DISCOVERY OF RESISTANCE-BREAKING CHEMISTRIES FOR VARROA MITE MANAGEMENT

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INTRODUCTION

The varroa mite is a primary driver for the periodical losses of bee colonies. The mite requires bees for food and reproduction and, in turn, elicits physiological deficiencies and disease transmission that compromise the health of bee colonies. The mite nervous system is a target site for existing acaricide chemistries. However, these acaricides not only have adverse health effects on bees, but resistance to these chemistries limits their use to reduce mite infestations and disease transmission in bee colonies. Voltage-gated chloride channels are involved the maintenance of nerve and muscle excitability in arthropod pests, which suggests these channels might be exploited as target sites for acaricide chemistries. Apistan® (the pyrethroid tau-fluvalinate), Checkmite+™ (the organophosphate coumaphos), and Apivar® (the foramidine amitraz) are among several control products for management of varroa mites (Martel et al. 2007). The effectiveness of these chemistries has diminished as a result of the increasing incidence of either target-site or metabolic detoxification resistance in varroa mite populations, worldwide (Van Leeuwen et al. 2010; González-Cabrera et al. 2013; Dmitryjuk et al. 2013). Here, we report a toxicological analysis of a natural stilbene product and related analogs against acaricide-susceptible and -resistant varroa mite populations. The specific aims of this study are: 1) to determine the field efficacy of stilbene chemistries to acaricide-susceptible and -resistant varroa mite populations and 2) to determine the mechanisms of resistance in acaricide-susceptible and -resistant varroa mite populations.

EXPERIMENTAL APPROACH AND RESULTS

TOXICITY AND FIELD EFFICACY BIOASSAYS OF ESTABLISHED AND EXPERIMENTAL ACARICIDES

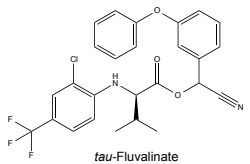
- Honey bee colonies are maintained at the Virginia Tech Price's Fork Apiary. Nurse bees (6-10 d old adults) were collected for each toxicity and field efficacy bioassay. Technical grade *tau*-fluvalinate, coumaphos, and amitraz were purchased from ChemService Inc. (West Chester, PA). 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), 2-methoxystilbene, and 3,5-dimethoxystilbene were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). (E)-2-(4-methoxystyryl)phenol was provided by Professor Jeffrey Bloomquist at the University of Florida. Apistan®, CheckMite+™, and Apivar® strips were purchased from Dadant & Sons Beekeeping Equipment (Chatham, VA).

- *tau*-Fluvalinate, coumaphos, amitraz, and stilbene toxicity was examined with honey bees using oral and topical exposure bioassays. Technical grade acaricides were orally administered in 50% sucrose solution or topically administered as a 1-µl aliquot to the dorsal thorax of each honey bee (ethanol carrier solvent). Each acaricide was tested at 10% active ingredient on 10 honey bees per treatment. Each treatment was replicated three times. Percent mortality was assessed 24 h post-treatment. Less than 10% mortality was observed for the honey bees exposed to each oral and topical treatment of acaricide.

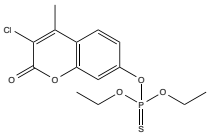
- Field efficacy of *tau*-fluvalinate, coumaphos, amitraz, and stilbenes was assessed with acaricide-susceptible and -resistant varroa mite populations using contact exposure bioassays. Bioassay arenas consisted of a 750-ml plastic container fastened to a wood platform with a removable sticky board. For each bioassay, *ca.* 300 honey bees were collected from the brood frame(s) of a hive. Bioassay arenas contained a 3 x 3 cm tab impregnated with *tau*-fluvalinate (Apistan®, 10.2% ai), coumaphos (CheckMite+™, 10.0% ai), or amitraz (Apivar®, 3.3% ai) or treated with the stilbenes (10% ai). Bioassay arenas were transported to the laboratory and maintained in a dark environmental chamber at 32 °C to measure the time-dependent efficacy of each acaricide. Number of varroa mites on the sticky boards were counted after a 3- and 6-h acaricide exposure period and the remaining varroa mites attached to the honey bees were counted after a 3-min ethanol wash.

