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Population structure and insecticide resistance status of *Tuta absoluta* populations from Turkey

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Abstract

BACKGROUND: *Tuta absoluta* is a devastating pest in tomato production areas worldwide. After its first introduction to Turkey in 2009, it quickly became the major pest of tomato-growing areas. Although some biocontrol agents have been used, especially in greenhouses, the main control of *T. absoluta* relies heavily on chemical insecticides. However, failure in chemical control has often been reported due to resistance development. In this study, we investigated (i) the population structure of 22 *T. absoluta* populations across Turkey by analysing haplotypes, based on the cytochrome oxidase subunit I gene; (ii) the efficacy of three registered insecticides from different classes (metaflumizone, chlorantraniliprole and spinosad) in real field-greenhouse conditions; and (iii) the geographic distribution of target-site mutations associated with insecticide resistance.

RESULTS: The efficacy of spinosad was higher than that of chlorantraniliprole and metaflumizone in the greenhouse trials, as documented by the mortality rates obtained, up to 14 days post application. Known resistance mutations in ryanodine receptors (RyR) (i.e. the I4790M/K and G4946E), nicotinic acetylcholine receptors (G275E), acetylcholinesterases (A201S) and voltage-gated sodium channels (F1845Y and V1848I) were found at various frequencies across the populations genotyped. The I4790K diamide resistance mutation in the RyR has been reported for the first time in *T. absoluta* populations. Although a total of eight haplotypes were found, the overall mean genetic distance was lower than 0.001, indicating the high genetic homogeneity among Turkish *T. absoluta* populations.

CONCLUSION: The results will contribute to design area-wide resistance management programs in *T. absoluta* control in Turkey. However, more monitoring studies are needed to implement evidence-based insecticide resistance management strategies in the frame of integrated pest management.

Supporting information may be found in the online version of this article.

Keywords: insecticide resistance; ryanodine receptors; *Tuta absoluta*; resistance mutations; nicotinic acetylcholine receptor

1 INTRODUCTION

Turkey is the fourth biggest tomato grower country in the world with over 10 million tonnes of annual tomato production.[1] However, crop losses due to biotic and abiotic factors are known to limit this production. The tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is an invasive pest threatening tomato production throughout the world.[2] This pest can cause direct and indirect damage resulting in up to 100% yield losses if not controlled properly.[3]

T. absoluta, originating from South America, was first reported outside its native continent in Spain at the end of 2006.[4] After 3 years, this pest was recorded in the Aegean region of Turkey,[5] and later found spread throughout the Asian continent.[6] The putative genetic bottleneck caused by the recent invasion of *T. absoluta* has been investigated in previous studies,[7,8] although additional studies may be required to fully elucidate the population structure worldwide.

Some biological control agents have been used to control *T. absoluta*, especially in greenhouses.[9–11] In addition, sex pheromone-based monitoring/control strategies, such as mating disruption and mass trapping, have also been considered as promising alternatives to control of this pest.[12] Nevertheless, the

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main control largely relies on chemical insecticides in Turkey.[13] However, due to developing resistance, registered chemicals have often failed to control this pest.[14,15]

Insecticide resistance development has long been known in *T. absoluta* populations even before the spread from their native origin, Chile.[16] This primarily includes quantitative changes in detoxification enzymes as well as altered target-site resistance mutations,[17] which have been recently reviewed by Guedes *et al.*[15] After invasion of large areas, decreasing insecticide efficacy has arisen as a global problem.[14,18] Insecticide resistance mechanisms in *T. absoluta* populations from different continents are quite similar due to low genetic variation probably caused by a single spreading point resulting in genetic bottleneck.[15]

Some insecticides are of particular interest in Turkey: the organophosphates (OPs), such as chlorpyrifos-methyl, the pyrazolines, such as metaflumizone and indoxacarb, the spinosyns, such as spinosad, and the diamides, such as chlorantraniliprole.

Along with carbamates, OPs are one of the oldest groups of insecticides with a long usage history in Turkey and worldwide. They inhibit acetylcholinesterase, which is a key enzyme for the hydrolytic metabolism of the neurotransmitter acetylcholin.[19] As a resistance mechanism to OPs, target-site insensitivity has been known for more than 60 years,[20] and various point mutations have been revealed to confer resistance in both insects and mites.[21,22] An alanine to serine substitution at position 201 (A201S) in *ace-1* has been reported to confer resistance to OPs in *T. absoluta*[23] and other important arthropod pests.[24–27]

Pyrazolines such as metaflumizone and indoxacarb block the voltage-dependent sodium channel, resulting in paralysis of insects.[28,29] Metaflumizone has been used for more than 10 years in Turkey and offers a good alternative in lepidopterous pest control. Two target-site mutations (F1845Y and V1848I) have been associated with indoxacarb resistance in *T. absoluta* [30] and also metaflumizone resistance in *Plutella xylostella*.[31] Both mutations have been functionally validated and found to confer moderate resistance to both insecticides; however, F1845Y caused substantially higher resistance levels to metaflumizone.[32]

Spinosad acts as an allosteric modulator of nicotinic acetylcholine receptors (nAChRs).[33,34] After spinosad resistance was first documented,[35] decreasing susceptibility level of *T. absoluta* populations were reported worldwide.[14,36,37] Resistance to spinosad has been associated with increasing P450 monooxygenase and esterase enzyme activity.[35] On the other hand, Silva *et al.* suggested a target-site mutation, G275E, in a conserved region towards the top of transmembrane domain 3 as a major resistance mechanism against spinosad, instead of metabolic resistance.[37] In addition, an exon 3-skipping event that produces a nonfunctional receptor and thus is unaffected by the spinosad has been described in Spinosad-resistant strains.[38] Also, genetic alterations in the $\alpha 6$ subunit which result in truncated nonfunctional protein have been observed in several insect pest species, including *T. absoluta*.[39–42]

