Project title:	**Characterising the molecular basis for insecticide resistance in the tomato leafminer *Tuta absoluta.***
Project number:	
Project leader:	Charles Grant. University of Exeter
Report:	2019 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

- Coragen remains effective in the UK although target-site resistance genes are present in UK populations.
- Spanish strains of *T. absoluta* show strong levels of resistance to Coragen.
- Over expression of detoxification genes confers metabolic resistance in Spanish strains.
- No quarantine on *T. absoluta* to the UK.
- Implementation of new control measure (Isonet-T - mating disruption) has been successful in control of *T. absoluta.*
- UK population could have the capacity to reproduce asexually.

Background

The tomato leaf miner *Tuta absoluta* is a highly destructive pest of economically important crops in the family solanaceae including tomatoes. It arrived in the UK from Spain in 2006(1) and is now so well established in tomato grow houses it is no longer identified as a quarentinable insect. The larvae of this moth are capable of devastating whole crops if left unchecked. Integrated Pest management (IPM) strategies have been extremely successful, however, control levels have fluctuated in glasshouses over the years due to arms-race dynamics initiated by population suppression through the introduction of novel control measures (insecticides and mating disruptors), followed by population resurgence driven by adaptive evolution (resistance).

Previous project reports describing the characterisation of the molecular basis of insecticide resistance in *T. absoluta* (2017, 2018) have clearly shown how the efficacy of the economically important bio-pesticide (conserve) was neutralised through evolution of novel target-site resistance, a resistance resulting in a >480-fold decrease in pesticide effectiveness. These reports also warned of how resistant genotypes to a second contemporary pesticide coragen (class: diamide), were not only present in UK populations but could be further selected for, resulting in a >4000-fold resistance increase (2).

Both previously described resistance mechanisms involve the alteration of the insecticides target-sites. Target-site resistance comes about through mutations that alter the chemical properties of transcribed proteins, typically nerve receptor proteins (3). This reduces the affinity of the pesticide inducing functional inhibition of the receptor. These mutations are then selectively amplified over subsequent generations to fixation culminating in the emergence of population-level resistance. However, a population of *T. absoluta*, originating from Spain, showed strong resistance to the pesticide but genetic analysis reviled an absence of any target-site mutations. This finding suggests other genes may be involved in generating resistance to coragen. The varying resistance phenotypes could potentially arise from differential expression of metabolic detoxification enzymes (4). One aim of this report is to identify the evident second mechanism of resistance to coragen in *T. absoluta*. We test for metabolic resistance, the detoxification of the compound, through the analysis of differentially expressed genes in the two Spanish strains of *T. absoluta*. These strains express either a resistant or susceptible phenotype. Identification of resistance mechanisms will allow for detection of resistant populations in the field informing the response of IPM strategies

IPM strategies have been greatly aided by the introduction of Isonet-T, a mating disruptor. Isonet-T was introduced to the market in 2017 works by inundating a closed glasshouse environments with a synthetic version of the female sex

pheromone. This prevents mate location and stops reproduction. Although highly successful some glasshouses reported lower levels of control. Previous reports into European population of *T. absoluta* show an ability to reproduce through a process called parthenogenesis (asexual reproduction)(5). This process could have the effect of reducing the level of control implemented by the mating disruptor as well as allowing population re-emergence in the absence of the control. Populations of *T. absoluta* were collected from UK glasshouses that had been using Isonet-T in their IPM and observed some variability in their results.

Summary

T. absoluta strains showing both a susceptible and resistant phenotypes were collected from Spain and evaluated for resistance. The ryanodine receptor, a nerve receptor and the target-site for coragen was assessed for mutations but was found to be free from any resistance associated alterations. This suggests other mechanisms of resistance must be functioning. To test for this second mechanism the transcriptomes (all the RNA) were sequenced from resistant and susceptible strains. This provided a list of all the genes that were expressed by the pest. These libraries of expressed genes were then mapped to the *T. absoluta* genome allowing the assessment of their levels of expression. The more RNA molecules of a particular gene, the greater the expression of that gene by the pest. A short list of candidate genes was created by comparing the expression profiles of susceptible and resistant strains of *T. absoluta.* This comparison provided a list of 20 genes that were significantly differentially expressed. These genes were then checked against online databases to assess their function. From this annotated list one gene stood out as a candidate resistance gene, a UGT-glucosyl transferase enzyme. The function of this gene is to detoxify compounds through the binding of a sugar molecule making the toxic compound highly water soluble and therefore easy to excrete. Further analysis of the function of this gene is under way to fully associate its role in the allowing resistance to diamides in *T. absoluta.*

