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RAPID COMMUNICATION

First report of *Tuta absoluta* resistance to diamide insecticides

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Abstract The tomato borer *Tuta absoluta* (Lepidoptera: Gelechiidae) is an invasive pest of tomato crops that is rapidly expanding around the world. It is considered a devastating pest and its control heavily relies on application of insecticides. Diamides are a novel class of insecticides acting on insect ryanodine receptors and are highly effective against lepidopteran pests. To date, chlorantraniliprole and flubendiamide have been registered in the market and they have been extensively used to manage *T. absoluta*. In this study, a survey was conducted in Greece and Italy monitoring diamide resistance. The populations originating from Sicily (Italy) exhibited LC_{50}s that ranged between 47.6–435 for chlorantraniliprole and 993–1.376 for flubendiamide, while for Crete (Greece) LC_{50}s ranged between 0.14–2.45 for chlorantraniliprole and 1.7–8.4 for flubendiamide (LC_{50}s in mg L^{-1}). Comparing this result to the susceptible reference strain, high resistance levels for the Italian populations were detected, i.e., up to 2,414- and 1,742-fold for chlorantraniliprole and flubendiamide, respectively. Resistance ratios for Greek populations were found up to 14-fold for chlorantraniliprole and 11-fold for flubendiamide, suggesting that diamide resistance is low but increasing considering monitoring data over time. Hereby, we report for the first time, cases of resistance development to diamide insecticides in *T. absoluta*. These findings underline the importance of committing to the resistance management strategies for diamide insecticides.

Keywords *Tuta absoluta* · Tomato leafminer · Resistance · Diamide insecticides · Chlorantraniliprole · Flubendiamide

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Key message

Resistance to diamides is reported for the first time in *Tuta absoluta*. Diamides are a new and highly potent class of insecticides extensively used for the control of the pest. High resistance levels (>100-fold) to diamides were reported in Italy, while low resistance levels were detected in Greece (>10-fold). The early warning in Greece was achieved due to the efficient resistance monitoring program.

Introduction

Diamides are a new group of insecticides that have been classified as ryanodine receptor modulators (MoA Group 28) (IRAC 2014). Insect ryanodine receptors (RyR) are calcium channels located in the sarcoplasmic reticulum. Diamides cause prolonged channel opening and uncoordinated muscle contraction in intoxicated pest insects subsequently leading to death (Ebbinghaus-Kintscher et al.

2006; Teixeira and Andaloro 2013). To date, two representatives of the diamide insecticide group are registered for pest control in Europe; flubendiamide, a phthalic acid diamide and chlorantraniliprole, an anthranilic acid diamide (Tohnishi et al. 2005; Lahm et al. 2007). Both compounds are particularly active against lepidopteran pests at fairly low rates and have an excellent safety profile. Eight years after market launch, diamide insecticides currently comprise 7 % of the global insecticide market, which underlines the importance of this chemistry (Sparks 2013).

Currently, diamide insecticides are used in a gradually increasing number of agricultural settings. However, after several years of field applications, cases of resistance development to diamides have been reported for some lepidopteran species. Cross resistance between flubendiamide and chlorantraniliprole has been reported for diamondback moth *Plutella xylostella* and smaller tea tortrix, *Adoxophyes honmai* (Wang and Wu 2012; Uchiyama and Ozawa 2014). In addition, resistance to chlorantraniliprole has been reported in the rice stem borer *Chilo suppressalis*, the cutworm *Spodoptera litura*, and the beet armyworm *Spodoptera exigua* (Su et al. 2012; Che et al. 2013; Gao et al. 2013). Finally, resistance has been reported for *C. suppressalis* to flubendiamide (Wu et al. 2014). Therefore, the implementation of resistance monitoring and management tactics is of utmost importance in order to prevent the development of resistance in frequently treated pests, such as the tomato borer, *T. absoluta* (Teixeira and Andaloro 2013).

Tuta absoluta is a major pest of tomato crops that in the past years challenged the global tomato production due to the high damage potential and rapid spread in Europe, Africa, and Asia (Desneux et al. 2011). Integrated control schemes implemented, including conservation of indigenous natural enemies (Zappalà et al. 2013) provided satisfactory results; however, in many cases, insecticide applications are still one the major pest control tactics (Roditakis et al. 2013a). It has been demonstrated that tomato borer can develop resistance to conventional as well as novel chemicals (Siqueira et al. 2000a, b, 2001; Lietti et al. 2005; Silva et al. 2011; Haddi et al. 2012; Gontijo et al. 2013; Campos et al. 2014a). Therefore, close monitoring of the insecticide susceptibility levels is of critical importance for sustainable pest control and early resistance detection. In Europe, baseline susceptibility data for major insecticides have been established for *T. absoluta* and can be used for resistance monitoring surveys (Roditakis et al. 2013a, b).