Diamides are a recently registered insecticide group.[43] These insecticides have been classified as ryanodine receptor (RyR) modulators and they have been considered as relatively safe to beneficial arthropods.[44,45] However, shortly after their introduction to the market, resistance appeared in European *T. absoluta* populations.[46] The diamide resistance has been associated with two target-site mutations (I4790M and G4946E/V; *Plutella xylostella* numbering) at RyR of *T. absoluta* that have been validated in further studies.[47–49] Metabolic resistance does not seem to have a major role in diamide resistance in *T. absoluta*.[50,51]

Despite huge economic importance, little is known about insecticide resistance status *T. absoluta* populations in Turkey. Uğurlu Karaağaç[52] reported up to 7.2- and 4-fold resistance ratios in Turkish *T. absoluta* populations to chlorantraniliprole and metaflumizone, respectively.[52] On the other hand, lower than 7.2-fold resistance to chlorantraniliprole, metaflumizone and spinosad was determined in *T. absoluta* populations collected from the Aegean region of Turkey.[53] However, it is not known whether resistance reaches 'practical resistance levels', i.e. if the reported resistance phenotypes reduces pesticide efficacy and has practical consequences for control.[54] Elevated detoxification enzyme activity has been associated with decreasing susceptibility to insecticides,[52,53] but there are no molecular studies available for the presence and frequency of insecticide resistance mutations in *T. absoluta* populations from Turkey so far.

In this study, first, a haplotype network analysis in *T. absoluta* populations using cytochrome oxidase I gene sequences herein obtained and some retrieved from the public GenBank database was performed, to determine the population structure of the pest across the world. We then conducted greenhouse trials using chlorantraniliprole, metaflumizone and Spinosad, which are the most commonly used insecticides in *T. absoluta* control. Finally, we screened the known resistance-associated mutation in *T. absoluta* populations from important tomato production areas of Turkey. The results of this study will contribute to the design of proper insecticide resistance management (IRM) programs in the country.

2 MATERIALS AND METHODS

2.1 *T. absoluta* populations

A total of 22 *T. absoluta* populations were collected from tomato greenhouses in different locations in Turkey. The tomato production of the sampled areas (6.8 million tonnes) accounts for more than half of the total production of the country (12.8 million tonnes). Detailed information about location, insecticide usage history and collection dates of field-collected populations is presented in Table S1 and Fig. 1.

2.2 Chemicals

Commercial formulations of three insecticides registered for *T. absoluta* control were used in field trials (see Table 1 for details).

2.3 Greenhouse insecticide trials

Four greenhouse trials were performed to determine the efficacy of selected insecticides in a randomized complete block design with three replications per treatment and each plot consisted of 20 m^2. Control plots were only sprayed with water. Before experiments, at least 100 tomato plants were checked and trials were performed when more than 10 plants were infected by larvae or

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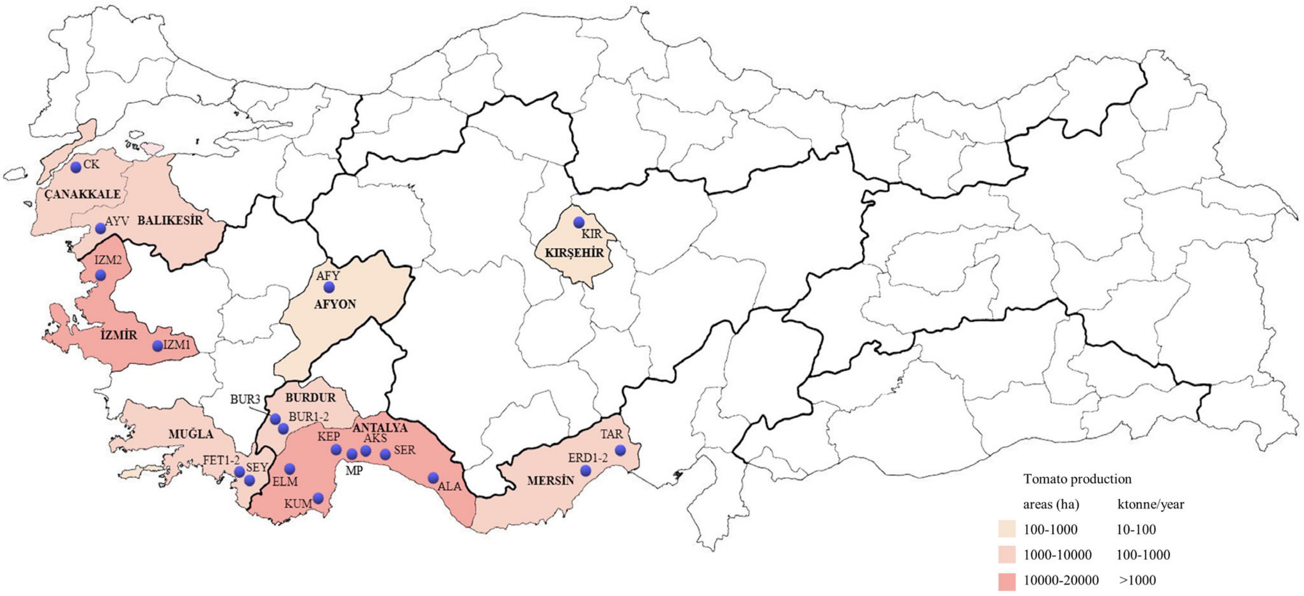

Figure 1. Map of the study area and sampling locations of 22 *Tuta absoluta* populations in Turkey.

eggs of *T. absoluta*. The registered field doses of chlorantraniliprole, metaflumizone and spinosad were applied to plants using a backpack sprayer. Alive larvae on five leaves of 10 plants were counted for each plot 3, 7 and 14 days after treatment (DAT) and the Henderson–Tilton formula was used to determine percentage mortality values.[55] Prior to analyses, equivalence of variance among groups was evaluated using the Levene's test and the Kolmogorov–Simirnov test was performed to test the normal distribution of the data. The data were analyzed using one-way ANOVA with the MINITAB Release 16 package program.[56] The mean differences were grouped using Tukey's test with $\alpha = 0.05$. Data are presented as the mean ± SEM, and $P < 0.05$ was considered statistically significant. More detailed information about greenhouse trials, such as location, application dates, varieties of tomatoes and climate data, is provided in Table S2.