The second aspect of this report was to assess the propensity of *T.absoluta* to reproduce parthenogenetically as this may influence the effectiveness of Isonet-T. The assessment showed that *T. absoluta* did indeed have the capcity to produce offspring in the absence of sex. Virgin females were place in isolated containers with a food source and tomato leaves. 91% of females laid eggs with an average of 14 eggs per female. The average life span of the females was 21 days. Only 4 of these females laid viable eggs resulting in 9 active mines. 4 of these larvae survived to adulthood producing 1 male and three females. These females were isolated at pupa (before sexually mature) and placed in test chambers with food and tomato leaves. 23, 27 and 41 eggs were laid by the females but no mines were detected. These results show that *T.absoluta* may persist in the absence of sex (induced by mating disruption) although with greatly reduced fecundity. This doesn't rule out the potential for the evolution of greater success of parthenogenesis if it has a genetic basis and is under strong selection from mating disruption.

Financial Benefits

- Assessment of current resistance status in UK populations of *T.absoluta* is vital to informing integrated pest management strategies.
- Early detection of resistance populations within the glasshouses can prevent misuse of control measures, inhibit fixation of resistance genes and slow the evolution of resistance.
- It ensures the adaptation of IPM minimising yield loss.

Action Points

- Monitor overall levels of control of current IPM.
- Continue to monitor resistance status of UK populations to diamides using the knowledge gained of resistance mechanisms evolved in *T. absoluta.*
- Where molecular resistance occurs suggest hiatus of diamide use to prevent total loss of the compound.
- Continue to monitor efficacy of Isonet-T. Reassess asexual reproductive capacity over time in the presence of the mating disruptor with a specific emphasis on glasshouses with *T. absoluta* presence.

SCIENCE SECTION

Introduction

The tomato leaf miner, *Tuta absoluta,* is an invasive pest species of economically important crops in the family solanacae. It radiated from South America, arriving in Europe in 2006(1). Upon arrival to the UK, in 2009, it became so well established in tomato glasshouses that it is no longer classified as a quarentinable pest. The larvae of this moth are capable of devastating whole crops if left unchecked. Development of Integrated pest management (IPM) strategies allowed for successful control of the pest - incorporating use of good farming practice, pesticides and bio control. However, continuous adjustments to IPMs are required to maintain control. Part of this process is developing an understanding of the pests' biology, specifically the adaptations of the pest that results in phenotypes resistant to contemporary control measures in order to inform reactive control.

In 2017 a new product, Isonet-T was developed by Shin Etsu Chemical Co. Ltd. and incorporated into *T. absoluta* IPM. Isonet-T is a mating disruptor and when deployed it emits high levels of a synthetic pheromone into the environment. This impedes the male moths assessment of semiochemical concentration gradients emitted by females. This inhibits location of a mate and thus prevents reproduction. The incorporation of Isonet-T into contemporary IPM was demonstrably successful, and was found to eradicate outbreaks in as little as one generation. Using an alternative mode of pest control isonet-T had the added benefit of reducing the reliance on existing chemical controls, relaxing selection for pesticide resistance. Some glasshouses however, reported a reduced efficacy of the product. The effectiveness of mating disruption in eliminating pest populations can be severely compromised if the target pest does not require sex to reproduce. Research by Megido and Verheggen (5) previously indicated that French populations of *T. absoluta* have the capacity to reproduce asexually

through deuterotokous parthenogenesis. The authors found that 4 out of 20 virgin females laid viable eggs. Therefore it is vital to assess the level of this process in UK populations, as this would inform IPM strategy.