As part of our ongoing resistance monitoring program, populations were collected in 2014 from greenhouse crops where pest control has been reported to be problematic after repeated application of diamide insecticides. In this study, we report the first cases of resistance to chlorantraniliprole and flubendiamide in *T. absoluta* globally. Resistance levels in Italian strains were found very high in some cases (>1,000-fold) for both molecules. The resistance in Greek strains was low (>10-fold). Our findings highlight the importance of resistance management tactic as recently described (Teixeira and Andaloro 2013).

Materials and methods

Insect strains

Populations for this study were collected during 2012 and 2014 from ten distinct sampling sites; six in Crete, Greece (Ierapetra, Arvi, and Tympaki), and four from Sicily, Italy (Pachino, Gela, and Acate). A detailed record for each population is given in Table 1.

Collections originated from infested greenhouse tomato crops. In 2014, individual growers reported problems in the control of the tomato borer, e.g., from Ierapetra and Sicily, but not from Tympaki. From each sampling site, tomato leaves infested with *T. absoluta* larvae were collected in large plastic bags. Approximately, 400–800 larvae were collected from each site. The samples were transferred to the lab in a cool box to avoid stressing the insects. Greek samples reached the lab within 1–2 h. Italian samples were delivered within 48 h. Insects from Sicily were obtained in excellent condition, suggesting that during transfer adequate nutrition was available and temperature never exceeded plant damage thresholds. The bags containing the infested leaves were opened in insect-proof rearing cages containing 2–3 insect-free potted tomato plants so that larvae could resume normal development.

The tomato plants (*Solanum lycopersicum,* cv. Valuro), used for the development of the strains, were maintained pest-free in large insect-proof cages under semi-field conditions. No insecticides were used during the plant development phase (for details see Roditakis et al. (2013a)).

The rearing cages were maintained at 26 ± 1 °C, 65 % RH and 16 h light:8 h dark photoperiod. When adequate numbers of adult insects were available, approximately 100 moths were collected and were allowed to oviposit on insect-free plants for 24–48 h. Those plants were incubated separately until the larvae reached the second instar.

Insecticides

Commercial formulations of the diamide insecticides chlorantraniliprole (Altacor 35WG, DuPont, France) and flubendiamide (Belt 24WG, Bayer CropScience AG, Germany) were used in the experiments.

Table 1 Identification codes and general information for the collected *T. absoluta* populations

Population	Location	Coordinates GPS	Sampling data	Crop	Generation tested	Application history	
						Total	Diamides
Lab strain			Aug-10	GH T	F1–F2		
GR-ARVI-12-1	Arvi, Kapsali	34°59′30.37″N 25°24′22.76″E	May-12	GH T	F1–F2	3	2
GR-TYMP-12-2	Tympaki, Lagolio	35°18′45.09″N 25°8′20.86″E	Jun-12	GH T	F1	5	2
GR-TYMP-14-1	Tympaki, Klima	35°5′41.09″N 24°45′27.05″E	Mar-14	GH T	F1–F2	4	1
GR-IER-14-1	Ierapetra, Kentri	35°2′9.06″N 25°44′55.69″E	Apr-14	GH T	F1	7	4
GR-IER-14-2	Ierapetra, Sopates	35°1′7.87″N 25°38′52.93″E	May-14	GH T	F0–F1	10	6
GR-IER-14-3	Ierapetra, Mpountoules	35°1′2.33″N 25°43′31.68″E	May-14	GH T	F0–F1	8	4
IT-PACH-14-1	Siracusa, Pachino	36°40′27.78″N 15°5′40.51″E	May-14	GH T	F0–F1	n.a.	n.a.
IT-PACH-14-2	Siracusa, Pachino	36°40′31.42″N 15°5′50.27″E	May-14	GH T	F0–F1	9	7
IT-GELA-14-1	Caltanissetta, Gela	37°1′3.04″N 14°19′30.04″E	May-14	GH T	F0–F1	12	8
IT-ACAT-14-1	Ragusa, Acate	36°59′7.44″N 14°23′22.03″E	May-14	GH T	F0–F1	10	5