2.4 RNA extraction and cDNA synthesis

RNA was extracted from 10 larvae for each population using a GeneMATRIX Universal RNA Purification Kit (Eurx, Gdańsk, Poland) following the manufacturer's instructions. The quality and purity of RNA were determined by a spectrophotometer (Thermo Scientific NanoDrop 2000). The RNA extracts were stored at −80 °C until further processing.

cDNA synthesis from RNAs isolated from *T. absoluta* populations was performed in two steps. In the first step, the reaction mixture consisted of 1 μg of total RNA and 0.2 $\mu g\ \mu L^{-1}$ random hexamer primer was completed to 12.5 μL with diethyl pyrocarbonate (DEPC)-treated water and denatured at 65 °C for 3 min. The tubes were kept on ice for 5 min. In the second stage, an enzyme mixture containing 100 units of Moloney murine leukemia virus (MMLV) reverse transcriptase enzyme (Thermo Fisher Scientific, Massachusetts, USA), 10 units of RNase inhibitor (Thermo Fisher Scientific, Massachusetts, USA) and 2 μL of 10 μM deoxynucleotide Triphosphates (dNTP) was added in denatured the RNAs mixture and was incubated at 42 °C for 1 h. cDNA was stored at −20 °C until required for further processing.

2.5 Primers and PCR protocols

The primers and PCR protocols used in this study are presented in Table S3. The accession numbers of the target gene sequences were KX519762 for Ryr, JQ701800 for voltage-gated sodium channel (VGSC), KP771859 for nAChR and KU985167 for *ace-1*.[23,37,49,57] The new primers were designed using Primer3.[58] All PCR reactions were performed with a thermal cycler (BioRad T100) in a total volume of 30 μL, including 2 μL of DNA template, 0.5 μL of forward primer, 0.5 μL of reverse primer, 21 μL of distilled water and 6 μL of FIREPol Master Mix (or 5x HOT FIREPol Blend Master Mix when the denaturation time was between 12 and 15 min (see Table S3) (Solis BioDyne, Tartu, Estonia). Electrophoresis on 2% agarose gel in 0.5X TAE buffer for 50 min at 100 V was performed to verify the

Table 1. General information on insecticides used in field trials against *Tuta absoluta*

Active ingredient	Trade name	Formulation	Field dose	AI field rate (g ha^{-1})	IRAC MoA*	Registered date
Metaflumizone 240 g/L	Alverde®	SC	100 mL/da	240	Group 22B	22.04.2009
Chlorantraniliprole 35%	Altacor 35 WG®	WG	10 g/100 L	35	Group 28	10.10.2008
Spinosad 480 g/L	Laser™	SC	25 mL/100 L	120	Group 5	19.11.1998

*Insecticide Resistance Action Committee (www.irac-online.org) mode-of-action classification.

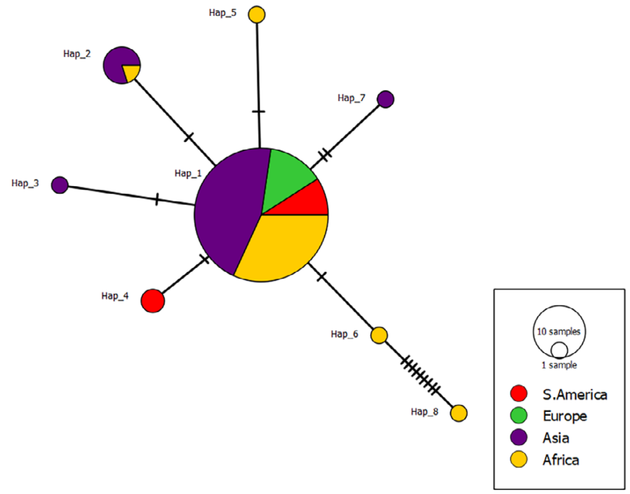

Figure 2. Haplotype network analysis of worldwide populations of *Tuta absoluta*. The sizes of the circles are proportional to haplotype frequency. Each solid line indicates a mutation step interconnecting two haplotypes.

size of the PCR products. All PCR products were sequenced in both directions with the same primers.

The presence and frequency of insecticide resistance mutations were determined based on the heights of fluorescence peaks from sequencing chromatograms as previously described[59] using BioEdit v.7.0.5.[60]

2.6 Analysis of haplotype networks

A total of 72 *COI* sequences herein obtained using Folmer primers[61] and retrieved from the public GenBank database were used to determine the number of haplotypes using DnaSP v6.[62] All the sequences were cleaned and aligned using BioEdit v.7.0.5[60] and MAFFT v.7.[63] PopArt v1.7 was used to draw haplotype networks based on the haplotypes generated by DnaSP.[64]

The *COI* sequences herein obtained were submitted to the National Center for Biotechnology Information database (accession numbers MW774415 to MW774436).

3 RESULTS AND DISCUSSION

3.1 Haplotype network of *T. absoluta* populations worldwide

After alignment, a total of 628 nucleotides belonging to partial sequences of the mitochondrial genes were included in the haplotype and subsequent network analysis, and 612 out of 628 nucleotides were conserved. Although a total of eight haplotypes was found, the overall mean genetic distance, the measure of the evolutionary divergence, was lower than 0.001, indicating the high genetic homogeneity among Turkish *T. absoluta* populations. Hap_1 was determined as the most frequent haplotype (Fig. 2) and all Turkish populations (included within the Asia continent, shown in purple) belong to Hap_1.

High genetic homogeneity among all analyzed populations was determined, similar to previous studies.[7,65] In line with Yükselbaba and Göçmen[66] all *COI* sequences of Turkish populations were identical, therefore no genetic variation among populations from different geographic backgrounds has been observed. Although some degree of genetic variation has been determined in native populations of *T. absoluta*, almost no genetic structuring in *T. absoluta* populations from invaded areas has been found.[8]

3.2 Greenhouse insecticide trials

The results of four greenhouse trials are given in Table 2. ANOVA results showed that the interaction between the efficacy of insecticides in all trials, except KUMLUCA, were found to be statistically significant (AKSU: df = 2, $F = 5.85$, $P = 0.009$; KEPEZ: df = 2, $F = 3.97$, $P = 0.032$; SERIK: df = 2, $F = 7.03$, $P = 0.004$; KUMLUCA: df = 2, $F = 2.42$, $P = 0.110$).