Complementing Isonet-T in current *T. absoluta* IPM is the diamide class of insecticides. Diamides have been in commercial use for over 10 years and the first report of diamide resistance in *T.absoluta* was in 2015 (6) . Furthermore studies have also reviled both the target-site of diamides (7), the ryanodine receptor, as well as point mutations resulting in amino acid substitutions (G4903E and I4746M) (3) in the receptor conferring resistance. The ryanodine receptor is located in the sarco/endoplasmic reticulum and is part muscular/nervous system (8). It is responsible for controlling calcium ion signalling by helping to control coordinated regulated muscular contractions. The binding of diamides to the ryanodine receptor results in extended excitation of the receptor preventing standard controlled release of Ca^{2+} into the muscle and central nervous system. The pesticide binds to the wild type receptor with high affinity resulting in prolonged association. This prolonged association causes functional impairment through extended periods of excitation, this excitation then results in uncontrolled Ca^{2+} release (9) leading to muscular spasms effectively rendering the insect paralyzed.

The above described resistance mutations result in the alteration of the insecticides target-site. This alters the biochemical properties of the gene product-pesticide interaction, decreasing the pesticides affinity for its specific binding site. With the repeat application of diamides these mutations are then selectively amplified throughout the population until reaching fixation (2) resulting in population level resistance. Resistant *T. absoluta* populations collected from a Spainsh glasshouse however, lacked any target-site mutations previously described in UK populations. This finding suggests that other mechanisms may contribute to the adapted arsenal of resistance mechanisms to IPM that have evolved in *T. absoluta*. One potential mechanism is the up regulation of metabolic enzymes, enzymes involved in detoxification of xenobiotics (4). These metabolic resistance enzymes would have historically, typically evolved to bio transform

plant toxins in a stepwise process nullifying the effects of the plants defensive secondary metabolites (10). The Functional cross over and phenotypic plasticity of these metabolic enzymes (through their differential expression) has long been associated with the ability to detoxify pesticides and is well documented in many insect species, for a broad range of pesticides and with varying modes of action. Evidence of new mechanisms is of concern to UK growers as these may be imported or evolve independently in the UK potentially limiting the efficacy of this pesticide class and overall IPM.

The aim of this report is to measure the rate of asexual reproduction in UK populations of *T. absoluta* in response to a reported reduced efficacy of Isonet-T and to identify the evident disparate second mechanism of resistance to diamides observed in *T. absoluta*. We will try to identify a candidate metabolic process for resistance through the analysis of differentially expressed genes. We will map the transcriptomes from two strains of *T. absoluta* expressing either a resistant or susceptible phenotype, but no target site resistance, to an assembled genome of *T.absoluta.*

Materials and methods

Insects

T.absoluta were collected from Sandylands nursery, Evesham (EVH). Diamide resistant (Sres) and suceptable (Ssus) *T absoluta* were aquired from Syngenta Switzerland. All 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).

Parthenogenesis.

Pupae were extracted from culture and sexed under a microscope. 100 female pupae were placed in individual chambers. A plastic beaker with a hole in the bottom was placed in another plastic beaker containing 100ml of water and fertilizer. The petiole of a tomato leaf was placed through the hole. A 1.5ml Eppendorf containing saturated sugar water and bunged with cotton wool was placed in the chamber as a food source for the adult moth. The chambers were covered with a double layer of fine cloth mesh. Longevity of females was recorded. Eggs were counted daily and leaves monitored for mining. Any offspring that pupated were sexed and placed in new chambers as described above and the same observations were recorded.

Bioinformatics

Next generation sequencing was conducted on DNA extractions from inbred lines of *T. absoluta* (6 generations). Genome assembly was generated using Illumina paired end reads and Pac-bio long read libraries. Transcriptome resources were acquired from Syngenta Switzerland and mapped to the genome using HiSat2. Differential gene expression analysis was conducted in Blast2Go pro. Mapped transcripts were visualised and assessed for pesticide target-site (ryanodine receptor) polymorphisms using Geneious R10.