Application history is also included if available

GH T Greenhouse tomato, *Total* total number of applications since January 2014 up to collection date, *Diamides* number of applications with diamides in the same period, *n.a.* not available

Bioassay method

The IRAC method 022 was adopted for the toxicological assays. The method protocol is described in detail in Roditakis et al. (2013a). Briefly, aqueous dispersions of commercial insecticide formulations were used in a leaf dip bioassay. All assays were performed in a 32 cell repli-dish (RT32W, Bioserve, US, www.insectrearing.com). Tomato leaflets, cut in square pieces were immersed in serial insecticide concentrations containing Triton X-100 (0.2 g L^{-1}) as non-ionic wetting agent. Treated leaf pieces were allowed to dry and then placed with their abaxial site on moist tissue paper cut to fit the wells of the repli-dish. Second instar *T. absoluta* larvae were carefully removed out of the galleries in infested tomato leaves. A single larva was placed in each well and then the repli-dish was sealed with transparent ventilated adhesive lids.

All treatments were placed in a large insect rearing room with controlled environment (26 (±1) °C, 50–60 % RH, 16 h L: 8 h D). Mortality was assessed after 72 h. A larva was considered dead if no movement could be observed. A larva was recorded as moribund if no coordinated movement or deficient response to external stimulus was observed (i.e., after gentle probing with a fine paint brush). Mortality was expressed by combining the total number of dead and moribund insects.

Data analysis

Mortality data from dose–response bioassays were subjected to probit analysis based on Finney (1964) using PriProbit 3.4 (Sakuma 1998). The software tests the linearity of dose-mortality response and provides the slope, the lethal concentrations (LC), and the 95 % fiducial limits (FL) of the lethal concentration for each mortality line. Using PriProbit, the relative potency ratio among responses was calculated. Responses were considered significantly different when the 95 % confidence interval of relative potency ratio did not include the value 1. Mortality was corrected for control mortality using Abbott's formula (Abbot 1925).

Results were compared to the reference strain to estimate the resistance ratio. In addition, the estimated LC_{80} and LC_{95} were compared to the maximum recommended label rate (RLR_{max}) to evaluate the potential of insecticide control failure based on the work of Silva et al. (2011) and Roditakis et al. (2013a). As indicated in the respective decision of the Hellenic Ministry of Rural Development and Agriculture the RLR_{max} for chlorantraniliprole was 35 mg L^{-1} and for flubendiamide 60 mg L^{-1}. Here, the authors would like to note that flubendiamide is not registered for the control of the tomato borer in Italy.

Pair wise comparisons were used to investigate the correlation between the responses of the strains to the diamide insecticides. A two-tailed test was applied to investigate the significance of Pearson product moment correlation. Analysis was conducted with the SPSS 11 statistical program (SPSS Inc., Chicago, IL).

Results

The probit analysis results are presented in Table 2. The responses of tested populations to both insecticides were homogenous and fitted the Log(Dose)/Probit(Mortality) model. The dose–response curves of tested populations exhibited high slopes (>1) in most cases. For chlorantraniliprole, similar average slopes were observed between the Italian and the 2014 Greek strains. More specifically, the slopes for the Italian strains ranged between 0.99 and 1.66 with an average of 1.36 (s.e. 0.16), while for Greek strains, the slopes ranged between 0.98 and 1.81 with an average of 1.29 (s.e. 0.18). The slopes for the Greek strains from 2012 were higher than 2 in all cases, potentially suggesting

Table 2 Log-dose probit-mortality data for *T. absoluta* populations with the insecticides chlorantraniliprole and flubendiamide

