Running title: mechanisms of spinosad resistance in *Tuta absoluta*

Mutation (G275E) of the nicotinic acetylcholine receptor α6 subunit is associated with high levels of resistance to spinosyns in *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

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Abstract

The tomato leafminer, *Tuta absoluta*, now a major pest of tomato crops worldwide, is primarily controlled using chemical insecticides. Recently, high levels of resistance to the insecticide spinosad have been described in *T. absoluta* populations in Brazil. Selection of a resistant field-collected strain led to very high levels of resistance to spinosad and cross-resistance to spinetoram, but not to other insecticides that target the nicotinic acetylcholine receptor (nAChR). In this study the mechanisms underlying resistance to spinosad were investigated using toxicological, biochemical and molecular approaches. Inhibition of metabolic enzymes using synergists and biochemical assessment of detoxification enzyme activity provided little evidence of metabolic resistance in the selected strain. Cloning and sequencing of the nAChR α6 subunit from *T. absoluta*, the spinosad target-site, from susceptible and spinosad-resistant strains was done to investigate the role of a target-site mechanism in resistance. A single nucleotide change was identified in exon 9 of the α6 subunit of the resistant strain, resulting in the replacement of the glycine (G) residue at position 275 observed in susceptible *T. absoluta* strains with a glutamic acid (E). A high-throughput DNA-based diagnostic assay was developed and used to assess the prevalence of the G275E mutation in 17 field populations collected from different geographical regions of Brazil. The resistant allele was found at low frequency, and in the heterozygous form, in seven of these populations but at much higher frequency and in the homozygous form in a population collected in the Iraquara municipality. The frequency of the mutation was significantly correlated with the mortality of these populations in discriminating dose bioassays. In summary our results provide evidence that the G275E mutation is an important mechanism of resistance to spinosyns in *T. absoluta*, and may be used as a marker for resistance monitoring in field populations.

Keywords: Tomato leafminer, insecticide resistance, nACh receptor, target-site alteration, metabolism

1. Introduction

The tomato leafminer, *Tuta absoluta* is a global threat to tomato production because of its great damage potential, short life cycle and high reproductive capacity [1-3]. *T. absoluta* originated in South America and was introduced to Brazil sometime between 1979 – 1980, after which it quickly spread nationwide causing serious damage to tomato crops [4, 5]. This pest arrived in Europe in 2006 and then subsequently spread to North Africa and the Middle East, and is now a serious problem for tomato cropping in a large number of countries [2].

The main method of *T. absoluta* control is through the application of chemical insecticides, however, the reliance on insecticides for control has led to the evolution of resistance [6]. The intensive use of organophosphates, carbamates, pyrethroids, benzoylureas, avermectin, indoxacarb and diamides has led to reports of resistance in *T. absoluta* populations in Brazil, Chile, Argentina, Greece and Italy [3, 7-13].

The insecticide spinosad was commercially introduced for pest control in 1997 [14]. The active ingredient of spinosad is a mixture of two compounds, spinosyn A and spinosyn D, produced by the microorganism *Saccharopolyspora spinosa* [15]. Spinosad acts on the nicotinic acetylcholine receptor (nAChR) causing a change in receptor conformation, leading to the opening of ion channels to the conduction of nerve stimulation, causing tremors, paralysis and death of the insect [16-18]. The nAChR is composed of five subunits, arranged symmetrically around a central pore with each subunit containing four transmembrane (TM1-TM4) domains and an extracellular N-terminal domain that includes the acetylcholine binding site [19]. Most insect genomes contain around 10-12 genes encoding nAChR subunit subtypes [20, 21]. Of these spinosad appears to specifically target the α6 subunit as studies on a number of insect

species have described an association between modification of this subunit subtype and spinosad resistance. For example, in the fruit fly, *Drosophila melanogaster* a strain with a Dα6 knockout was shown to be highly resistant to spinosad [22]. In the crop pests, the diamondback moth, *Plutella xylostella* and the oriental fruit fly *Bactrocera dorsalis* spinosad resistance was linked to mutations in the nAChR α6 subunit that result in truncated transcripts [23, 24]. Recently, spinosad resistance in western flower thrips, *Frankliniella occidentalis*, and melon thrips, *Thrips palmi*, was reported to be associated with a single nucleotide change in the nAChR α6 subunit, resulting in the replacement of a glycine (GGG) residue in susceptible insects with a glutamic acid (GAG) in resistant insects [25, 26] at position 275, which is located in a conserved region towards the top of TM3.

In *T. absoluta*, high levels of resistance to spinosad were recently described in a field population from Brazil [27]. Further selection led to a strain which exhibited extremely high levels of resistance to spinosad [28]. The resistance of this strain was autosomal, recessively inherited, monofactorial, and showed strong cross-resistance to spinetoram (spinosoid) but not to thiamethoxam (a neonicotinoid) suggesting resistance is mediated by a target-site mechanism [28]. Despite this finding, the underlying mechanisms of resistance to spinosyns in *T. absoluta* remain to be characterized.

The aim of the current study was to investigate the molecular and biochemical mechanisms underlying resistance to spinosyns in resistant strains. The possible involvement of detoxification enzymes such as cytochrome P450-dependent monooxygenases ($P450_s$), glutathione *S*-transferase (GST_s) and carboxylesterases (CE_s) was examined using insecticide synergists and enzymatic assays. To explore the role of a target-site alteration in resistance the *T. absoluta* nAChR *α*6 subunit (*Taα6*) was PCR amplified, cloned and sequenced from resistant and susceptible strains. Finally, a

molecular diagnostic tool was developed that allowed sensitive detection of a resistance-associated mutation in individual *T. absoluta* and used to screen 17 field populations collected from different geographical regions of Brazil.

2. Material and Methods

2.1 Chemicals

Spinosad (Tracer 480 g AL/l concentrated suspension) was obtained from Dow AgroSciences industrial Ltda, Franco da Rocha, SP, Brazil. The insecticide synergists used in this study were 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).

2.2. Insects

The susceptible strain of *T. absoluta* (Pelota – RS, named here as PLT-Sus) was collected and maintained in the laboratory without exposure to insecticide. A strain of *T. absoluta* from Iraquara-BA, previously reported as resistant to spinosad [27], was divided into two cultures: one without exposure (named as IRA-Unsel) and the other (named as IRA-Sel) subjected to further selection with spinosad and maintained indefinitely under selection with 500 mg Al/l of spinosad under laboratory conditions [28]. For phenotyping and genotyping, 15 other populations collected from different geographical regions of Brazil were used as detailed in Table 1.