Results

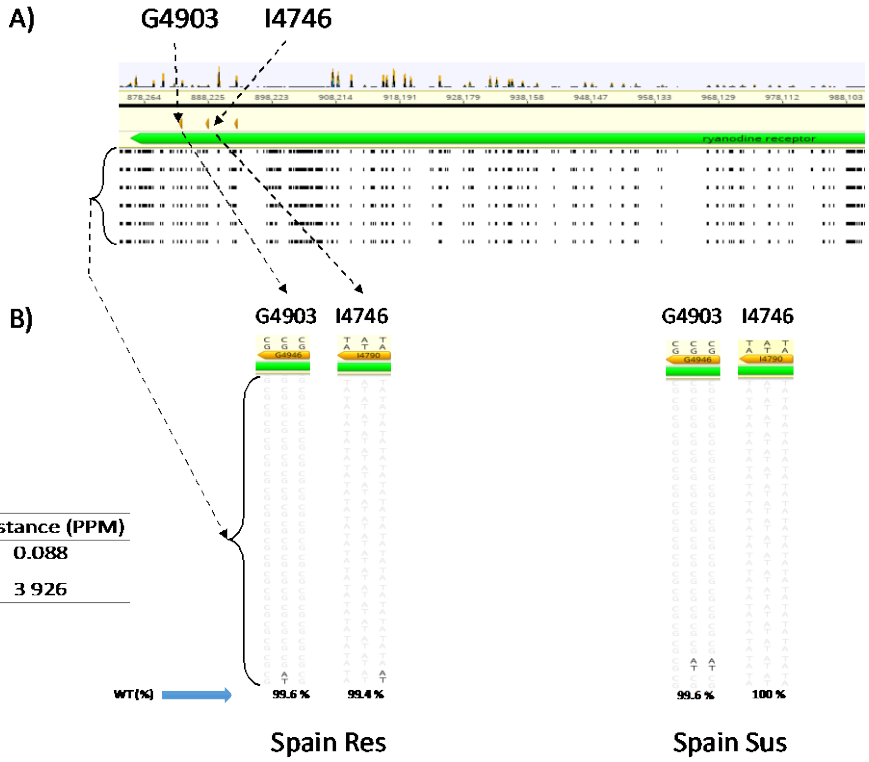
Parthenogenesis in EVH

Of the 100 female pupae sexed, 92 eclosed successfully. Of the 92 adult females, 84 laid eggs with an average of 14.27 per female, and lived for an average of 21.29 days. 4 females laid viable eggs resulting in 9 active mines being detected. Of these 9 mines, 4 larvae survived to adulthood, each from a different parent with the sex ratio being 1:3 male to female. The F1 virgin females laid 23, 27 and 41 eggs and lived for an average of 24.67 days. No mines were detected from these females.

Target site resistance

Alignment of transcripts to genomic resources showed no mis-sence mutations (see fig 1) suggesting no target-site alterations were contributing to the resistant phenotype. Search for a resistance candidate gene in the absence of target-site resistance gave a shortlist of 20 genes based on a fold change greater than 2. Sequence alignments to a blast database were used to identify gene function (fig. 2). From this list, gene 995, (UDP-glucosyl transferase) was identified as the most likely candidate for a resistance associated gene. This was based on differences in expression between the resistant and susceptible Spanish strains (fold change - 117), the significance of the fold change (<0.05) and the genes function (detoxification enzyme).

Fig 1. A) Coverage of transcripts (black lines) mapped to the ryanodine receptor (green bar) with positions of target-site alterations G4903 and I4746 indicated with dashed arrows. B) Close up of mapped regions for both resistant and susceptible phenotypes showing the sequence and its percentage identity to the wild type. C) Table showing resistance to diamides for the two phenotypes in parts per million (ppm)



C)

Strain	Location	Resistance (PPM)
Spain-Sus	Spain	0.088
Spain-Res	Spain	3 926

Fig 2. Table of genes identified as potential targets including sequence description regulation pattern fold change and significance.