CYTOCHROME P450 MONOOXYGENASE, ESTERASE, GLUTATHIONE *S*-TRANSFERASE, AND ACETYLCHOLINESTERASE BIOASSAYS

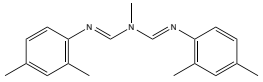
- Cytochrome P450 monooxygenase, esterase, and glutathione *S*-transferase enzyme activities were measured in varroa mites from 20 honey bee colonies using the methods described by Anderson and Zhu (2004) and Jin-Clark et al. (2008). Acetylcholinesterase activity was measured in varroa mites using the methods of Adelman et al. (2012). Total protein was measured according to the method of Smith et al. (1985). Enzyme and protein measurements were performed on a SpectraMax M2 multimode microplate reader (Molecular Devices, Sunnyvale, CA).



tau-Fluvalinate
Cyano(3-phenoxyphenyl)methyl N-[2-chloro-4-(trifluoromethyl)phenyl]-D-valinate



Coumaphos
O-(3-Chloro-4-methyl-2-oxo-2H-chromen-7-yl) O,O-diethyl phosphorothioate



Amitraz
N'-(2,4-Dimethylphenyl)-N-[(E)-[(2,4-dimethylphenyl)imino]methyl]-N-methylimidoformamide

Table 1. Field efficacy bioassays of *tau*-fluvalinate (Apistan®), coumaphos (CheckMite+™), and amitraz (Apivar®) against acaricide-susceptible and –resistant varroa mite populations. Acaricide efficacy was assessed for the varroa mite populations after a 3- and 6-h exposure period. Varroa mites remaining on the honey bees were removed after a 3-min ethanol wash. Acaricide efficacy < 60% suggests the possibility of resistance after a 6-h exposure period. Each test was considered valid based on the presence of > 5 varroa mites per 100 honey bees. The honey bee colonies have not been treated with acaricides.

Acaricide	Hive ID	3 h Mite Drop	6 h Mite Drop	3 min EtOH Mite Count	Total Mite Count	3 h Efficacy	6 h Efficacy	Total Bee Count	Mite Count / 100 Bees	Resistant < 60% Efficacy	Valid Test > 5 Mites / 100 Bees
tau-Fluvalinate, 10.2% ai	VT15	13	1	23	37	35.14%	37.84%	458	8.08	Yes	Yes
	VT22	64	7	11	82	78.05%	86.59%	302	27.15	No	Yes
	VT26	24	2	9	35	68.57%	74.29%	388	9.02	No	Yes
	VT36	27	2	3	32	84.38%	90.63%	343	9.33	No	Yes
	VT37	14	3	3	20	70.00%	85.00%	318	6.29	No	Yes
	VT39	17	3	18	38	44.74%	52.63%	163	23.31	Yes	Yes
	VT52	62	6	32	100	62.00%	68.00%	493	20.28	No	Yes
	VT61	16	4	4	24	66.67%	83.33%	311	7.72	No	Yes
	VT67	28	3	5	36	77.78%	86.11%	396	9.09	No	Yes
	VT100	13	5	6	24	54.17%	75.00%	236	10.17	No	Yes
	VT112	6	4	12	22	27.27%	45.45%	240	9.17	Yes	Yes
Coumaphos, 10.0% ai	VT13	12	4	3	19	63.16%	84.21%	379	5.01	No	Yes
	VT15	3	1	11	15	20.00%	26.67%	217	6.91	Yes	Yes
	VT22	84	10	6	100	84.00%	94.00%	357	28.01	No	Yes
	VT26	15	3	17	35	42.86%	51.43%	321	10.90	Yes	Yes
	VT36	5	1	7	13	38.46%	46.15%	257	5.06	Yes	Yes
	VT37	8	6	9	23	34.78%	60.87%	314	7.32	No	Yes
	VT39	8	15	7	30	26.67%	76.67%	313	9.58	No	Yes
	VT42	3	3	8	14	21.43%	42.86%	254	5.51	Yes	Yes
	VT52	20	5	15	40	50.00%	62.50%	283	14.13	No	Yes
	VT61	38	4	4	46	82.61%	91.30%	355	12.96	No	Yes
	VT67	11	1	12	24	45.83%	50.00%	243	9.88	Yes	Yes
	VT100	18	12	4	34	52.94%	88.24%	364	9.34	No	Yes
	VT112	27	6	1	34	79.41%	97.06%	288	11.81	No	Yes
Amitraz, 3.3% ai	VT15	32	1	0	33	96.97%	100.00%	436	7.57	No	Yes
	VT22	76	0	0	76	100.00%	100.00%	363	20.94	No	Yes
	VT26	41	8	0	49	83.67%	100.00%	577	8.49	No	Yes
	VT36	20	0	0	20	100.00%	100.00%	343	5.83	No	Yes
	VT37	39	0	0	39	100.00%	100.00%	445	8.76	No	Yes
	VT39	28	0	0	28	100.00%	100.00%	385	7.27	No	Yes
	VT42	20	0	0	20	100.00%	100.00%	349	5.73	No	Yes
	VT52	94	0	0	94	100.00%	100.00%	525	17.90	No	Yes
	VT61	34	0	0	34	100.00%	100.00%	413	8.23	No	Yes
	VT67	61	0	0	61	100.00%	100.00%	391	15.60	No	Yes
	VT100	45	0	0	45	100.00%	100.00%	352	12.78	No	Yes
	VT112	19	0	0	19	100.00%	100.00%	342	5.56	No	Yes