2.3 Bioassays

Full dose response toxicological bioassays were conducted using the PLT-Sus, IRA-Unsel, and IRA-Sel strains using a completely randomized design with two replications per treatment, with the whole bioassay repeated twice. Seven to eight concentrations of spinosad that resulted in mortality between 0 and 100% were used, with bioassays performed as described previously [29]. The spinosad solutions were diluted in water containing 0.01% Triton X-100. Control solution consisted of diluent minus insecticide. Spinosad-treated (or diluent treated in the case of controls) tomato leaflets were placed in Petri dishes (80 mm diameter) with ten 2nd instar larvae of *T. absoluta* and bioassays were maintained under controlled environmental conditions (25 ± 1 ºC temperature, 65 ± 5% relative humidity 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.

Discriminating dose bioassays were performed using the doses identified previously by Campos et al. [28]. To discriminate heterozygous and homozygous resistant insects the doses 0.25 and 5 mg AI /l of spinosad prepared as above were used respectively. Five replicates each comprising ten 2nd instar larvae of *T. absoluta* + a control treatment were used. Mortality was assessed 48 hours after exposure.

2.4 Synergism bioassays

Second instar larvae of spinosad susceptible (PLT-Sus), unselected (IRA-Unsel) and selected (IRA-Sel) strains were exposed to spinosad + PBO, + DEF or + DEM in concentration-mortality bioassays to determine if metabolism is involved in the resistance. The bioassays were performed essentially as described for the concentration-mortality bioassays, but with all larvae topically treated (0.2 µL larvae^{-1}) with 1.0 mg Al/ml of either PBO, DEM, or DEF before exposure to spinosad. The selected synergist concentrations caused no mortality when used alone on *T. absoluta.*

2.5 Sample extraction for enzyme assays

For enzyme assays, 10 L2 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 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 to separate cell debris 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) to obtain microsomes with the resulting microsomal pellet resuspended in homogenization buffer containing 20% glycerol [30]. Quantitation of protein was determined by the bicinchoninic acid method using bovine serum albumin (BSA) as standard [31].

2.6 Esterase assays

Esterase activity was measured with a method adapted from van Asperen [32]. Stock solutions (250mM) of α-naphthyl acetate and β-naphthyl acetate were prepared in acetone. For esterase analysis using α-naphthyl acetate as substrate each reaction consisted of 2 µL α-naphthyl acetate, 10 µL of sample diluted to 1:100 and 188 µL of sodium phosphate buffer (0.02M, pH 7.2). The same procedure was carried out for esterase analysis using β-naphthyl acetate as substrate, however the samples were diluted to 1:10. Samples were then incubated at 30ºC for 15 minutes and reactions stopped using 33.2 µL of 0.3% FAST Blue B. Absorbance was read at 595 nm 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 mmol min^{-1} μg^{-1} of $protein^{-1}$.

2.7 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 [33]. 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 30ºC for 5 minutes before 2 µL of CDNB (150 mM) was added to the reaction. The formation of 2,4-dinitrophenyl-*S*-glutathione was immediately measured using a biophotometer (Eppendorf) at 340 nm with the reaction monitored for 5 minutes with read intervals of 30 sec. Each sample was analyzed in triplicate, and measurements comprised a total of nine replicates. Absorbance data was 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 $mM^{-1}.cm^{-1}$).

2.8 Cytochrome P_{450}–dependent mono-oxygenase (O-demethylase) assays

Activity of cytochrome P_{450} was determined through *O* – demethylation by monitoring the conversion of substrate *p* – nitroanisole ($O_2N–C_6H_4–O–CH_3$) to nitrophenol [34]. The 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 before 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 and read at 405 ηm on a microplate reader. Each sample was analysed in triplicate, and measurements 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 nmoles min^{-1} $\mu g^{-1}$of $protein^{-1}$.

2.9 RNA extraction and cDNA synthesis

Total RNA was isolated from three independent pools of 10 larvae of susceptible and resistant *T. absoluta* strains, using Trizol reagent (Invitrogen® Life Technologies) following the manufacturer's protocol. SuperScript® III First Strand Synthesis Kit for RT-PCR system (Invitrogen® Life Technologies) was used for cDNA synthesis. Reactions comprised 200 ng of RNA, 1µL of random hexamers (50 ng μL^{-1}) and 1 µL dNTPs (10mM). Samples were incubated at 65ºC for 5 min placed on ice for 1 min and the following reagents 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), samples were then incubated for 25ºC for 10 min, 50ºC for 50 min and 85ºC for 15 min. Any remaining RNA was then removed from cDNA preparations by adding 1 µL of RNA H to each reaction and heating at 37ºC for 20 min.

2.9.1 Molecular cloning of the T. absoluta nAChR α6 subunit (Taα6)

Nested PCR was used to amplify the α6 subunit of the nAChR receptor in two amplicons using cDNA prepared from susceptible, unselected and selected *T. absoluta* strains. Specific primers were based on a nAChR α6 sequence from a *de novo* assembled transcriptome of *T. absoluta* (unpublished). Primary PCR reactions were performed with 1 µL of cDNA containing 10 pmol of each primer pair: Spod_a6_F3 (TGC CCG TRT CGG AGC AAG) and Tuta_nachr_mid_R1 (GAG TCT GGT GGC AGT GTA) were used to amplify the first half of the subunit and Tuta_nachr_mid_F1

(GGA GGC GAT TTA TCA GAC T) and Tuta _nachr_R1 (AAT AGT GTG AAC ACG AAC AGG) to amplify the second half. In secondary reactions, 1µL of the primary PCR product was used, containing the primers Spod_a6_F3 and Tuta_nachr_QPCR_R1 (AACACATGGCACGATCAGGT) for the first half and Tuta_nachr_mid_F2 (TGG CGA ATG GTA TTT GAT AGG) and Tuta_nachr_R2 (ACC TGT CAA CAA CCA TCG C) for the second half. PCR reactions also contained 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. 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 products purified from gel slices using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's recommendations. Amplified fragments were cloned into pCR® 2.1-TOPO® TA vector (Invitrogen® Life Technologies) and sequenced on a ABI 3500 sequencer (Applied Biosystems, Cleveland, Ohio, USA). Analysis of the sequencing results was performed using Geneious R7.1 (Biomatters Ltd., New Zealand).