In addition, significant differences were determined between different time points after treatments in all trials (AKSU: df = 2, $F = 10.22$, $P = 0.001$; KEPEZ: df = 2, $F = 13.41$, $P = 0.000$; SERIK: df = 2, $F = 11.00$, $P = 0.000$; KUMLUCA: df = 2, $F = 11.70$, $P = 0.000$).

The results for each insecticide are discussed below.

3.3 Resistance status against neurotoxic insecticides (metaflumizone and OPs)

In general, metaflumizone showed the lowest toxicity in field conditions, especially 7 DAT the efficacy of metaflumizone was

Table 2. Efficacy (% mortality ± SEM) of metaflumizone (M), chlorantraniliprole (C) and spinosad (S) against *Tuta absoluta* in tomato greenhouses of Turkey

Population	Insecticide	3 DAT*	7 DAT	14 DAT
AKSU	M	32.39 ± 5.13Cb	65.92 ± 13.87Aa	56.25 ± 12.62Aab
	C	45.59 ± 4.71Bc	76.50 ± 1.78Aa	60.33 ± 4.22Ab
	S	65.45 ± 3.57Ab	81.30 ± 2.34Aa	75.48 ± 4.46Aa
KUMLUCA	M	49.86 ± 12.39Aa	67.21 ± 5.23Ba	59.10 ± 7.15Ba
	C	60.43 ± 8.71Aa	75.30 ± 5.94ABa	67.50 ± 5.77ABa
	S	49.30 ± 11.08Ab	85.04 ± 3.06Aa	78.87 ± 4.43Aa
KEPEZ	M	36.46 ± 7.91Aa	54.06 ± 11.73Ba	52.21 ± 12.30Ba
	C	45.38 ± 14.20Ab	77.89 ± 1.70Aa	68.55 ± 2.07ABa
	S	46.31 ± 9.60Ab	83.57 ± 1.95Aa	72.49 ± 2.34Aa
SERIK	M	44.83 ± 3.45Bc	67.92 ± 4.33Ba	55.33 ± 0.99Ab
	C	56.86 ± 4.60Ab	78.55 ± 4.19Aa	69.79 ± 8.82Aab
	S	65.92 ± 2.64Ab	82.33 ± 2.70Aa	72.30 ± 8.50Aab

*Day after treatment.
Different capital letters show statistically significant differences among insecticides for each time level ($P < 0.05$, Tukey test).
Different lower case letters show statistically significant differences among times for each insecticide level ($P < 0.05$, Tukey test).

Table 3. The presence and frequency of resistance-associated mutations (mean ± SE) in ryanodine receptors (RyRs), nicotinic acetylcholine receptor (nAChR), acetylcholinesterase (AChE) and voltage-gated sodium channel (VGSC) in 22 *Tuta absoluta* populations from Turkey

	RyRs		nAChR	AChE	VGSC	
Population	I4790M/K	G4946E	G275E	A201S	F1845Y	V1848I
KUM*	100	0	0	65.2 ± 1.5	67.5 ± 4.1	15.6 ± 4.8
AKS*	I/M/K	0	0	64.1 ± 1.7	0	0
SER*	62.7 ± 2.5	0	0	57.5 ± 2.8	0	0
KEP*	53.6 ± 3.4	0	0	66.7 ± 3.3	20.6 ± 3.5	44.9 ± 2.3
ELM	57.6 ± 1.8	31.2 ± 1.3	0	56.9 ± 0.9	31.4 ± 0.2	0
MP	68.5 ± 0.4	28.0 ± 0.6	0	62.8 ± 6.8	74.9 ± 2.4	0
CK	79.5 ± 6.3	0	0	45.6 ± 2.9	0	0
ERD1	100	0	0	37.1 ± 5.1	23.4 ± 1.9	35.5 ± 1.6
ERD2	100	0	0	81.6 ± 2.2	59.9 ± 0.4	44.7 ± 2.2
TAR	80.6 ± 2.1	0	0	59.6 ± 1.6	0	41.6 ± 4.5
KIR	100 (K)	0	100	70.6 ± 3.4	52 ± 3.6	29.1 ± 0.7
IZM1	66.7 ± 1.3	0	0	61.4 ± 0.4	0	0
SEY	71.8 ± 4.2	33.2 ± 2.9	0	55.6 ± 4.0	0	61.4 ± 1.3
FET1	65.0 ± 1.6	0	0	56.9 ± 2.5	0	0
FET2	0	54.4 ± 5.7	0	57.0 ± 1.1	56.2 ± 2.8	49.9 ± 1.6
ALA	64.0 ± 3.1	60.7 ± 9.8	0	46.4 ± 0.6	0	0
BUR1	100	0	0	63.7 ± 4.2	53.5 ± 1.1	40.2 ± 2.7
BUR2	68.9 ± 5.4	0	0	68.5 ± 1.8	34.7 ± 2.6	42.8 ± 5.6
BUR3	73.0 ± 1.9	25.6 ± 1.3	0	71.4 ± 7.9	69.6 ± 1.9	50.2 ± 8.4
DIK	77.0 ± 0.7	0	0	55.2 ± 3.2	0	0
AYV	54.6 ± 3.5	80.2 ± 0.7	0	63.9 ± 5.3	0	0
AFY	81.6 ± 2.2	0	87.6 ± 4.9	66.3 ± 2.7	72.3 ± 2.0	14.6 ± 0.8

*Populations used in greenhouse trials.

significantly lower than other tested insecticides for all populations,except AKS. In short-term efficacy (3 DAT), metaflumizone caused lower mortality rates (<50%) when compared with spinosad and chlorantraniliprole. In addition, the toxicity significantly increased in 7 DAT compared with 3 DAT in half of the trials. Although AKS and SER did not contain known resistance mutations in VGSC, the toxicity of metaflumizone was low, especially 3 DAT, which might reflect the involvement of other resistance mechanisms. On the other hand, KUM and KEP, which contained both mutations (but not fixed), showed 67% and 54% mortality rates at most, respectively.