Year	Strain	N	LC_{50}	FL 95 %	RR	LC_{80}	FL 95 %	LC_{95}	Slope	s.e.	X^2	d*f*	*p*
Chlorantraniliprole													
2014	Lab	187	0.18	0.13–0.30a		0.72	0.42–1.7	2.6	1.43	0.27	0.4	3	0.930
2012	Lab	144	0.16	0.08–0.23A		0.34	0.23–0.63	0.73	2.46	0.58	1.0	1	0.320
2010	Lab*	379	0.42	0.33–0.53		0.99	0.78–1.4	2.2	2.28	0.25	4.1	9	0.906
Italy													
2014	IT-PACH-14-1	189	47.6	30.8–77.1c	265	243	136–611	1,147	1.19	0.17	8.0	4	0.090
2014	IT-PACH-14-2	126	63.7	42.1–128c	354	204	108–1,123	622	1.66	0.42	2.4	1	0.120
2014	IT-ACAT-14-1	192	435	165–1,193d	2,414	3,022	1,124–79,653	19,242	0.99	0.30	4.4	33	0.220
2014	IT-GELA-14-1	191	225	135–343d	1,250	762	493–1,369	2,438	1.58	0.24	3.0		0.380
Greece													
2014	GR-TYMP-14-1	189	0.38	0.17–0.57a	2	1.1	0.73–2.0	3.0	1.81	0.42	1.6	3	0.650
2014	GR-IER-14-1	159	2.45	1.24–17.0b	14	17.6	5.0–2,476	115	0.98	0.31	0.1	2	0.940
2014	GR-IER-14-2	189	1.34	0.77–2.3b	7	7.8	4.0–27.7	41.6	1.10	0.21	4.4	3	0.220
2014	GR-IER-14-3	242	1.91	0.97–3.2b	11	8.8	4.8–28.9	37.6	1.27	0.28	0.9	4	0.910
2012	GR-ARVI-12-1	191	0.17	0.12–0.23A	1	0.31	0.23–0.49	0.55	3.26	0.69	0.3	3	0.957
2012	GR-TYMP-12-2	190	0.14	0.09–1.98A	1	0.36	0.25–0.56	0.85	2.12	0.32	1.3	3	0.726
Flubendiamide													
2014	Lab	186	0.79	0.3–1.5a		4.65	2.4–13.3	25.2	1.09	0.22	1.2	3	0.740
2010	Lab*	120	1.31	0.78–2.2		5.68	3.1–15.7	23.1	1.32	0.23	6.2	4	0.188
Italy													
2014	IT-PACH-14-1	127	993	384–1649c	1,257	2,944	1,762–10,243	8,306	1.78	0.53	2.5	1	0.110
2014	IT-PACH-14-2	128	1,376	792–2772c	1,742	59,718	2,891–47,759	23,813	1.32	0.36	0.0	1	0.910
2014	IT-GELA-14-1	190	1,019	500–2130c	1,289	5,776	2,622–33,336	30,267	1.11	0.26	1.5	3	0.690
Greece													
2014	GR-TYMP-14-1	186	1.7	0.85–2.6a	2	4.7	3.1–9.2	12.2	1.94	0.47	1.8	3	0.610
2014	GR-IER-14-1	222	3.8	2.1–6.7b	5	22.0	11.1–90.3	117	1.10	0.24	7.3	4	0.120
2014	GR-IER-14-2	192	3.6	1.5–7.4b	5	31.8	13.9–154	254	0.89	0.18	1.6	3	0.670
2014	GR-IER-14-3	192	8.4	3.6–17.0b	11	45.1	21.4–239	223	1.15	0.29	0.2	3	0.970

Lab strain is the susceptible reference strain tested at different time periods

n number of larvae tested, *FL* fiducial limits, *RR* resistance ratio. LC_{50} in mg L^{-1}

[a] Different letters indicate significant differences in the responses ($P < 0.05$, see text for details). Capital letters indicate comparisons with the respective reference strain

[b] Chi-square testing linearity of dose-mortality response

* Data from Roditakis et al. (2013b) with optimized analysis by PriProbit (Sakuma 1998)

higher homogeneity in the populations of 2012 (Table 2). Similar average slopes were also observed for flubendiamide for the Italian and the Greek strains. Slopes for the Italian strains ranged between 1.11 and 1.78 with an average of 1.40 (s.e. 0.20), while for Greek strains, the slopes ranged between 0.89 and 1.95 with an average of 1.27 (s.e. 0.23).

The susceptibility levels of the laboratory susceptible reference strain (Lab strain) to flubendiamide and chlorantraniliprole were evaluated over a period of 5 years (Table 2). For flubendiamide, no differences were observed between 2010 and 2014. For chlorantraniliprole, a slight difference was observed between 2010, 2012, and 2014 assays (Fig. 1).

In 2012, the LC_{50}s ranged between 0.14–0.17 mg L^{-1} for chlorantraniliprole and no differences could be observed when comparing with the response of the susceptible reference strain. No data are available for flubendiamide in 2012.