2.9.2 DNA extraction

Insects were placed in liquid nitrogen and homogenized individually in a 1.5 ml microfuge tube using a mini pestle. After the addition of 200 µl of DNAzol® (Invitrogen® Life Technologies) homogenates were centrifuged at 15,000 *g* at 4°C for 15 min. 100µl of 100% ethanol was then added to precipitate DNA and samples were centrifuged at 15,000 g at 4°C for 20 min. Pellets were washed with 70 % ethanol, air-dried for 5 min at room temperature and dissolved in 30 µl of nuclease-free H_20. The

quality and quantity of DNA was assessed using a spectrophotometer (NanoQuant Infinite 200, Tecan, Switzerland).

2.9.3 Intron amplification

To facillitate the development of a DNA-based diagnostic assay long PCR enzyme mix (Thermo Scientific, USA) was used to amplify intronic sequence upstream of exon nine/downstream of exon 8. 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 purpose. PCR reactions consisted of 2.5 µl of 10X Long PCR (Fermentas, Life Sciences) 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 µl 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 seconds, 55°C/20 seconds, 68°C /25 minutes, with a final extension of 68°C/ 20 min) was performed. PCR products were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR products were sequenced by Eurofins Genomics, Germany.

2.9.3 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 mutation site. For all assays the probe labelled with VIC (TGG CAG GGA CTTAC), was specific for the wild-type allele, while a second probe, labelled with FAM (TGG CAG AGA CTT AC) was specific for the mutant allele. Each probe also carried a 3' non-fluorescent quencher. PCR reactions (15 µl) contained 2 µL of genomic DNA extracted from

281 individual insects using DNAzol reagent, 7.5 µl of SensiMix DNA kit (Quantace), 800
282 nM of each primer and 200 nM of each probe. Samples were run on a Rotor-Gene 6000
283 (Corbett Research) using the temperature cycling conditions of: 10 min at 95°C
284 followed by 40 cycles of 95°C for 10 s and 65°C for 45 s. The increase in fluorescence
285 of the two probes was monitored in real time by acquiring each cycle on the yellow
286 (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and
287 510 nm emission) of the Rotor-Gene respectively.
288 *2.9.4 Data analysis*
289 Mortality data obtained from concentration–response bioassays were corrected with the
290 mortality observed in the control treatment [35] and analysed by probit analysis at $P >$
291 0.05 [36] using the program Polo-Plus ® [37]. Resistance ratios were calculated through
292 the "lethal ratio test" and were considered significant if 95 % confidence interval (CI)
293 did not include the value 1.0 [38]. Data on the activity of esterases, glutathione *S*-
294 transferases, and cytochrome P450-dependent monooxygenases were analysed using
295 SAS [39]. The assumptions of normality and homoscedasticity were tested using PROC
296 UNIVARIATE and PROC GLM [39]. The activity data were subjected to an analysis of
297 variance (ANOVA) using PROC ANOVA and the Tukey's test (HSD) at $P < 0.05$ for
298 grouping the means [39]. The R allele frequency from genotyping data was subjected to
299 correlation analysis with the mortality data from discriminating dose bioassay of each
300 population, using PROC CORR [39].
301 **3. Results**
302 *3.1 Bioassays*
303 Diagnostic bioassays detected low levels of resistance in Brazilian populations of *T.*
304 *absoluta*, except in Iraquara-BA where significantly higher levels of resistance were
305 observed. Mortality ranged from 7 ± 3 to 100 % using a concentration of 0.25 mg

306 spinosad L^{-1} and when a concentration of 5mg spinosad L^{-1} was used only 2 populations
307 showed any survivorship (Table 1). In the dose-response bioassays the PLT-Sus
308 population had an LC_{50} of 0.020 mg spinosad L^{-1} and the IRA-Unsel population had an
309 LC_{50} of 5.8 spinosad L^{-1}, while the IRA-Sel population presented an LC_{50} of 1001 mg
310 spinosad L^{-1} (Table 2). The IRA-Unsel population had a resistance ratio of 284-fold,
311 while the population subjected to selection pressure showed a resistance ratio of 48,900-
312 fold (Table 2).
313 *3.2 Synergism Assays*
314 The synergistic ratio of PBO, DEF and DEM for spinosad was 1.1-, 3.5- and 1.6-fold
315 respectively in the PLT-Sus population, and 2.4-, 3.3- and 4.1-fold in the IRA-Unsel
316 population (Table 3). Synergism of spinosad was statistically significant for all three
317 synergists for the unselected population but only for DEF in the PLT-sus population. In
318 the IRA-Sel population, synergistic ratios were 0.5- 0.6- and 0.6-fold for PBO, DEF and
319 DEM respectively, which were not statistically significant suggesting no involvement of
320 metabolism in resistance to spinosad.
321 *3.3 Enzyme Assays*
322 Biochemical assays of esterase activity differed significantly among populations of *T.*
323 *absoluta* using both α-naphthyl acetate and β-naphthyl acetate as substrates. The α-
324 esterase activity was 0.02 ± 0.004 mmol/ min/μg^{-1}, 0.05 ± 0.005 mmol/min/μg^{-1} and
325 0.03 ± 0.008 mmol/min/μg^{-1} for the PLT-Sus, IRA-Usel and IRA-Sel strains
326 respectively (Table 4). While the β-esterase activity varied from 0.050 ± 0.010
327 mmol/min/μg^{-1} (IRA-Sel) to 0.09 ± 0.010 mmol/min/μg^{-1} (PLT-Sus) (Table 4). Assays
328 of glutathione S-transferase (GST) activity showed significant differences between the
329 strains with variation of 2.4-fold observed. The IRA-Sel strain had the greatest GST
330 activity (72.6 ± 1.1 µmol/ min/μg^{-1}) while the PLT-Sus strain had the lowest activity (30

± 3.6 µmol/ min/µg^{-1}) (Table 4). The activity of cytochrome P450–dependent monooxygenases differed significantly between the strains tested with variation of 3 - fold. Activity of cytochrome P450–dependent monooxygenases mediated by *O*-demethylase, ranged from 0.02 ± 0.004 ηmoles/min/µg^{-1} for the susceptible population to 0.06 ± 0.002 ηmoles min/µg^{-1} for the selected population (Table 4).

3.4 Cloning the nAChR alpha 6 subunit of T. absoluta (Taα6)

The nAChR α6 subunit of the PLT-Sus, IRA-Unsel and IRA-sel populations was PCR amplified, cloned, sequenced and deposited with Genbank under accession number KP771859. Comparison of the sequence obtained from the resistant IRA-Unsel and IRA-sel strains with that of the susceptible strain 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 position 275 in exon 9 of the α6 subunit (see figure 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. A TaqMan diagnostic assay was developed (see below) and used to determine the exact frequency of the mutation in the IRA-sel strain (69%) and IRA-Unsel strain (67.5%).