In the VGSC, more than half of the populations harboured F1845Y and/or V1848I mutations, but the frequency of mutations varied substantially according to populations and locations, from 0–74.9% for F1845Y and 0–61.4% for V1848I (Table 3). More than half of the sampled populations (12 out of 21) contained one of these two mutations (Table 3). Ten out of 22 populations harboured both mutations together, but not fixed. Most of the population from the Mediterranean region of Turkey had both target-site mutations, therefore repeated use of pyrazoline insecticide should be avoided and rotation of insecticides should be favoured to create an area-wide chemical control program.

All *T. absoluta* populations contained the A201S (heterozygous for all) mutation, possibly reflecting the use of OP compounds in tomato crops, particularly in the last decade. Interestingly, the mutation was neither fixed nor absent in sampled *T. absoluta* populations and the frequency was between 37.1% and 81.6%. Similar to our study, a high frequency of A201S mutation was determined in *T. absoluta* populations from Iran.[67] Although many OP insecticides have been banned or restricted worldwide,[68] others are still used to control various pests. The high frequency of the A201S mutation indicates a high resistance risk, suggesting that OPs may not be suitable for the control of *T. absoluta* in Turkey.

3.4 Resistance status against chlorantraniliprole

In general, chlorantraniliprole showed better efficiency than metaflumizone but lower than spinosad in field conditions. In short-term efficacy (3 DAT), chlorantraniliprole caused lower mortality rates (45.38–60.43%) when compared with spinosad (46.31–65.92%), except for KUM. In all greenhouse experiments, decreasing mortality rates after the seventh day of treatment were observed, probably because of a reduced residue effect of insecticides and newly emerged larvae. All populations tested in greenhouse conditions had the I4790M/K mutation, even fixed for KUM, but mortality rates were higher than 75% 7 DAT, indicating that this mutation is not enough to cause high resistance levels to chlorantraniliprole on its own. In line with our results, Douris *et al*. reported that the I4790M mutation confers only low chlorantraniliprole resistance.[48]

Both screened diamide resistance-associated mutations were found in the Turkish *T. absoluta* populations, but the frequency of resistance mutations largely varied among populations sampled across the country (0–100% for I4790M/K, 0–80.2% for G4946E; Table 3). All sampled populations, except FET2, harboured I4790M/K mutations and I4790M has been known to confer moderate resistance to flubendiamide.[48,69] The I4790M mutation was fixed for four populations, while KIR was determined as the only population containing I4790K mutation fixed. The I4790K mutation has recently been reported in *P. xylostella*,[70] but to our knowledge this is the first report of this mutation in *T. absoluta*. A calcium imaging study recently elucidated the mechanism of this novel mutation in diamide insecticide resistance, including cyantraniliprole.[70] However, the relationship between I4790K and resistance to diamide insecticides in *T. absoluta* remains unknown. Functional validation studies should be performed to reveal the role of this mutation in diamide resistance.

Seven out of 22 populations (31.8%) contained E (glutamic acid), but not being fixed, at position 4903 in RyR. Although six populations harboured the combination of I4790M + G4946E mutations, both mutations were present as heterozygous. In cases where the I4790M/K mutation was fixed in certain populations, the G4946E mutation was not present, indicating that there was no combination of these two mutations in a single haplotype.

Although the introduction of the G4946E mutation using CRISPR-Cas9 in *Drosophila melanogaster* resulted in a lethal phenotype, G4946V mutation has been known to confer a high level of chlorantraniliprole and flubendiamide resistance.[48] Considering the frequency of both mutations and the bioassay data, chlorantraniliprole and flubendiamide should only be used in rotation with insecticides belonging to different mode of action groups in sampled areas. Since lack of cross-resistance against cyantraniliprole and target-site mutations confer only low cyantraniliprole resistance,[48] use of cyantraniliprole may be favored among diamides, if needed. In addition, the absence of cross-resistance between diamides and traditional insecticides is known,[51] therefore rotation of insecticides should be implemented in tomato-growing areas when designing resistance management programs.

3.5 Resistance status against spinosad

Although the toxicity rates caused by spinosad and chlorantraniliprole did not differ statistically, the efficacy of spinosad was higher in most cases (Table 2). Since the mortality rates were always higher than 80% 7 DAT, spinosad seems to be the only insecticide that did not cause control failure in field conditions according to Guedes.[71] Among all screened mutations, G275E was the least frequent mutation in Turkish *T. absoluta* populations (two out of 22, fixed for one; see Table 3). This mutation has previously been reported in important thrips species[72,73] and has also been validated using CRISPR/Cas9 gene editing in *Drosophila melanogaster*.[74]

Considering the high efficiency and very low frequency of the G275E mutation, spinosad seems to offer a good alternative for chemical control of *T. absoluta* in Turkey. Similarly, spinosad showed excellent efficacy against Cypriot populations of *T. absoluta*.[75] In addition, since spinosad showed no cross-resistance to other widely used insecticides registered in *T. absoluta* control and resistance to spinosad has been known to decrease in the absence of selection pressure indicating the fitness cost of resistance development,[76] it should be implemented in resistance management programs.

4 CONCLUDING REMARKS AND RECOMMENDATIONS

Based on the determination of the efficacy of insecticides in greenhouse conditions, spinosad showed higher mortality rates followed by chlorantraniliprole and metaflumizone in both short- and long-term efficacy. All the previously known resistance-associated mutations were found in the Turkish *T. absoluta* populations, but the frequency of resistance mutations differed. Three out of 22 populations (AFY, KIR, BUR3) contained five resistance mutations together, indicating the difficulties in choosing chemical alternatives in multiple resistant populations. In addition, high genetic homogeneity seems to cause evolution of similar insecticide resistance mechanisms in *T. absoluta* populations from different geographic regions.

Based on mortality rates and the low frequency of target-site mutation conferring resistance, spinosad seems to be the best rotation alternative in chemical control of *T. absoluta* from Turkey. More monitoring studies are needed to implement evidence-based IRM strategies in the frame of integrated pest management. In addition, chemical insecticides should be combined with alternative management strategies such as mating disruption methods and biological control to decrease the frequency of resistant alleles and for sustainable pest control.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 FAOSTAT. http://www.fao.org/faostat/en. Accessed 10 March 2021.

