Project title:	**Characterising the molecular basis for insecticide resistance in the tomato leafminer *Tuta absoluta.***
Project number:	CP 162
Project leader:	Charles Grant. University of Exeter
Report:	2018 annual report
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Key staff:	Professor Chris Bass University of Exeter
Location of project:	Penryn Campus. University of Exeter
Industry Representative:	Dr. Rob Jacobson
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[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headlines

Conserve (Spinosad)

- Strong Conserve insecticide resistance prevalent in UK populations of *Tuta absoluta.*
- Two unique mechanisms of resistance Identified.
- Development of diagnostic for one mechanism is completed and shows good detection of resistance genes.

Coragen (Chlorantraniliprole)

- Low level of resistance detected
- Selected lines showed potent resistance
- All known UK and European resistant strains of *Tuta absoluta* sequenced for robust diagnostic capable of rapidly detecting any resistance-associated mutation.

Background

The UK consumes around 500 000 tonnes of fresh tomatoes each year, of which 92 000 tonnes are UK grown and these have a retail value of approximately £190 million. The invasion of *Tuta absoluta* into tomato crops can result in massive yield losses. Developing, implementing and maintaining effective control measures has been an urgent requirement for the UK tomato growing industry. This control has come about through the development of an integrated pest management strategy (IPM), incorporating biocontrol agents with pesticide applications and pheromone mating disruptors. However, in recent years, this IPM has struggled to take total control of the pest in certain glasshouses. This

is due to, either the evolution of resistance genes within populations or the importing of populations already resistant to the chemistries used, Conserve (spinosad) and Coragen (chlorantraniliprole). Furthermore, the loss of effectiveness of mating disruptors has been reported. These outcomes are alarming and bring into question the long-term effectiveness of this contemporary IPM.

Summary

Conserve

Bioassay results have confirmed the widespread presence of resistance to Conserve in UK populations. Last year's report identified the mechanism of resistance in two populations, an RNA alteration resulting in a truncated acetylcholine receptor (a receptor in the nervous system which is the target of Conserve). A molecular approach to identifying this is possible but currently slow and expensive. We are currently assembling the genome of *T. absoluta* which will help us elucidate the genomic mutation that caused the truncation of the nerve receptor protein. Once this is known, simple, cheap and fast diagnostics will allow rapid assessment of the gain/loss of resistance genes in glasshouse populations allowing 'realtime' resistance assessments.

This year we identified a second mechanism of resistance to Conserve. This mechanism was also an alteration in the same nerve receptor, however this alteration is a deletion of three genomic nucleotides, resulting in exclusion of one single amino acid. The position of this amino acid in the finished folded protein is in a region of the receptor suspected to be the binding site of Conserve. To associate the amino acid deletion with survival to the application of Conserve, individual *T.absoluta* larvae were exposed to a dose of the pesticide expected to kill about 50 % of the population. The dead and alive individuals were then collected and analysed using a specially designed target mutation assay (TaqMan ®). Genotype was scored by the colour of fluorescence

from one of two specific probes which bind discriminately to either the genotype, with the deletion or without the deletion. There was a strong correlation between individuals surviving the test and having the deletion present, as well as between individuals dying in the test and them lacking the deletion. The overall frequency of resistance genes in the population was 0.57. Thirty one individuals were homozygous susceptible, 88 were heterozygous and 56 were homozygous resistant.

Coragen

The low-level presence of Coragen resistance was identified in UK populations using leaf dip bioassays. A low-frequency mutation in the ryanodine receptor (a nerve-muscle junction-receptor in the nervous system) at a location associated with Coragen binding was observed. Suggestion that this was the mechanism of resistance was confirmed when the strain was selected for resistance. The mutation rose in frequency to 100% in the population along with their resistance to the pesticide, which after the final selection experiment was over 4000 times more resistant than susceptible strains. Growers have reported control failure of *T. absoluta* by Coragen in this season's crops. Samples from these glasshouses will be tested to assess any rise in frequency of this mutation within the population as predicted by the selection experiment.

The development of a molecular diagnostic is under way. The genetic mutation we are targeting with diagnostic is already present at low frequency in the population and so, it is likely that this resistance mechanism was imported from European populations. To ensure the robustness of the diagnostic we are collaborating with research groups in Europe in an attempt to incorporate all known resistance associated mutations into one diagnostic. This would mean the diagnostic would be sensitive to any further imported resistant genotypes.