In 2014, the LC_{50}s ranged between 0.14–2.45 for chlorantraniliprole and 1.7–8.4 for flubendiamide (LC_{50}s in mg L^{-1}). Calculated resistance ratios were found up to 14- and 11-fold for chlorantraniliprole and flubendiamide,

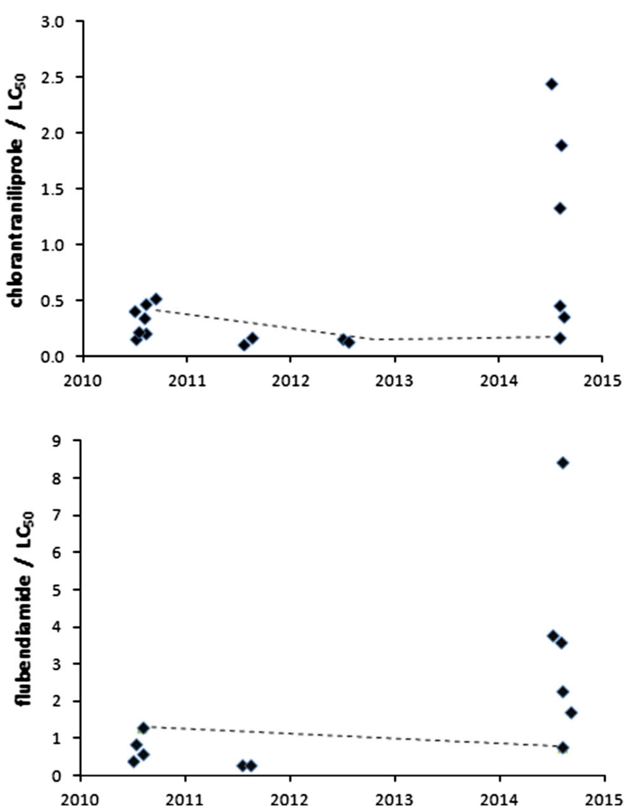

Fig. 1 The LC_{50}s for chlorantraniliprole and flubendiamide from field collected Greek strains over a period of 5 years. *Dashed line* represents the baseline toxicity as estimated by the LC_{50} values of the Lab strain over time. Data of 2010 and 2011 were taken from Roditakis et al. (2013a)

respectively, suggesting development of low resistance levels in GR-IER-14-1 and GR-IER-14-3. Considering the LC_{50}s obtained between 2010 and 2012, a significant divergence from the baseline toxicity was observed for the first time in 2014 (Fig. 1), potentially suggesting that the observed resistance levels developed only recently. Nevertheless, LC_{80}s were lower than the RLR_{max}, in all cases, indicating that field efficacy levels of both chemicals are still sufficiently high on the tested strains.

All Italian strains were collected in 2014. The LC_{50}s ranged between 47.6–435 for chlorantraniliprole and 993–1.376 for flubendiamide (LC_{50}s in mg L^{-1}). Comparisons to the susceptible reference strain revealed resistance ratios ranging from 265- to 2.414-fold and 1.257- to 1.742-fold for chlorantraniliprole and flubendiamide, respectively. In addition, LC_{80}s were also found significantly higher than 35 and 60 mg L^{-1}, the maximum recommended label rates (RLR_{max}) for chlorantraniliprole and flubendiamide, respectively.

Investigation of cross resistance with pair wise comparisons within regional groups of strains was not conducted due to the low numbers of pairs (3–4 pairs). Comparing all 2014 strains together, significant correlation was observed between the resistance ratios for chlorantraniliprole and flubendiamide ($r = 0.97$, $t = 8.48$, $n = 7$, $P < 0.01$, Pearson Correlation, Two-tailed test, log transformed data). It is suggested that the resistance mechanisms involved in the tested strains may confer resistance to both chlorantraniliprole and flubendiamide.

A direct association with application history could not be demonstrated in this study, possibly due to the lack of a full application history record. However, there is one important point regarding the pest control tactics adopted in the concerned farms; more than half of the recorded applications were performed with a diamide insecticide during 2014. More specifically, the percent (%) of diamide usage based on the application history was 52 % for Crete and 64 % for Sicily, denoting the extreme reliance of pest control on the particular mode of action in farms of Southern Europe.