3.5 TaqMan diagnostic assays

After optimization the TaqMan assay allowed sensitive detection of the G275E mutation in individual insects (see figure 2). The assay uses 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 produced a strong increase in FAM fluorescence, whilst a homozygous wild-type individual produces a strong increase in VIC fluorescence. Heterozygous individuals produce an intermediate increase in both channels (Fig. 2). The TaqMan assay was used to screen

340 *T. absoluta* individuals collected from different regions in Brazil. This revealed that the frequency of the G275E allele is present at only low frequency in populations of *T. absoluta* in Brazil (Table 1). The resistant allele was only observed, exclusively in the heterozygous form, in populations of Anápolis - GO, Brasília - DF, Gameleira II - DF, João Dourado I - BA, João Dourado III - BA, Lagoa Grande - PE, Paulínia -SP e Sumaré – SP ranging in frequency from 2.5-12.5%. However, the Iraquara-BA population was the exception to this trend where the mutation was detected at high frequency (67.5%) and in the homozygous form (Table 1). The frequency of the resistant allele was found to correlate strongly with the level of mortality observed for each population in discriminating dose bioassay with increasing R allele frequency correlated with a decrease in mortality in bioassays (DD_1, r=-0.835, P<0.0001, N=17) and (DD_2, r=-0.958, P<0.0001, N=17).

4. Discussion

The indiscriminate use of insecticides in Brazil for the control of *T. absoluta* has resulted in the rapid emergence of resistant populations [10, 11, 27]. The loss of efficacy of traditional, cheaper products to resistance has in turn caused an increase in the use of newer chemistry such as spinosad. As a result resistance to this compound has now been described in *T. absoluta*, with a recent study describing high levels of spinosad resistance in certain *T. absoluta* populations from Brazil [27]. Furthermore, laboratory selection of a spinosad resistant field strain from Brazil led to a rapid increase in resistance to this insecticide [28]. The primary objective of the current study was to characterize the molecular and biochemical basis of spinosad resistance in *T. absoluta* with the aim of developing a mechanism-specific molecular diagnostic that can be used to rapidly screen populations across the country.

379 The three enzyme systems we analyzed in the present study are those most commonly
380 involved in resistance to several insecticides in a range of different insect species [40-
381 43]. Increased monooxygenase (*O*-demethylase) activity has been associated with
382 resistance to spinosad in *S. exigua* and *H. armigera* [44, 45]. In the case of *T. absoluta*
383 Reyes et al. [46] showed that resistance to spinosad in Chilean populations was
384 associated with increased cytochrome P450-dependent monooxygenases. However, we
385 did not find enhanced P450 activity or synergism of spinosad using a P450 inhibitor in
386 the IRA-Sel population, which would explain the very high level of resistance,
387 suggesting metabolism may be associated with lower levels of resistance. However,
388 significant synergism of spinosad was observed in the IRA-Unsel founder population
389 using all three inhibitors. Campos et al. [28] observed a decreased activity of
390 cytochrome P450-dependent monooxygenases and esterases following the selection
391 course of the IRA-Unsel colony. Other studies report that the synergists DEF and PBO
392 did not enhance the toxicity of spinosad in *P. xylostella*, *M. domestica*, *S. exigua* and *F.*
393 *occidentalis* [47-49].
394 Cloning and sequencing of the spinosad target-site, the nAChR α6 subunit revealed a
395 single non-synonymous change in the IRA-sel and IRA-Unsel strain compared to the
396 susceptible strain that results in an amino acid substitution, G275E, predicted to lie at
397 the top of the third α-helical transmembrane domain. This amino acid substitution has
398 been previously reported in two thrip species, *F. occidentalis* and *T. palmi* where it was
399 also associated with resistance to spinosad [25, 26]. In *F. occidentalis* the G275E
400 substitution was identified in a laboratory-selected strain displaying high levels of
401 resistance (resistance ratio > 350,000) to spinosad that was selected from a field
402 population collected in Almeria, Spain, that had been subjected to intensive treatment
403 with spinosad [47]. Resistance to spinosad in this strain was reported to be autosomal,

404 almost completely recessive and controlled by a single locus [50]. The effect of this
405 mutation on nAChR function was characterized through the expression of the analogous
406 mutation (A275E) in the human nAChR α7 subunit in *Xenopus oocytes* where it was
407 found to abolish the modulatory effects of spinosad but had no significant effect upon
408 activation by the natural ligand acetylcholine [26]. The G275E mutation has also been
409 recently associated with spinosad resistance in two strains of *T. palmi* collected from
410 Japan, although synergist bioassays provided evidence that metabolic mechanisms may
411 also contribute to resistance in at least one of these strains [25].
412 In *P. xylostella* resistance to spinosad has been associated with a truncated nAChR α6
413 subunit sequence in resistant individuals [23]. Rinkevich et al. [51] also reported that
414 resistance to spinosad in this species is associated with mutations that generate
415 premature stop codons shortly after TM3. Truncated nAChR α6 subunits were also
416 associated with resistance to spinosad in *B. dorsalis* and *D. melanogaster* [24, 52]. In
417 contrast to these studies our findings represent an example of spinosad resistance in a
418 lepidopteran species resulting from a point mutation that may not lead to receptor loss
419 of function as found for *F. occidentalis*.
420 The G275E mutation was identified in this study in two spinosad resistant lab strains of
421 *T. absoluta* and its presence also associated with resistance in field strains (see below).
422 However, it is unlikely to fully explain the extreme resistance phenotype exhibited by
423 the spinosad-selected IRA-sel strain as the frequency of the G275E mutation in this
424 strain was essentially the same as that of the unselected IRA-Unsel strain, suggesting
425 additional mechanisms contribute to resistance in the IRA-sel strain. One potential
426 alternative mechanism is metabolic resistance. However, as detailed above bioassays
427 using inhibitors of the three main enzyme systems frequently involved in resistance and
428 biochemical assessment of enzyme activity failed to provide evidence of metabolic

resistance in the IRA-sel strain. Furthermore, no additional mutations or indels were consistently observed in the gene encoding the nAChR alpha 6 between the IRA-Unsel and IRA-sel resistant strain. Further molecular characterisation of the resistance observed in this strain is therefore required with investigation of alternative xenobiotic detoxification systems and reduced insecticide penetration two possible areas of future research.