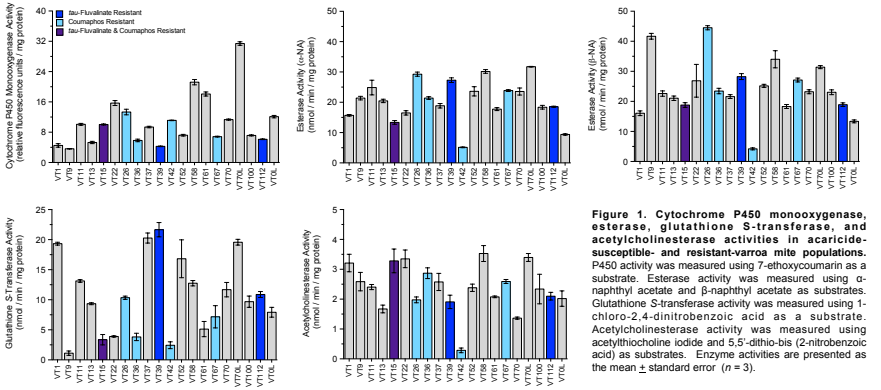


Figure 1. Cytochrome P450 monooxygenase, esterase, glutathione S-transferase, and acetylcholinesterase activities in acaricide-susceptible- and resistant-varroa mite populations. P450 activity was measured using 7-ethoxycoumarin as a substrate. Esterase activity was measured using α-naphthyl acetate and β-naphthyl acetate as substrates. Glutathione *S*-transferase activity was measured using 1-chloro-2,4-dinitrobenzoic acid as a substrate. Acetylcholinesterase activity was measured using acetylthiocholine iodide and 5,5'-dithio-bis (2-nitrobenzoic acid) as substrates. Enzyme activities are presented as the mean ± standard error ($n = 3$).

Table 2. Field efficacy bioassays DIDS, 2-methoxystilbene, 3,5-dimethoxystilbene, and (E)-2-(4-methoxystyryl)phenol) against acaricide-resistant varroa mite populations. Acaricide efficacy was assessed for the varroa mite populations after a 3- and 6-h exposure period. Varroa mites remaining on the honey bees were removed after a 3-min ethanol wash. Acaricide efficacy < 60% suggests the possibility of resistance after a 6-h exposure period. Each test was considered valid based on the presence of > 5 varroa mites per 100 honey bees. Varroa mite populations were selected based on their resistance to *tau*-fluvalinate and/or coumaphos.