Discussion

The susceptibility of the tomato borer *T. absoluta* to insecticides has been monitored over the past years and was recently published (Roditakis et al. 2013a, b). In 2014, for the very first time tomato borer cross resistance between chlorantraniliprole and flubendiamide was clearly detected in Italian populations, with resistance ratios greater than 1.000-fold for both compounds. The resistance ratios in Greek populations are at a much lower level but seem to run in parallel, too. Similar studies in other regions of the world indicated variations of less than 10-fold to diamide insecticides in *T. absoluta* (Roditakis et al. 2013b; Campos et al. 2014b). Although the present study is to our knowledge the first report of high-level resistance to diamide insecticides in *T. absoluta,* cross resistance to diamides in other lepidopteran pests has been previously reported, in *Plutella xylostella* and *Adoxophyes honmai* (Wang and Wu 2012; Uchiyama and Ozawa 2014). Troczka et al. (2012) demonstrated that resistance in *P. xylostella* was associated with a glycine to glutamic acid substitution (G4946E) in the C-terminal membrane-spanning domain of the RyR. Metabolic detoxification has also been implicated to some extent; however, these initial findings have not been investigated further yet (Wang et al. 2013; He et al. 2014). The mechanisms involved in diamide resistance for *T. absoluta* are unknown and under investigation by our research group.

Monitoring of susceptibility levels in Greece allowed the early detection of low-level resistance in *T. absoluta*, prior to extensive complains on the performance of insecticides or documented control failures. This is a major advantage in pest management as appropriate and targeted guidelines can now be disseminated with a clear warning that incompliance may result in potentially reduced performance of the investigated chemicals.

In Italy, the resistance levels where found >1.000-fold higher and control failures could be associated with diamide resistance. One of the key questions now in Sicily is the extent of the resistance phenomenon in greenhouse tomato crops and the level of impact this may have on the pest management in the area. This could be only answered by extensive resistance monitoring surveys in the hope to identify large niches of susceptible populations. A second key question concerning the Italian populations is on the resistance mechanisms in the particular environment. Studies in diamide resistant *P. xylostella* have shown that reversal of resistance may occur in absence of selection pressure (Wang et al. 2013; Ribeiro et al. 2014). On the other hand, other *P. xylostella* strains have shown long term stability suggesting absence of fitness cost (Steinbach et al. 2014). In some cases, late detection of resistance almost fixed homozygous resistance alleles in an area, thus eliminating susceptible phenotypes and working against resistance reversal. This has been demonstrated for pyrethroid resistance in *T. absoluta and Bemisia tabaci*: few/no susceptible insects to pyrethroids have been identified after extensive surveys (Tsagkarakou et al. 2009; Haddi et al. 2012). In these cases, tested populations possess high pyrethroid resistance levels although the particular chemical class has not been used (Roditakis et al. 2009, 2013a).

Diamide insecticides in several regions, such as Crete and Sicily, are considered the basis for tomato borer control due to their high efficacy and selective profile for beneficial insects (i.e., predators and pollinators) (Biondi et al. 2012; Larson et al. 2014). This can be clearly noted by the high usage % of diamides in the sampled farms. Development of high resistance levels to diamides might have tremendous impact on current management practices and potentially on tomato production. It is of critical importance that the guidelines on the rational use of diamides in pest management schemes for *T. absoluta* should be followed before any further development and/or expansion of resistance occurs in both countries (see product label). It is also important to mention the unprecedented efforts done proactively by the industry (IRAC, http://www.irac-online.org/) and the whole scientific community to prevent the onset of diamide resistance via strict label writing and unprecedented, joint educational activity (Teixeira and Andaloro 2013). For this scope, simplified illustrated guidelines were made public to facilitate adoption of basic resistance management tactics (IRAC 2011). The resistance mechanisms and cross-resistance pattern in those strains are currently under investigation in order to check for alternative effective molecules. However, reliance solely on chemicals will not provide the flexibility required for a rational resistance management scheme as part of an integrated pest management scenario. Potentially introgression of chemical control with the use of beneficials, such as predators and parasitoids (Zappalà et al. 2012; Chailleux et al. 2013; Mollá et al. 2014), and the suitable cultural, physical, and semiochemical means should be further investigated and implemented, reducing the number of required applications with chemicals in order to keep selection pressure to a minimum. All possible tools need to be exploited to suppress further spread of diamide resistance in a devastating resistance-prone pest such as *T. absoluta*. Mode of action rotation is one of the options, but should be part of a wider IPM program.

Author contribution

ER, RN, MG3, and AB conceived and designed research, EV, MG1, and MS conducted experiments, ER, RN, and AB wrote the manuscript, ER and MG1 analyzed data.

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