Financial Benefits

- Molecular diagnostics are cheaper and faster than traditional bioassays.
- Early detection of resistance populations within the glasshouses.
- Inform whether pesticide application will have the desired effect.
- Reduce mismanagement of pesticides.
- Slow evolution of resistance genes.

Action Points

- Stop the use of Conserve (spinosad) and continue to monitor resistance in the field to see if there is any return of efficacy.
- Monitor effectiveness of Coragen (chlorantraniliprole) closely and report any application failures so resistance to those populations can be assessed.

SCIENCE SECTION

Introduction

Resistance of *Tuta absoluta* to both Conserve and Coragen has been reported and confirmed in European populations. In light of reported control failures by these compounds in the UK, it was suspected that resistance was now present. Resistance could have arisen either through the importing of individuals from resistant populations in Europe or could have evolved independently in non-resistant populations.

Previously, this project has confirmed that resistance to Conserve and Coragen is present in UK populations and uncovered mechanisms of resistance of both compounds. Resistance to Coragen was explained through the identification of a genomic mutation in the ryanodine receptor gene. The ryanodine receptor is situated at the interface between nerve and muscle cells and its excitation by Coragen causes muscle spasms and death. The mutation was located at the binding site of Coragen, a site previously associated with resistance in European populations.

Resistance to Conserve was attributed to a major alteration in RNA transcripts that encode a nerve receptor protein (nicotinic acetylcholine receptor). A sub-unit of this receptor (the α6 sub-unit) is the target site of Conserve and intoxication by the compound results in uncontrolled nerve messaging, paralysis and death. One site in the UK however showed resistance to Conserve but no RNA alterations. Recent developments in this project uncovered a second mechanism of Conserve resistance, also a novel mechanism of resistance. This population from Lancashire (LAN) had a triplet deletion in the genomic DNA of a suspected Conserve target site on the nAChR gene. In this current report we carry out phenotypic and molecular characterisation of this new mechanism.

Materials and methods

Insect strains

Strains of *Tuta. absoluta* were collected from Flavourfresh Salads Ltd, Lancashire (LAN). Insects were housed in controlled environment rooms at 25°C, 60% R.H., 16:8 light-dark cycle and fed *ad libitum* on tomato plants (var. Money Maker).

Leaf-dip assays

Leaf-dip bioassays were completed according to test method 22 guidelines produced by the Insecticide Resistance Action Committee (IRAC http://www.irac-online.org/content/uploads/Method_022_Tuta.pdf).

RNA extraction and cDNA synthesis

All *T. absoluta* were snap frozen in liquid nitrogen and stored at -80°C prior to RNA extractions. RNA extractions were carried out using ISOLATE II RNA Mini Kit (Bioline). cDNA was then synthesized from 1µg of total RNA using Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific) according to their protocol.

Nicotinic acetylcholine receptor amplification (nAChR)

nAChR amplification was conducted using DreamTaq Green PCR master mix (Thermo Fisher Scientific) with a nested PCR protocol. Both primer pairs were designed using Geneious (v8.1.9, Biomatters Ltd) and synthesized by Sigma Aldrich. 1µg (1µl) of cDNA was used in the first cycle procedure. This product was then diluted 1:10 in water. 1µl of the diluted solution was used in the second reaction. Both PCR cycle protocols were as above.

PCR purification

PCR products were purified with GeneJET PCR purification kit (Thermo Scientific) in accordance with their protocol. Purified DNA was then quantified using a spectrophotometer (NanoDrop, Thermo-Fisher).

Sequencing

All sequencing was completed by Eurofins Genomics with samples submitted in accordance with their guidelines and analyzed using Geneious (v8.1.9, Biomatters Ltd).

Association studies - Spinosad

10 larvae were placed on a tomato leaf treated with 150 ppm of spinosad, this was replicated 20 times. For the control 10 larvae were placed on a tomato leaf treated only with dilutant, this was replicated 5 times. Mortality was scored after 72 h. all larvae were snap-frozen in liquid nitrogen and stored at -80°C.