Name	Sequence Description	Regulation	Fold change	Significance
g995	UDP-glucosyl transferase	[UP]	117	1.84E-14
g26926	U3 small nucleolar ribonucleoprotein protein MPP10	[UP]	15	1.00E-17
g23043	succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial-like	[UP]	13	5.98E-09
g31908	uncharacterized protein LOC113235291	[UP]	12	4.19E-16
g24845	uncharacterized protein LOC113226571 isoform X2	[UP]	12	0.002669776
g21016	guanine nucleotide-binding protein G(s) subunit alpha isoform X2	[UP]	11	1.32E-10
g2288	esterase FE4-like	[UP]	10	1.64E-06
g1159	uncharacterized protein LOC113398902 isoform X2	[UP]	5	9.01E-10
g6361	uridine diphosphate glucose pyrophosphatase-like	[UP]	4	2.39E-08
g33646	hypothetical protein g.15997	[UP]	4	5.72E-05
g33632	cytochrome P450 9e2-like	[UP]	4	3.16E-05
g33634	cytochrome P450 9e2-like	[UP]	4	0.000237
g6681	Alpha-endosulfine	[UP]	4	1.98E-06
g33631	cytochrome P450 9e2-like	[UP]	3	0.012399409
g33645	probable cytochrome P450 9f2	[UP]	3	0.00115187
g33633	cytochrome P450 9e2-like	[UP]	2	0.003166875
g3890	hypothetical protein BSV51_6126, partial	[UP]	2	9.13E-05
g13729	zonadhesin-like	[DOWN]	-2	0.000408
g13074	CLUMA_CG007581, isoform A	[DOWN]	-3	2.79E-05
g4451	uncharacterized protein LOC113403262	[DOWN]	-3	3.55E-10
g33948	membrane-bound alkaline phosphatase-like	[DOWN]	-4	6.94E-11
g27147	hypothetical protein g.4678	[DOWN]	-4	3.70E-05
g16717	UDP-glucuronosyltransferase 2B19-like	[DOWN]	-4	5.33E-07
g16718	hypothetical protein g.3773, partial	[DOWN]	-4	7.15E-06
g21426	vanin-like protein 2 isoform X2	[DOWN]	-4	5.86E-11
g14844	—NA—	[DOWN]	-5	0.000954
g25933	hypothetical protein BSV51_255	[DOWN]	-7	1.50E-10
g16403	piggyBac transposable element-derived protein 4-like	[DOWN]	-8	8.27E-10
g1122	uncharacterized protein LOC110369752	[DOWN]	-9	1.25E-36

Discussion

The main findings of this report show; Firstly, that parthenogenesis is present in UK population and secondly metabolic resistance to diamides is present in European populations. The presence of parthenogenesis is of concern for the continued effectiveness of isonet-T. While the product has been an overarching success in the UK these results show that it may potentially be incapable of eradicating the pest from the crop meaning a potential resurgence of the pest in the absence of the control measure. Furthermore, if parthenogenesis has a genetic basis then it may be selected for in the presence of mating disruption. Over time a shift in reproductive strategy may result in reduced efficacy of Isonet-T. Trials are currently under way to assess any potential shift in parthenogenetic capacity within populations that have been exposed to Isonet-T for many generations.

In the Spanish populations of *T.absoluta* showing diamide resistance, no target-site alterations were observed. When comparing transcriptomic profiles of resistant vs susceptible phenotypes one gene stood out as a *bona fide* candidate gene. The

resistant phenotype had A 117-fold increase in expression of gene g995. Blast hits matched this sequence to that of a UDP-glucosyl transferrase, a gene that belongs to a large family of detoxification genes. This family of detoxification has been preveiously correlated to diamide resistance lepidopteran species including the diamondback moth (*Plutella xylostella*) (11) and the striped rice stem borer (*Chilo suppressalis*) (12). Functional validation of this gene is currently under way and an important step in understanding its causal nature in detoxification of diamides. Transgenic manipulations of *D. melanogaster* will allow expression of this gene and be used to confirm its role in detoxification of diamides. This will aid understanding of cause of resistance in any emerging diamide resistant UK populations.

Isonet-T and diamides are currently extremely successful in the control of *T. absoluta* in UK populations. The results of this report, however, do suggest that the monitoring of these control measures, in conjunction with each other, would be of value to the industry, as any populations that persist in the face of mating disruption could not be controlled if the evolution/importation of diamide resistance *T. absoluta* resulted in a high prevalence of resistance in the UK. This problem would worsen if the overall success of mating disruption diminished over time.

Conclusions

- UK populations of *T. absoluta* exhibit parthenogenetic capacity.
- This may allow pest populations to persist within crops.
- Metabolic resistance has evolved in European populations of *T. absoluta.*
- UDP-glycosyl transferase enzyme identified as candidate gene.

Knowledge and Technology Transfer

- AHDB 2019 student conference.

- CLES annual student research talk.
- Second manuscript for Pest Management Science currently in preparation.

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