To determine the current frequency of the G275E mutation in populations of *T. absoluta* in Brazil and its association with resistance we developed a high-throughput DNA-based diagnostic assay that can be used to screen individual insects for the presence of the mutation. This platform has been used previously to screen global populations of *T. absoluta* for *kdr* mutations associated with pyrethroid resistance [53, 54]. In the current study the TaqMan assay was used to screen 17 field-collected populations for the G275E mutation and the results obtained were compared with the observed mortality of these strains in two discriminating dose bioassays. Overall the monitoring revealed that the frequency of the resistance-associated mutation is low or zero in most populations of *T. absoluta* in Brazil. In populations where it was found it was usually observed in the heterozygous form. The exception to this was the population collected from Iraquara where 45% of the insects tested were homozygous for the G275E mutation. It is also noteworthy that this strain was the only population to display low levels of mortality in discriminating dose bioassays using 5mg/L spinosad. Although the mutation monitoring and discriminating dose bioassays reveal that resistance to spinosad is currently low in *T. absoluta* populations in Brazil, the results for the Iraquara population provide a worrying demonstration that mutation frequency and resistance can reach much higher levels in local hotspots where selection pressure is higher. Furthermore, it has recently been demonstrated that much higher levels of spinosad resistance can be selected in *T.*

absoluta after just a few generations of selection [28]. Previous research has also demonstrated that spinosad resistance in *T. absoluta* is associated with cross-resistance to other spinosyns [27].

To avoid the development of spinosad resistance in populations of *T. absoluta* across Brazil it is now paramount that a resistance management strategy be developed based on rotation of spinosad with insecticides of different modes of action that currently retain efficacy, such as the diamides and chlorfenapyr. It will also be important to regularly monitor the distribution and frequency of resistance in national populations. In this regard the use of diagnostic concentration bioassays, to detect novel resistance, in combination with high-throughput diagnostic assays can allow resistance to be detected at an early stage and help guide the implementation of informed control and resistance management strategies.

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References

[1] M.C.F. Coelho, F.H. Franca, Biologia e quetotaxia da larva e descrição da pupa e adulto da traça-do-tomateiro, Pesq. Agropec. Bras. 22 (1987) 129-135.

480 [2] N. Desneux, E. Wajnberg, K.A.G. Wyckhuys, G. Burgio, S. Arpaia, C.A. Narvaez-
481 Vasquez, J. Gonzalez-Cabrera, D.C. Ruescas, E. Tabone, J. Frandon, J. Pizzol, C.
482 Poncet, T. Cabello, A. Urbaneja, Biological invasion of European tomato crops by *Tuta*
483 *absoluta*: ecology, geographic expansion and prospects for biological control, J. Pest
484 Sci. 83 (2010) 197-215.
485 [3] R.N.C. Guedes, M.C. Picanço, A.L. Matioli, D.M. Rocha, Efeito de inseticidas e
486 sistemas de condução do tomateiro no controle de *Scrobipalpuloides absoluta*
487 (Meyrick) (Lepidoptera: Gelechiidae), An. Soc. Entomol. Bras. 23 (1994) 321-325.
488 [4] F.H. França, M. Castelo Branco, Ocorrência de traça-do-tomateiro (*Tuta absoluta*)
489 em solanáceas silvestres no Brasil Central, Hortic. Bras. 10 (1992) 6-10.
490 [5] R.N.C. Guedes, M.C. Picanço, The tomato borer *Tuta absoluta* in South America:
491 pest status, management and insecticide resistance, EPPO Bull. 42 (2012) 211-216.
492 [6] M. Picanço, R.N.C. Guedes, G.L.D. Leite, F.P.C. R, E.A. Silva, Incidência de
493 *Scrobipalpuloides absoluta* em tomateiro sob diferentes sistemas de tutoramento e de
494 controle químico, Hortic. Bras. 13 (1995) 180-183.
495 [7] M.M.M. Lietti, E. Botto, R.A. Alzogaray, Insecticide resistance in Argentine
496 populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), Neotrop. Entomol.
497 34 (2005) 113-119.
498 [8] E. Roditakis, E. Vasakis, M. Grispou, M. Stavrakaki, R. Nauen, M. Gravouil, A.
499 Bassi, First report of *Tuta absoluta* resistance to diamide insecticides, J. Pest Sci. 88
500 (2015) 9-16.
501 [9] E.R. Salazar, J.E. Araya, Respuesta de la polilla del tomate, *Tuta absoluta*
502 (Meyrick), a insecticidas en Arica, Agric.Téc. 61 (2001) 429-435.

503 [10] G.A. Silva, M.C. Picanço, L. Bacci, A.L.B. Crespo, J.F. Rosado, R.N.C. Guedes,
504 Control failure likelihood and spatial dependence of insecticide resistance in the tomato
505 pinworm, *Tuta absoluta*, Pest Manage. Sci. 67 (2011) 913-920.
506 [11] H.Á.A. Siqueira, R.N.C. Guedes, M.C. Picanço, Insecticide resistance in
507 populations of *Tuta absoluta* (Lepidoptera: Gelechiidae), Agric. For. Entomol. 2 (2000)
508 147-153.
509 [12] H.A.A. Siqueira, R.N.C. Guedes, M.C. Picanço, Cartap resistance and synergism in
510 populations of *Tuta absoluta* (Lep., Gelechiidae), J. Appl. Entomol. 124 (2000) 233-
511 238.
512 [13] H.A.A. Siqueira, R.N.C. Guedes, D.B. Fragoso, L.C. Magalhaes, Abamectin
513 resistance and synergism in Brazilian populations of *Tuta absoluta* (Meyrick)
514 (Lepidoptera: Gelechiidae), Int. J. Pest Manage. 47 (2001) 247-251.
515 [14] T.C. Sparks, J.E. Dripps, G.B. Watson, D. Paroonagian, Resistance and cross-
516 resistance to the spinosyns – A review and analysis, Pestic. Biochem. Physiol. 102
517 (2012) 1-10.
518 [15] V.L. Salgado, T.C. Sparks, 6.5 - The Spinosyns: Chemistry, Biochemistry, Mode
519 of Action, and Resistance, in: L.I. Gilbert (Ed.) Comprehensive Molecular Insect
520 Science, Elsevier, Amsterdam, 2005, pp. 137-173.
521 [16] J. Cisneros, D. Goulson, L.C. Derwent, D.I. Penagos, O. Hernández, T. Williams,
522 Toxic Effects of Spinosad on Predatory Insects, Biol. Control 23 (2002) 156-163.
523 [17] V.L. Salgado, J.J. Sheets, G.B. Watson, A.L. Schmidt, Studies on the mode of
524 action of spinosad: the internal effective concentration and the concentration
525 dependence of neural excitation, Pestic. Biochem. Physiol. 60 (1998) 103-110.
526 [18] G.D. Thompson, R. Dutton, T.C. Sparks, Spinosad – a case study: an example from
527 a natural products discovery programme, Pest Manage. Sci. 56 (2000) 696-702.