TaqMan PCR

To associate the genotype with survival TaqMan assays were used to assess dead and alive individuals. DNA was extracted from over 200 individual larvae using DNAzol. Primers and MGB probes were designed by Thermo Fisher. Each probe was labelled with a 5' reporter dye specific to both wild-type and mutant alleles. VIC reporter dyes detected the wild-type allele and 6FAM reporter dye detected the mutant allele. Each probe also had a 3' non-fluorescent quencher. Dissociation of the reporter dye from the probe, and therefore the non-fluorescent quencher, results in emission of a photon of wavelength specific to the reporter dye used and therefore specific to each allele. PCR reactions contained 2 µl genomic DNA, extracted from individual insects using DNAzol reagent, 7.5 µl of SensiFAST SYBR No-ROX kit (Bioline), 800nM of each primer and 200nM of each probe. Samples were run on CFX96 system (Bio-Rad) using

the temperature cycling conditions of 10 minutes at 95°C followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. The increase in fluorescence of the two probes VIC and FAM was monitored in real time. In all runs, at least one control for each genotype (mutant homozygous, heterozygous and wild-type homozygous) was included. Genotype was then scored by the fluorescence of either reporter dye for homozygous individuals or the fluorescence of both reporter dyes for heterozygotes. The significance of the association between the triple deletion to the survival of spinosad was examined using Chi-square analysis.

Results

Leaf dip assay

LAN strain showed tolerance to spinosad with an LC 50 (149 ppm) higher than the recommended field application rate of around 87-100 ppm.

nAChR sequencing

Amplification and sequencing of the nAChR cDNA exposed one novel alteration in resistant population LAN that had not been reported in other resistant UK populations, a triplet deletion in at position F238 (fig. 1).

Association studies - LAN triplet deletion

193 individuals were successfully exposed to 150 ppm of spinosad; 108 died, 30 were affected (alive but showed no coordinated response to stimuli) and 55 survived the treatment. This was in contrast to the control treatment in which, out of 50 individuals, 2 died and 48 survived. The application of spinosad is significantly associated with the survival of the individual. (Fishers exact test of independence, $p < 0.01$).

TaqMan assay

175 individuals from the discriminating dose assay were successfully assayed with Taqman using MGB probes designed to bind to the wildtype and mutant alleles discriminately. The resistant allele frequency in the population was 0.57. 31 individuals were homozygous wildtype, 88 were heterozygous and 56 were homozygous mutant (fig. 2). The application of spinosad is significantly associated with the survival of the individual ($\chi 2$ (4) = 78.499, $p < 0.001$). Further analysis showed individuals in the dead group had a lower than expected proportion of the resistant genotype ($p < 0.001$) and a greater than expected proportion of susceptible ($p < 0.001$) and heterozygous genotypes ($p < 0.001$). In contrast, individuals surviving the assay had a higher than expected proportion of the resistant genotype ($p < 0.001$) and a lower proportion of susceptible ($p < 0.001$) and heterozygous ($p < 0.001$) genotypes. The genotype frequencies of the affected group did not deviate statistically from those expected by chance.

Figure 1. A) Amplification of nAChR gene from cDNA showing location of triplet deletion in the red dashed circle. B) Zoomed in sequence view of triplet deletion in LAN strain compared to strains with previously characterised mechanisms. C) Table showing conservation of F238 region among Arthropoda, the pink box indicated the triplet deletion in the LAN strain.