2 Biondi A, Guedes RNC, Wan F-H and Desneux N, Ecology, worldwide spread, and management of the invasive South American tomato pinworm, *Tuta absoluta*: past, present, and future. *Annu Rev Entomol* **63**:239–258 (2018).

3 Desneux N, Wajnberg E, Wyckhuys KA, Burgio G, Arpaia S, Narváez-Vasquez CA *et al.*, Biological invasion of European tomato crops by Tuta absoluta: ecology, geographic expansion and prospects for biological control. *J Pest Sci* **83**:197–215 (2010).

4 Urbaneja A, Vercher R, Navarro Llopis V, Porcuna Coto JL and García Marí F, La polilla del tomate, *'Tuta absoluta'*. *Phytoma Espana* **194**: 16–23 (2007).

5 Kılıç T, First record of *Tuta absoluta* in Turkey. *Phytoparasitica* **38**:243–244 (2010).

6 Han P, Bayram Y, Shaltiel-Harpaz L, Sohrabi F, Saji A, Esenali UT *et al.*, *Tuta absoluta* continues to disperse in Asia: damage, ongoing management and future challenges. *J Pest Sci* **92**:1317–1327 (2019).

7 Cifuentes D, Chynoweth R and Bielza P, Genetic study of Mediterranean and South American populations of tomato leafminer *Tuta absoluta* (Povolny, 1994)(Lepidoptera: Gelechiidae) using ribosomal and mitochondrial markers. *Pest Manage Sci* **67**:1155–1162 (2011).

8 Guillemaud T, Blin A, Le Goff I, Desneux N, Reyes M, Tabone E *et al.*, The tomato borer, *Tuta absoluta*, invading the Mediterranean Basin, originates from a single introduction from Central Chile. *Sci Rep* **5**:1–5 (2015).

9 Zappala L, Biondi A, Alma A, Al-Jboory IJ, Arno J, Bayram A *et al.*, Natural enemies of the South American moth, *Tuta absoluta*, in Europe, North Africa and Middle East, and their potential use in pest control strategies. *J Pest Sci* **86**:635–647 (2013).

10 Keçeci M and Öztop A, Possibilities for biological control of *Tuta absoluta* (Meyrick, 1917)(Lepidoptera: Gelechiidae) in the western Mediterranean Region of Turkey. *Turk Entomol Derg* **41**:219–230 (2017).

11 Mansour R, Brévault T, Chailleux A, Cherif A, Grissa-Lebdi K, Haddi K *et al.*, Occurrence, biology, natural enemies and management of *Tuta absoluta* in Africa. *Entomol Gen* **38**:83–112 (2018).

12 Caparros Megido R, Haubruge E and Verheggen F, Pheromone-based management strategies to control the tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae). A review. *Biotechnologie, Agronomie. Soc Environ* **17**:475–482 (2013).

13 Ünlü L, Ögür E and Uulu TE, Control of *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae) in open field tomato crops using the mating disruption technique. *Phytoparasitica* **49**:385–396 (2021).

14 Roditakis E, Vasakis E, Garcia-Vidal L, del Rosario M-AM, Rison JL, Haxaire-Lutun MO *et al.*, A four-year survey on insecticide resistance and likelihood of chemical control failure for tomato leaf miner *Tuta absoluta* in the European/Asian region. *J Pest Sci* **91**:421–435 (2018).

15 Guedes R, Roditakis E, Campos M, Haddi K, Bielza P, Siqueira H *et al.*, Insecticide resistance in the tomato pinworm *Tuta absoluta*: patterns, spread, mechanisms, management and outlook. *J Pest Sci* **92**:1329–1342 (2019).

16 Salazar ER and Araya JE, *Tomato Moth, Tuta Absoluta (Meyrick) Response to Insecticides in Arica*. Agricult Tec, Chile (2001).

17 Li X, Schuler MA and Berenbaum MR, Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52**:231–253 (2007).

18 Campos MR, Biondi A, Adiga A, Guedes RN and Desneux N, From the Western Palaearctic region to beyond: *Tuta absoluta* 10 years after invading Europe. *J Pest Sci* **90**:787–796 (2017).

19 Colovic MB, Krstic DZ, Lazarevic-Pasti TD, Bondzic AM and Vasic VM, Acetylcholinesterase inhibitors: pharmacology and toxicology. *Curr Neuropharmacol* **11**:315–335 (2013).

20 Smissaert H, Cholinesterase inhibition in spider mites susceptible and resistant to organophosphate. *Science* **143**:129–131 (1964).

21 Fournier D, Mutations of acetylcholinesterase which confer insecticide resistance in insect populations. *Chem Biol Interact* **157**:257–261 (2005).

22 Van Leeuwen T and Dermauw W, The molecular evolution of xenobiotic metabolism and resistance in chelicerate mites. *Annu Rev Entomol* **61**:475–498 (2016).

23 Haddi K, Berger M, Bielza P, Rapisarda C, Williamson M, Moores G *et al.*, Mutation in the ace-1 gene of the tomato leaf miner (*Tuta absoluta*) associated with organophosphates resistance. *J Appl Entomol* **141**: 612–619 (2017).

24 Andrews M, Callaghan A, Field L, Williamson M and Moores G, Identification of mutations conferring insecticide-insensitive AChE in the cotton-melon aphid, *Aphis gossypii* Glover. *Insect Mol Biol* **13**:555–561 (2004).

25 Hsu J-C, Haymer DS, Wu W-J and Feng H-T, Mutations in the acetylcholinesterase gene of *Bactrocera dorsalis* associated with resistance to organophosphorus insecticides. *Insect Biochem Mol Biol* **36**:396–402 (2006).

26 Lee D-W, Choi JY, Kim WT, Je YH, Song JT, Chung BK *et al.*, Mutations of acetylcholinesterase1 contribute to prothiofos-resistance in *Plutella xylostella* (L.). *Biochem Biophys Res Commun* **353**:591–597 (2007).