528 [19] S. Sine, A. Engel, Recent advances in Cys-loop receptor structure and function,
529 Nature 440 (2006) 448 - 455.
530 [20] M. Grauso, R. Reenan, E. Culetto, D. Sattelle, Novel Putative Nicotinic
531 Acetylcholine Receptor Subunit Genes, Dalpha5, Dalpha6 and Dalpha7, in Drosophila
532 melanogaster Identify a New and Highly Conserved Target of Adenosine Deaminase
533 Acting on RNA-Mediated A-to-I Pre-mRNA Editing, Genetics 160 (2002) 1519 - 1533.
534 [21] A. Jones, V. Raymond-Delpech, S. Thany, M. Gauthier, D. Sattelle, The nicotinic
535 acetylcholine receptor gene family of the honey bee, Apis mellifera, Genome Res. 16
536 (2006) 1422 - 1430.
537 [22] T. Perry, J. McKenzie, P. Batterham, A Dalpha6 knockout strain of *Drosophila*
538 *melanogaster* confers a high level of resistance to spinosad, Insect Biochem. Mol. Biol.
539 37 (2007) 184-188.
540 [23] S.W. Baxter, M. Chen, A. Dawson, J.Z. Zhao, H. Vogel, A.M. Shelton, D.G.
541 Heckel, C.D. Jiggins, Mis-spliced transcripts of nicotinic acetylcholine receptor α6 are
542 associated with field evolved spinosad resistance in *Plutella xylostella* (L.), PLoS
543 Genet. 6 (2010).
544 [24] J.C. Hsu, H.T. Feng, W.J. Wu, S.M. Geib, C.H. Mao, J. Vontas, Truncated
545 transcripts of nicotinic acetylcholine subunit gene Bdα6 are associated with spinosad
546 resistance in *Bactrocera dorsalis*, Insect Biochem. Mol. Biol. 42 (2012) 806-815.
547 [25] W.X. Bao, Y. Narai, A. Nakano, T. Kaneda, T. Murai, S. Sonoda, Spinosad
548 resistance of melon thrips, *Thrips palmi*, is conferred by G275E mutation in α6 subunit
549 of nicotinic acetylcholine receptor and cytochrome P450 detoxification, Pestic.
550 Biochem. Physiol. 112 (2014) 51-55.

551 [26] A.M. Puinean, S.J. Lansdell, T. Collins, P. Bielza, N.S. Millar, A nicotinic
552 acetylcholine receptor transmembrane point mutation (G275E) associated with
553 resistance to spinosad in *Frankliniella occidentalis*, J. Neurochem. 124 (2013) 590-601.
554 [27] M.R. Campos, T.B.M. Silva, W.M. Silva, J.E. Silva, H.A.A. Siqueira, Spinosyn
555 resistance in the tomato borer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), J.
556 Pest Sci. 88 (2015) 405-412.
557 [28] M.R. Campos, A.R.S. Rodrigues, W.M. Silva, T.B.M. Silva, V.R.F. Silva, R.N.C.
558 Guedes, H.A.A. Siqueira, Spinosad and the Tomato Borer *Tuta absoluta*: A
559 Bioinsecticide, an Invasive Pest Threat, and High Insecticide Resistance, PLoS One 9
560 (2014) e103235.
561 [29] M.R. Campos, T.B.M. Silva, W.M. Silva, J.E. Silva, H.A.A. Siqueira,
562 Susceptibility of *Tuta absoluta* (Lepidoptera: Gelechiidae) Brazilian populations to
563 ryanodine receptor modulators, Pest Manage. Sci. 71 (2015) 537-544.
564 [30] R.J. Wright, M.E. Scharf, L.J. Meinke, X. Zhou, B.D. Siegfried, L.D. Chandler,
565 Larval susceptibility of an insecticide-resistant Western Corn Rootworm (Coleoptera:
566 Chrysomelidae) population to soil insecticides: laboratory bioassays, assays of
567 detoxification enzymes, and field performance, J. Econ. Entomol. 93 (2000) 7-13.
568 [31] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D.
569 Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of
570 protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76-85.
571 [32] K. van Asperen, A study of housefly esterases by means of a sensitive colorimetric
572 method, J. Insect Physiol. 8 (1962) 401-416.
573 [33] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases. The first
574 enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130-7139.

575 [34] R.L. Rose, W.A. Brindley, An evaluation of the role of oxidative enzymes in
576 Colorado potato beetle resistance to carbamate insecticides, Pestic. Biochem. Physiol.
577 23 (1985) 74-84.
578 [35] W.S. Abbott, A method of computing the effectiveness of an insecticide, J. Econ.
579 Entomol. 18 (1925) 265-267.
580 [36] D.J. Finney, Probit Analysis, London, England, 1971.
581 [37] LeOra-Software, POLO-Plus, POLO for Windows computer program, version 2.0.
582 LeOra-Software, Petaluma, CA., 2005.
583 [38] J.L. Robertson, R.M. Russell, H.K. Preisler, Savin, N.E., Bioassays with
584 arthropods, 2ª ed., CRC Press Taylor & Francis Group, Boca Raton, FL, 2007.
585 [39] SAS Institute, SAS user's guide: statistics, version 8.2. SAS Institute, Cary, NC,
586 2001.
587 [40] R. Feyereisen, Insect P450 Enzymes, Annu. Rev. Entomol. 44 (1999) 507-533.
588 [41] J.D. Hayes, J.U. Flanagan, I.R. Jowsey, Glutathione transferases, Annu. Rev.
589 Pharmacol. Toxicol. 45 (2005) 51-88.
590 [42] R. Heidari, A.L. Devonshire, B.E. Campbell, S.J. Dorrian, J.G. Oakeshott, R.J.
591 Russell, Hydrolysis of pyrethroids by carboxylesterases from *Lucilia cuprina* and
592 *Drosophila melanogaster* with active sites modified by in vitro mutagenesis, Insect
593 Biochem. Mol. Biol. 35 (2005) 597-609.
594 [43] W. Hongchun, L. Jun, L. Bo, L. Yuanlai, D. Liyao, The role of cytochrome P450
595 monooxygenase in the different responses to fenoxaprop-P-ethyl in annual bluegrass
596 (*Poa annua* L.) and short awned foxtail (*Alopecurus aequalis* Sobol.), Pestic. Biochem.
597 Physiol. 107 (2013) 334-342.