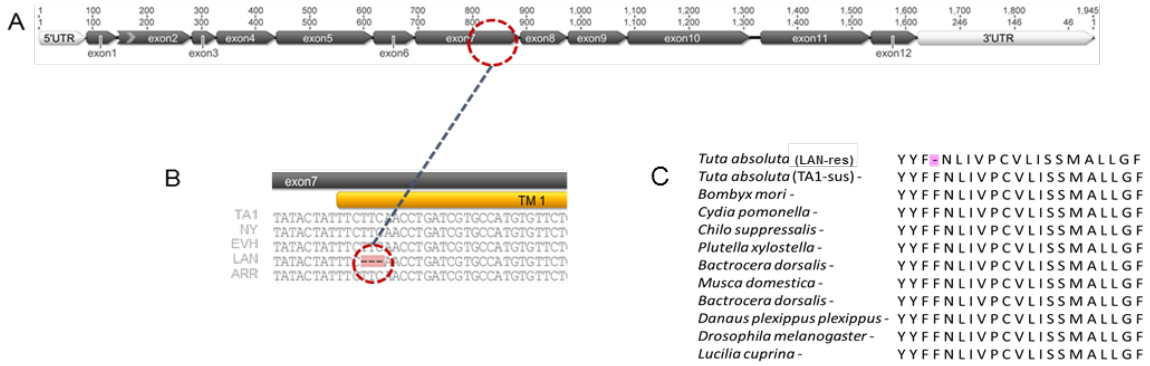
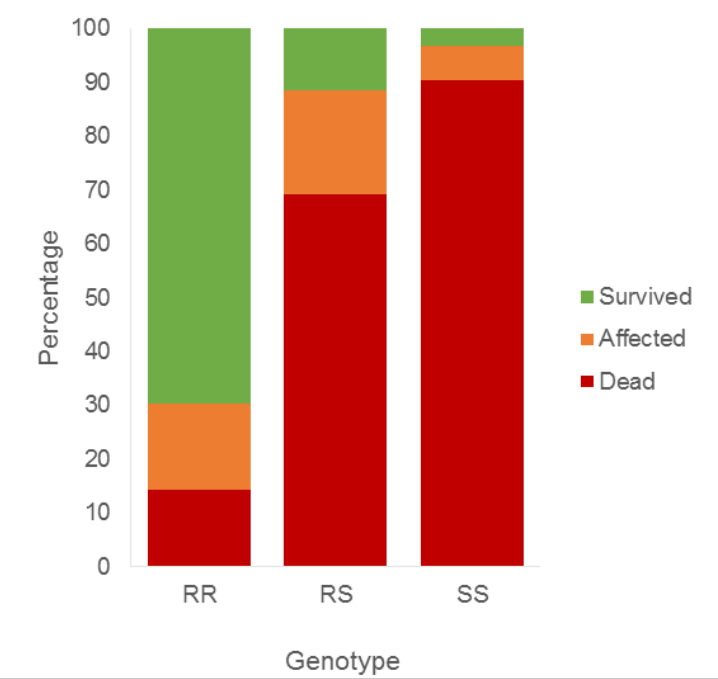


Figure 2. A graph showing the percentage of Dead, Affected and Survived individual for each genotype when exposed to 150ppm of spinosad. RR = Homozygous resistant, RS = Heterozygotes and SS = Homozygous susceptible.



Discussion

The triplet deletion observed in transcripts of *nAChR* is previously undescribed and results in the loss of a phenylalanine amino acid at position F238. Statistical support of the causal role of this mutation in conferring resistance was revealed with the co-segregation of the F238 deletion with the resistant phenotype in survival bioassays.

The location of the deletion provides further evidence of its causal role in resistance. F238 is positioned in a trans-membrane region of the nAChR α sub-unit. Amongst Arthropoda transmembrane regions are highly conserved (fig. 1C). This suggests functional constraints on this region of the gene, preventing natural variation. This makes any alteration extremely unlikely and consistent with typical resistance mutations. This finding is consistent with previous work on spinosad that shows it binds to transmembrane sites and it is therefore likely that the F238 deletion directly modifies the spinosad binding site (Puinean *et. al.* 2013). Furthermore, transmembrane domains have been associated with pesticide binding in other nerve receptors with similar structures to the nAChR, such as the binding of ivermectin to the glutamate-gated chloride channel transmembrane domains (Hibbs *et. al.* 2011).

The F238del mutation has only been observed in UK strains of *T. absoluta.* This suggests that the mutations identified in this study arose independently in UK populations rather than being imported via infected plant material from outside the UK. The fact that evolution of resistance to spinosad has occurred many times independently, by multiple mechanisms and that the insects with these resistance-alterations show no great loss of fitness suggests that the nAChR α6 subunit is a poor target site for pesticides.

Conclusions

- Novel mechanism for spinosad resistance found in *T. absoluta* populations from Lancashire.
- F238 deletion mutation was detected in trans-membrane spinosad binding region.
- F238 deletion was statistically associated with resistant phenotype.
- Multiple resistance mechanisms to spinosad have rapidly evolved in the field potentially rendering spinosad redundant.

Knowledge and Technology Transfer

- AHDB 2018 student conference.
- CLES Student research talk.
- Article published in the AHDB Grower Journal.
- Manuscript for Pest Management Science currently in preparation.

References

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spinosad in *Frankliniella occidentalis*. *Journal of Neurochemistry*; 124(590-601 DOI Electronic Resource Number (2013).

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