Acaricide	Hive ID	3 h Mite Drop	6 h Mite Drop	3 min EtOH Mite Count	Total Mite Count	3 h Efficacy	6 h Efficacy	Total Bee Count	Mite Count / 100 Bees	Tolerant < 60% Efficacy	Valid Test > 5 Mites / 100 Bees
DIDS, 10.0% ai	VT15	1	9	36	46	2.17%	21.74%	431	10.67	Yes	Yes
	VT26	4	7	7	18	22.22%	61.11%	344	5.23	No	Yes
	VT36	3	12	7	22	13.64%	68.18%	284	7.75	No	Yes
	VT39	1	3	17	21	4.76%	19.05%	216	9.72	Yes	Yes
	VT42	1	2	9	12	8.33%	25.00%	425	2.82	Yes	No
	VT112	8	13	9	30	26.67%	70.00%	315	9.52	No	Yes
2-methoxystilbene, 10.0% ai	VT15	1	4	40	45	2.22%	11.11%	296	15.20	Yes	Yes
	VT26	3	3	12	18	16.67%	33.33%	180	10.00	Yes	Yes
	VT36	0	1	13	14	0.00%	7.14%	262	5.34	Yes	Yes
	VT39	1	1	14	16	6.25%	12.50%	162	9.88	Yes	Yes
	VT42	1	1	14	16	6.25%	12.50%	316	5.06	Yes	Yes
	VT112	4	1	27	32	12.50%	15.63%	309	10.36	Yes	Yes
3,5-dimethoxystilbene, 10.0% ai	VT15	5	0	25	30	16.67%	16.67%	276	10.87	Yes	Yes
	VT26	3	2	15	20	15.00%	25.00%	198	10.10	Yes	Yes
	VT36	5	3	8	16	31.25%	50.00%	309	5.18	Yes	Yes
	VT39	7	0	29	36	19.44%	19.44%	236	15.25	Yes	Yes
	VT42	3	0	9	12	25.00%	25.00%	200	6.00	Yes	Yes
	VT112	1	1	20	22	4.55%	9.09%	401	5.49	Yes	Yes
(E)-2-(4-methoxystyryl)phenol, 10.0% ai	VT15	15	7	23	45	33.33%	48.89%	303	14.85	Yes	Yes
	VT26	2	1	15	18	11.11%	16.67%	223	8.07	Yes	Yes
	VT36	7	2	8	17	41.18%	52.94%	307	5.54	Yes	Yes
	VT39	5	3	21	29	17.24%	27.59%	243	11.93	Yes	Yes
	VT42	6	3	15	24	25.00%	37.50%	318	7.55	Yes	Yes
	VT112	3	1	18	22	13.64%	18.18%	395	5.57	Yes	Yes

SUMMARY AND FUTURE DIRECTIONS

- Apistan® (the *tau*-fluvalinate pyrethroid), Checkmite+™ (the organophosphate coumaphos), and Apivar® (the foramidine amitraz) were used as acaricide standards. Both *tau*-fluvalinate and coumaphos have lower field efficacy than amitraz to the tested varroa mite populations. This suggests that the varroa mites are resistant to *tau*-fluvalinate and coumaphos based on the percent efficacies being less than 60% (Table 1).

- Cytochrome P450 monooxygenases, esterases, glutathione *S*-transferases, and acetylcholinesterase exhibit differential activities across the varroa mite populations (Fig. 1). The differences in enzyme activities may confer metabolic detoxification resistance in the varroa mite populations with reduced *tau*-fluvalinate and coumaphos field efficacy; however, these use of acaricide synergists will be required to confirm the possibility of metabolic detoxification resistance and the involved mechanisms.

- *tau*-Fluvalinate- and coumaphos-resistant varroa mite populations appear to be tolerant to the stilbenes 2-methoxystilbene, 3,5-dimethoxystilbene, and (E)-2-(4-methoxystyrl)phenol with field-efficacy percentages less than 60% (Table 2). However, the stilbene DIDS exhibited field efficacy higher than 60% against the acaricide-resistant varroa mite populations compared to *tau*-fluvalinate and coumaphos (Table 2). These data suggest that DIDS might serve as a candidate chemistry for the development of resistance-breaking acaricides for varroa mite management.

- Our current research activities are focused on the acaricide-resistance monitoring and management, identification of metabolic detoxification and target-site resistance mechanisms, and discovery of unique resistance-breaking acaricides for the management of varroa mite population and the protection of honey bee colony health.

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