598 [44] W. Wang, J. Mo, J.a. Cheng, P. Zhuang, Z. Tang, Selection and characterization of
599 spinosad resistance in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), Pestic.
600 Biochem. Physiol. 84 (2006) 180-187.
601 [45] D. Wang, X. Qiu, X. Ren, F. Niu, K. Wang, Resistance selection and biochemical
602 characterization of spinosad resistance in *Helicoverpa armigera* (Hübner) (Lepidoptera:
603 Noctuidae), Pestic. Biochem. Physiol. 95 (2009) 90-94.
604 [46] M. Reyes, K. Rocha, L. Alarcón, M. Siegwart, B. Sauphanor, Metabolic
605 mechanisms involved in the resistance of field populations of *Tuta absoluta* (Meyrick)
606 (Lepidoptera: Gelechiidae) to spinosad, Pestic. Biochem. Physiol. 102 (2012) 45-50.
607 [47] P. Bielza, V. Quinto, J. Contreras, M. Torné, A. Martín, P.J. Espinosa, Resistance
608 to spinosad in the western flower thrips, *Frankliniella occidentalis* (Pergande), in
609 greenhouses of south-eastern Spain, Pest Manage. Sci. 63 (2007) 682-687.
610 [48] N. Liu, X. Yue, Insecticide Resistance and Cross-Resistance in the House Fly
611 (Diptera: Muscidae), J. Econ. Entomol. 93 (2000) 1269-1275.
612 [49] J.Z. Zhao, Y.X. Li, H.L. Collins, L. Gusukuma-Minuto, R.F. Mau, G.D.
613 Thompson, A.M. Shelto, Monitoring and characterization of diamondback moth
614 (Lepidoptera: Plutellidae) resistance to spinosad, J. Econ. Entomol. 95 (2002) 430-436.
615 [50] P. Bielza, V. Quinto, E. Fernández, C. Grávalos, J. Contreras, Genetics of Spinosad
616 Resistance in *Frankliniella occidentalis* (Thysanoptera: Thripidae), J. Econ. Entomol.
617 100 (2007) 916-920.
618 [51] F.D. Rinkevich, M. Chen, A.M. Shelton, J.G. Scott, Transcripts of the nicotinic
619 acetylcholine receptor subunit gene Pxyla6 with premature stop codons are associated
620 with spinosad resistance in diamondback moth, *Plutella xylostella*, Invertebr. Neurosci.
621 10 (2010) 25-33.

622 [52] G.B. Watson, S.W. Chouinard, K.R. Cook, C. Geng, J.M. Gifford, G.D. Gustafson,
623 J.M. Hasler, I.M. Larrinua, T.J. Letherer, J.C. Mitchell, W.L. Pak, V.L. Salgado, T.C.
624 Sparks, G.E. Stilwell, A spinosyn-sensitive *Drosophila melanogaster* nicotinic
625 acetylcholine receptor identified through chemically induced target site resistance,
626 resistance gene identification, and heterologous expression, Insect Biochem. Mol. Biol.
627 40 (2010) 376-384.
628 [53] K. Haddi, M. Berger, P. Bielza, D. Cifuentes, L.M. Field, K. Gorman, C.
629 Rapisarda, M.S. Williamson, C. Bass, Identification of mutations associated with
630 pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner
631 (*Tuta absoluta*), Insect Biochem. Mol. Biol. 42 (2012) 506-513.
632 [54] W.M. Silva, M. Berger, C. Bass, V.Q. Balbino, M.H.P. Amaral, M.R. Campos,
633 H.A.A. Siqueira, Status of pyrethroid resistance and mechanisms in Brazilian
634 populations of *Tuta absoluta*, Pestic. Biochem. Physiol. 122 (2015) 8-14.
635

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

Population	DD[a]$_1$ 0.25 mg Al/l	DD[b]$_2$ 5 mg Al/l	R-Allele Freq (%)	Gen Freq - SS(%)	Gen Freq - RS(%)	Gen Freq - RR(%)
América Dourada – BA	100	100	0	100	0	0
Anápolis – GO	97 ± 3	100	2.5	95	5	0
Brasília – DF	83 ± 9	100	2.5	95	5	0
Gameleira 2 – BA	83 ± 7	100	0	100	0	0
Gameleira 1 – BA	61 ± 21	100	10	80	20	0
Guaraciaba do Norte - CE	71 ± 14	100	0	100	0	0
Iraquara – BA	7 ± 3	21 ± 4	67.5	10	45	45
João Dourado - BA I	54 ± 33	97 ± 3	10.5	78.9	21.1	0
João Dourado - BA II	69 ± 7	100	0	100	0	0
João Dourado - BA III	93 ± 3	100	12.5	75	25	0
Lagoa Grande – PE	97 ± 3	100	5	90	10	0
Paulínia – SP	100	100	12.5	75	25	0
Pelotas – RS	100	100	0	100	0	0
Pesqueira – PE	100	100	0	100	0	0
Sumaré – SP	100	100	7.5	85	15	0
Tianguá – CE	100	100	0	100	0	0
Venda Nova – ES	83 ± 7	100	0	100	0	0

[a] Diagnostic doses – 0.25 mg Al/l. [b] diagnostic doses-5 mg Al/l *: susceptible allele - S; resistant allele - R

Table 2. Susceptibility of *Tuta absoluta* strains to spinosad.

Population	N[a]	Slope ± SE[b]	LC_{50} (CI95%)[c]	LC_{80} (CI95%)[c]	χ^2 DF[d]	RR_{50} (CI95%) [e]	RR_{80} (CI95%) [e]
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)

[a] Total number of larvae bioassayed. [b] Standard error. [c] Milligrams spinosad per liter water. [d] Chi-squared and Degree of Freedom. [e] Resistance ratio: ratio between LC_{50} resistant and LC_{50} susceptible and confidence of interval at 95%, calculated through Robertson at al., (2007) method. * Resistance ratio non-significant if the confidence interval brackets the value 1.0.

Table 3. Synergism of spinosad in susceptible and resistant strains of *Tuta absoluta*.

Population	Treatment	N[a]	Slope ± SE[b]	LC_{50} (CI95%)[c]	χ^2 DF[d]	SR_{50} (CI95%) [e]
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)

[a] Total number of larvae bioassayed. [b] Standard error. [c] Milligrams spinosad per liter water. [d] Chi-squared and Degree of Freedom. [e] Synergism ratio: ratio between LC_{50} non synergized and LC_{50} synergized for each population and confidence of interval at 95%, calculated through Robertson at al., (2007) method. * 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 (± SE) activity of detoxification enzymes in three *T. absoluta* strains.

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

*Means followed by the same letter within column are not statistically different by Tukey's test at 5% probability.

