5]
PLT-Sus	Spinosad	338	2.16 ± 0.20	0.020 (0.016 – 0.026)	7.7 (7)	1.0 (0.7 – 1.4)
	+ PBO	266	1.70 ± 0.17	0.018 (0.012 – 0.030)	10.2 (7)	1.1 (0.8 – 1.6)
	+ DEF	225	1.22 ± 0.19	0.006 (0.003 – 0.009)	5.3 (6)	3.5 (2.0 – 6.1)*
	+ DEM(†)	213	1.55 ± 0.24	0.26 (0.11 – 0.41)	5.1 (5)	1.6 (0.9 – 3.1)
IRA-Unsel	Spinosad	209	1.12 ± 0.21	5.87 (2.82 – 9.52)	3.7 (5)	1.0 (0.5 – 2.1)
	+ PBO	320	1.19 ± 0.19	1.96 (0.88 – 3.14)	2.1 (5)	2.4 (1.1 – 5.3)*
	+ DEF	375	0.84 ± 0.12	1.42 (0.40 – 2.90)	7.6 (7)	3.3 (1.4 – 8.1)*
	+ DEM	221	0.78 ± 0.17	1.42 (0.21 – 3.47)	4.8 (6)	4.1 (1.1 – 15.7)*
IRA-Sel	Spinosad	210	2.12 ± 0.31	1001 (729 – 1311)	3.2 (6)	1.0 (0.7 – 1.4)
	+ PBO	316	1.53 ± 0.16	1941 (1369 – 2710)	7.1 (6)	0.5 (0.4 – 0.8)
	+ DEF	226	0.73 ± 0.15	1806 (260 – 4337)	9.8 (6)	0.6 (0.3 – 1.1)
	+ DEM	319	1.42 ± 0.17	1583 (776 – 2503)	7.5 (5)	0.6 (0.4 – 1.0)

[1]Total number of larvae bioassayed. [2]Standard error. [3]Milligrams spinosad per liter water. [4]Chi-squared and Degree of Freedom. [5]Synergism ratio: ratio between LC_{50} non synergized and LC_{50} synergized for each population, calculated through Robertson and Preisler (1992) method, and confidence of interval at 95%. Synergism ratio non-significant if the confidence interval brackets the value 1.0. (†) This response line was compared with the response line without diethyl maleate [LC_{50}= 0.41 (0.24 – 0.62)], using a different lot of spinosad.

Table 4. Mean activity of detoxification enzymes from *Tuta absoluta* populations.

Population	α esterase mmol/min/µg	β esterase mmol/min/µg	GST µmoles/min/µg	CypO ηmoles/min/µg
PTL-Sus	0.02 ± 0.004 b[1]	0.09 ± 0.010 a	30 ± 3.6 a	0.02 ± 0.004 c
IRA-Unsel	0.05 ± 0.005 ab	0.06 ± 0.001 b	67 ± 4.4 b	0.04 ± 0.005 b
IRA-Sel	0.03 ± 0.008 a	0.05 ± 0.003 b	72 ± 13.5 b	0.06 ± 0.002 a

[1]Means followed by the same letter are not statistically different by Tukey's test at 5% probability.