27 Khajehali J, Van Leeuwen T, Grispou M, Morou E, Alout H, Weill M *et al.*, Acetylcholinesterase point mutations in European strains of *Tetranychus urticae* (Acari: Tetranychidae) resistant to organophosphates. *Pest Manage Sci* **66**:220–228 (2010).

28 Salgado V and Hayashi J, Metaflumizone is a novel sodium channel blocker insecticide. *Vet Parasitol* **150**:182–189 (2007).

29 Silver KS, Du Y, Nomura Y, Oliveira EE, Salgado VL, Zhorov BS *et al.*, Voltage-gated sodium channels as insecticide targets. *Adv Insect Phys* **46**:389–433 (2014).

30 Roditakis E, Mavridis K, Riga M, Vasakis E, Morou E, Rison JL *et al.*, Identification and detection of indoxacarb resistance mutations in the para sodium channel of the tomato leafminer, *Tuta absoluta*. *Pest Manage Sci* **73**:1679–1688 (2017).

31 Wang XL, Su W, Zhang JH, Yang YH, Dong K and Wu YD, Two novel sodium channel mutations associated with resistance to indoxacarb and metaflumizone in the diamondback moth, *Plutella xylostella*. *Insect Sci* **23**:50–58 (2016).

32 Samantsidis G-R, O'Reilly AO, Douris V and Vontas J, Functional validation of target-site resistance mutations against sodium channel blocker insecticides (SCBIs) via molecular modeling and genome engineering in Drosophila. *Insect Biochem Mol Biol* **104**:73–81 (2019).

33 Chatzidaki A and Millar NS, Allosteric modulation of nicotinic acetylcholine receptors. *Biochem Pharmacol* **97**:408–417 (2015).

34 Crossthwaite AJ, Bigot A, Camblin P, Goodchild J, Lind RJ, Slater R *et al.*, The invertebrate pharmacology of insecticides acting at nicotinic acetylcholine receptors. *Pestic Sci* **42**:67–83 (2017).

35 Reyes M, Rocha K, Alarcón L, Siegwart M and Sauphanor B, Metabolic mechanisms involved in the resistance of field populations of *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae) to spinosad. *Pestic Biochem Physiol* **102**:45–50 (2012).

36 Campos MR, Silva TB, Silva WM, Silva JE and Siqueira HA, Spinosyn resistance in the tomato borer *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae). *J Pest Sci* **88**:405–412 (2015).

37 Silva WM, Berger M, Bass C, Williamson M, Moura DM, Ribeiro LM *et al.*, Mutation (G275E) of the nicotinic acetylcholine receptor α6 subunit is associated with high levels of resistance to spinosyns in *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae). *Pestic Biochem Physiol* **131**: 1–8 (2016).

38 Berger M, Puinean AM, Randall E, Zimmer CT, Silva WM, Bielza P *et al.*, Insecticide resistance mediated by an exon skipping event. *Mol Ecol* **25**:5692–5704 (2016).

39 Baxter SW, Chen M, Dawson A, Zhao J-Z, Vogel H, Shelton AM *et al.*, Mis-spliced transcripts of nicotinic acetylcholine receptor α6 are associated with field evolved spinosad resistance in *Plutella xylostella* (L.). *PLoS Genet* **6**:e1000802 (2010).

40 Rinkevich FD, Chen M, Shelton AM and Scott JG, Transcripts of the nicotinic acetylcholine receptor subunit gene Pxylα6 with premature stop codons are associated with spinosad resistance in diamondback moth, *Plutella xylostella*. *Invertebr Neurosci* **10**:25–33 (2010).

41 Hsu J-C, Feng H-T, Wu W-J, Geib SM, C-h M and Vontas J, Truncated transcripts of nicotinic acetylcholine subunit gene Bdα6 are associated with spinosad resistance in *Bactrocera dorsalis*. *Insect Biochem Mol Biol* **42**:806–815 (2012).

42 Grant C, Jacobson R, Ilias A, Berger M, Vasakis E, Bielza P *et al.*, The evolution of multiple-insecticide resistance in UK populations of tomato leafminer, *Tuta absoluta*. *Pest Manage Sci* **75**:2079–2085 (2019).

43 Sparks TC, Crossthwaite AJ, Nauen R, Banba S, Cordova D, Earley F *et al.*, Insecticides, biologics and nematicides: Updates to IRAC's mode of action classification-a tool for resistance management. *Pestic Biochem Physiol* **167**:104587 (2020).

44 Dinter A, Brugger K, Bassi A, Frost N-M and Woodward MD, Chlorantraniliprole (DPX-E2Y45, DuPont™ Rynaxypyr®, Coragen® and Altacor® insecticide)-a novel anthranilic diamide insecticide-demonstrating low toxicity and low risk for beneficial insects and predatory mites. *IOBC/wprs Bulletin* **35**:128–135 (2008).

45 Brugger KE, Cole PG, Newman IC, Parker N, Scholz B, Suvagia P *et al.*, Selectivity of chlorantraniliprole to parasitoid wasps. *Pest Manage Sci* **66**:1075–1081 (2010).

46 Roditakis E, Vasakis E, Grispou M, Stavrakaki M, Nauen R, Gravouil M *et al.*, First report of *Tuta absoluta* resistance to diamide insecticides. *J Pest Sci* **88**:9–16 (2015).

47 Troczka BJ, Williams AJ, Williamson MS, Field LM, Lüemmen P and Davies TE, Stable expression and functional characterisation of the diamondback moth ryanodine receptor G4946E variant conferring resistance to diamide insecticides. *Sci Rep* **5**:1–11 (2015).

48 Douris V, Papapostolou K-M, Ilias A, Roditakis E, Kounadi S, Riga M *et al.*, Investigation of the contribution of RyR target-site mutations in diamide resistance by CRISPR/Cas9 genome modification in Drosophila. *Insect Biochem Mol Biol* **87**:127–135 (2017).

49 Roditakis E, Steinbach D, Moritz G, Vasakis E, Stavrakaki M, Ilias A *et al.*, Ryanodine receptor point mutations confer diamide insecticide resistance in tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae). *Insect Biochem Mol Biol* **80**:11–20 (2017).