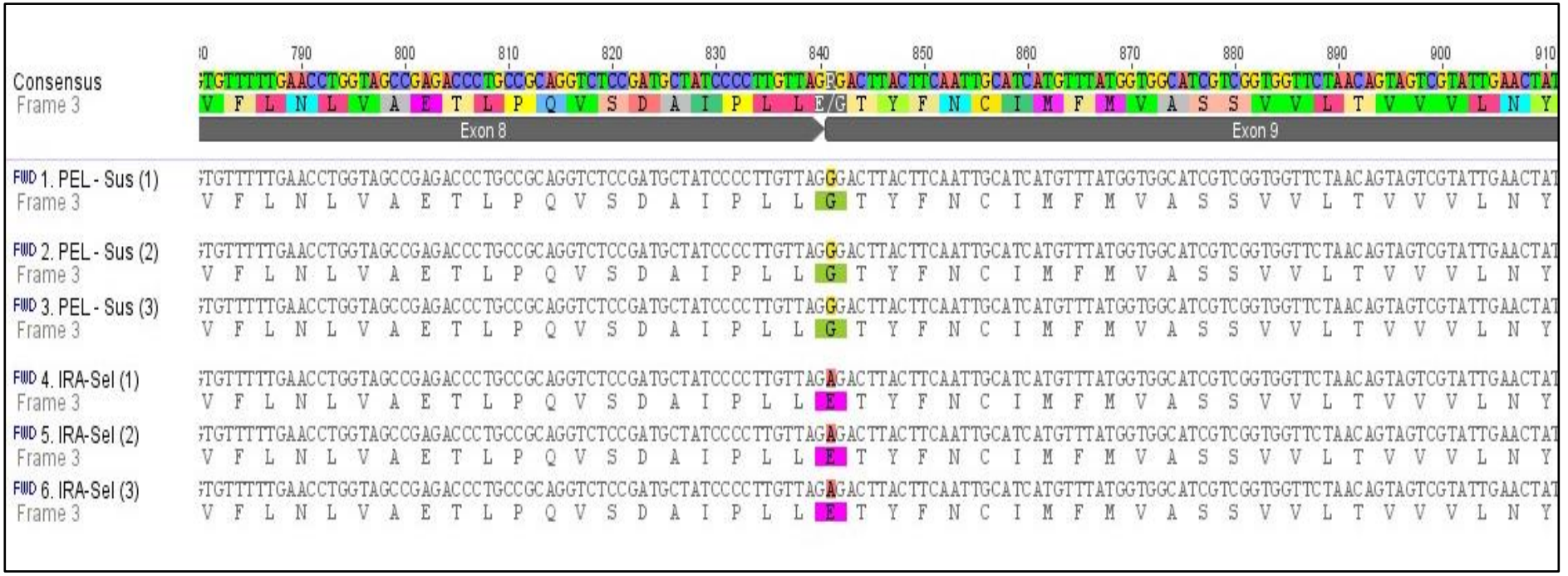

Figure 1. Alignment of nAChr alpha six (Taα6) subunit sequences from the IRA-Sel (spinosad resistant) and Pel (spinosad susceptible) strains of *T. absoluta* showing the presence of an amino acid substitution (G275E) in the resistant strain.

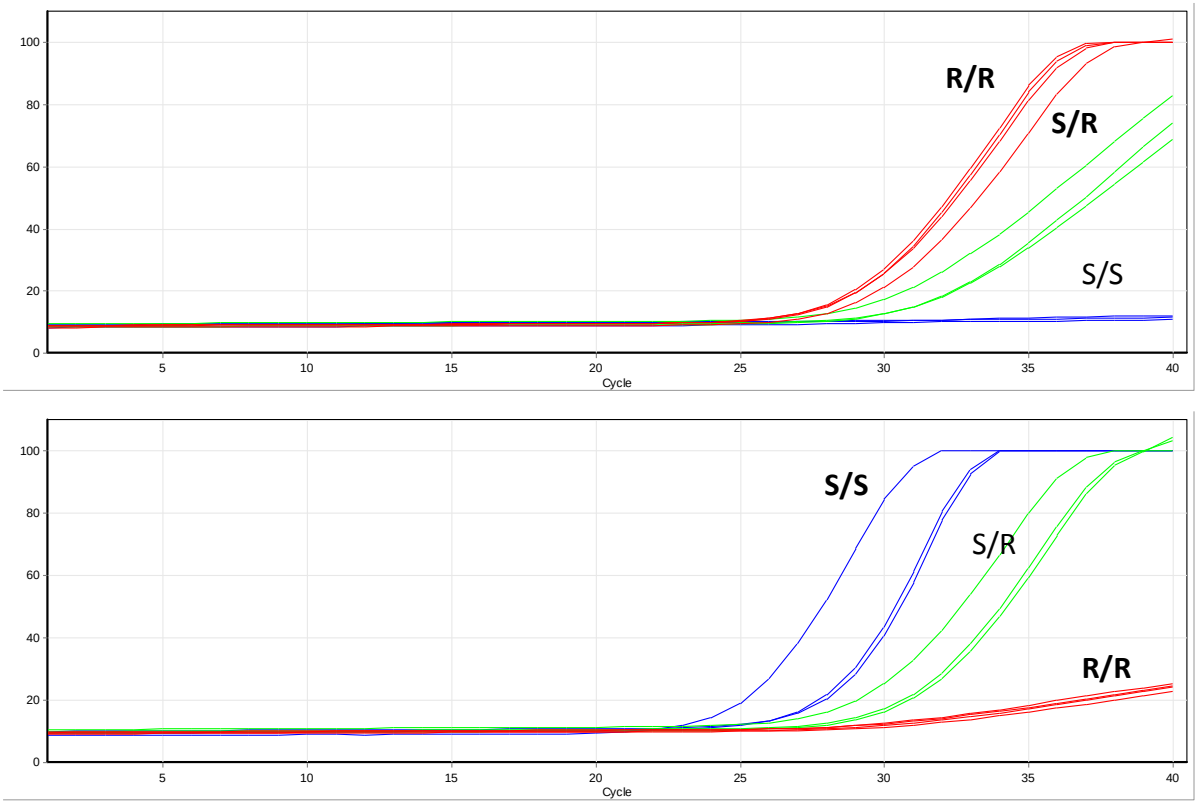

Figure 2. Real-time TaqMan detection of the G275E in *Tuta absoluta*. The top graph shows the FAM-labelled probe specific for the mutant allele, and the bottom graph shows the VIC-labelled probe specific for the wild-type allele. S: wild-type allele; R: resistant allele.