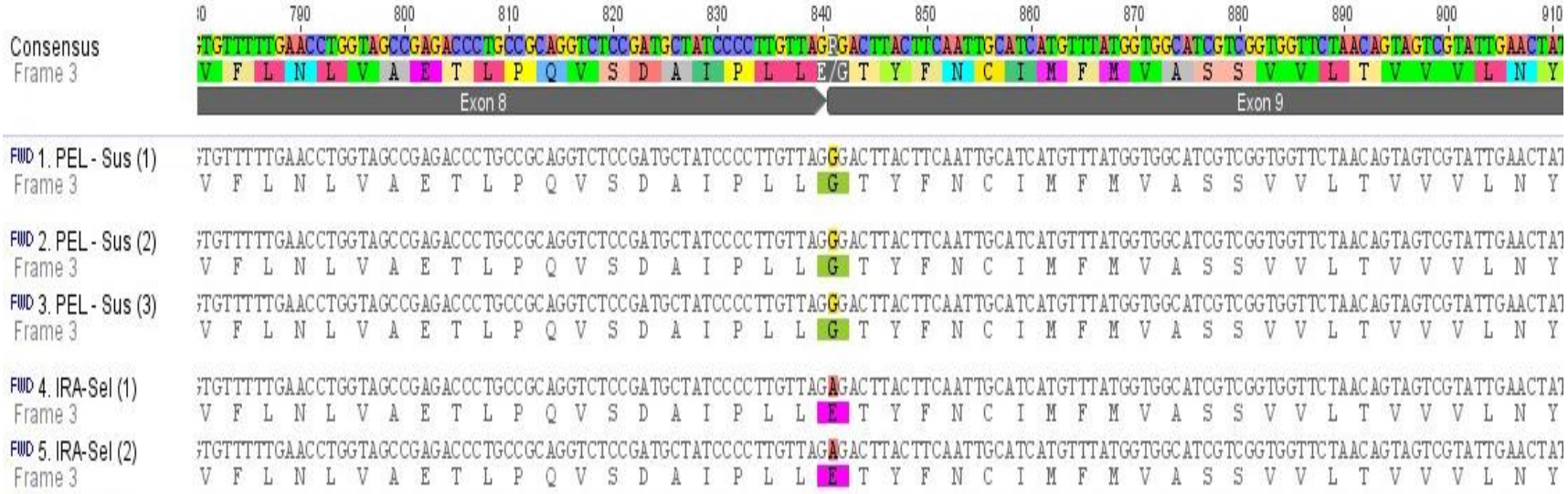

Figure 1. Alignment of nAChR alpha 6 subunit sequences from the IRA-Sel (spinosad resistant) and PEL (spinosad susceptible) strains of *Tuta absoluta* showing the presence of an amino acid substitution (G275E) in the resistant strain.

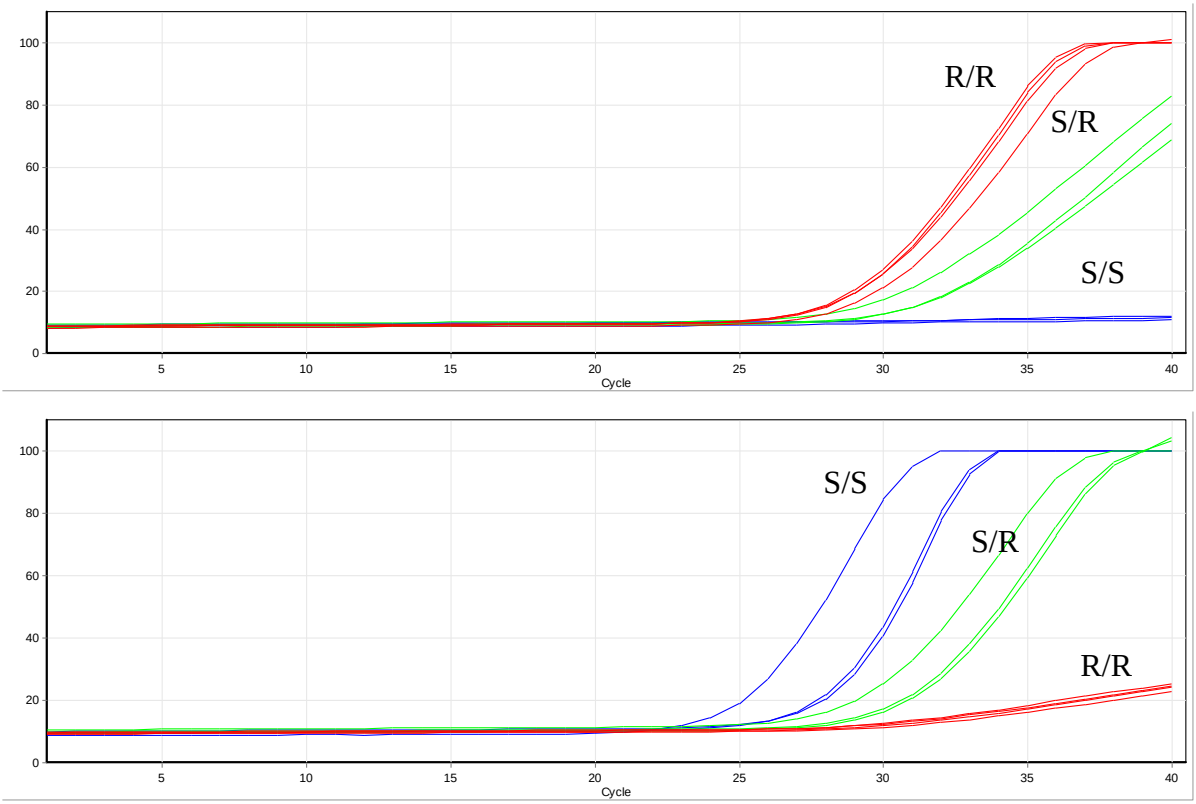

Figure 2. Real-time TaqMan detection of the G275E in *Tuta absoluta*. For each pair of graphs, the top graph shows the FAM-labelled probe specific for the mutant allele, and the bottom graph shows the VIC-labelled probe specific for the wild-type allele. S: wild-type allele; R: resistant allele.