50 Nauen R and Steinbach D, Resistance to diamide insecticides in lepidopteran pests, in *Advances in Insect Control and Resistance Management, ed. by Horowitz AR and Ishaaya I*. Cham: Springer, pp. 219–240 (2016).

51 Silva JE, LMdS R, Vinasco N, RNC G and HÁA S, Field-evolved resistance to chlorantraniliprole in the tomato pinworm *Tuta absoluta*: inheritance, cross-resistance profile, and metabolism. *J Pest Sci* **92**:1421–1431 (2019).

52 Uğurlu Karaağaç S, Enzyme activities and analysis of susceptibility levels in Turkish *Tuta absoluta* populations to chlorantraniliprole and metaflumizone insecticides. *Phytoparasitica* **43**:693–700 (2015).

53 Yalcin M, Mermer S, Kozaci LD and Turgut C, Insecticide resistance in two populations of *Tuta absoluta* (Meyrick, 1917)(Lepidoptera: Gelechiidae) from Turkey. *Turk Entomol Derg* **39**:137–145 (2015).

54 Tabashnik BE, Mota-Sanchez D, Whalon ME, Hollingworth RM and Carrière Y, Defining terms for proactive management of resistance to Bt crops and pesticides. *J Econ Entomol* **107**:496–507 (2014).

55 Henderson CF and Tilton EW, Tests with acaricides against the brown wheat mite. *J Econ Entomol* **48**:157–161 (1955).

56 Ryan BF, Joiner BL and Cryer JD, *MINITAB Handbook: Update for Release*. Cengage Learning, Boston, USA (2012).

57 Haddi K, Berger M, Bielza P, Cifuentes D, Field LM, Gorman K *et al*., Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (*Tuta absoluta*). *Insect Biochem Mol Biol* **42**:506–513 (2012).

58 Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M *et al*., Primer3—new capabilities and interfaces. *Nucleic Acids Res* **40**:e115–e115 (2012).

59 Van Leeuwen T, Vanholme B, Van Pottelberge S, Van Nieuwenhuyse P, Nauen R, Tirry L *et al*., Mitochondrial heteroplasmy and the evolution of insecticide resistance: non-Mendelian inheritance in action. *Proc Natl Acad Sci USA* **105**:5980–5985 (2008).

60 Hall TA, *BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT*. Nucleic Acids Symposium Series [London]. Information Retrieval Ltd, UK, c1979-c2000, pp. 95–98 (1999).

61 Folmer O, Black M, Hoeh W, Lutz R and Vrijenhoek R, DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* **3**:294–299 (1994).

62 Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE *et al*., DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol Biol Evol* **34**:3299–3302 (2017).

63 Katoh K, Rozewicki J and Yamada KD, MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform* **20**:1160–1166 (2019).

64 Leigh JW and Bryant D, popart: full-feature software for haplotype network construction. *Methods Ecol Evol* **6**:1110–1116 (2015).

65 Mehrkhou F, Güz N, Korkmaz EM and Çağatay NS, Analysis of genetic variation in an important pest, *Tuta absoluta*, and its microbiota with a new bacterial endosymbiont. *Turk J Agric For* **45**:111–123 (2021).

66 Yükselbaba U and Göçmen H, Determination of genetic variation of tomato leafminer [*Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae)] populations from west Mediterreanean region of Turkey based on mitochondrial cytochrome oxidase I (mtCOI). *Mediterr Agric Sci* **29**:5–7 (2016).

67 Zibaee I, Mahmood K, Esmaeily M, Bandani A and Kristensen M, Organophosphate and pyrethroid resistances in the tomato leaf miner *Tuta absoluta* (Lepidoptera: Gelechiidae) from Iran. *J Appl Entomol* **142**:181–191 (2018).

68 IARC, Some organophosphate insecticides and herbicides. Lyon:International Agency for Research on Cancer (2017).

69 Wang X, Cao X, Jiang D, Yang Y and Wu Y, CRISPR/Cas9 mediated ryanodine receptor I4790M knockin confers unequal resistance to diamides in *Plutella xylostella*. *Insect Biochem Mol Biol* **125**:103453 (2020).

70 Jouraku A, Kuwazaki S, Miyamoto K, Uchiyama M, Kurokawa T, Mori E *et al*., Ryanodine receptor mutations (G4946E and I4790K) differentially responsible for diamide insecticide resistance in diamondback moth, *Plutella xylostella* L. *Insect Biochem Mol Biol* **118**: 103308 (2020).

71 Guedes RNC, Insecticide resistance, control failure likelihood and the First Law of Geography. *Pest Manage Sci* **73**:479–484 (2017).

72 Puinean AM, Lansdell SJ, Collins T, Bielza P and Millar NS, A nicotinic acetylcholine receptor transmembrane point mutation (G275E) associated with resistance to spinosad in F rankliniella occidentalis. *J Neurochem* **124**:590–601 (2013).

73 Bao WX, Narai Y, Nakano A, Kaneda T, Murai T and Sonoda S, Spinosad resistance of melon thrips, *Thrips palmi*, is conferred by G275E mutation in α6 subunit of nicotinic acetylcholine receptor and cytochrome P450 detoxification. *Pestic Biochem Physiol* **112**:51–55 (2014).

74 Zimmer CT, Garrood WT, Puinean AM, Eckel-Zimmer M, Williamson MS, Davies TE *et al*., A CRISPR/Cas9 mediated point mutation in the alpha 6 subunit of the nicotinic acetylcholine receptor confers resistance to spinosad in *Drosophila melanogaster*. *Insect Biochem Mol Biol* **73**: 62–69 (2016).

75 Michaelides G, Seraphides N, Pitsillou M and Sfenthourakis S, Susceptibility of Cypriot *Tuta absoluta* populations to four targeted insecticides and control failure likelihood. *J Appl Entomol* **143**:508–517 (2019).

76 Campos MR, Rodrigues ARS, Silva WM, Silva TBM, Silva VRF, Guedes RNC *et al*., Spinosad and the tomato borer *Tuta absoluta*: a bioinsecticide, an invasive pest threat, and high insecticide resistance. *PLoS One* **9**:e103235 (2014).