DISCOVERY OF STILBENE 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 (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 were: 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 STANDARD USE AND STILBENE ACARICIDES

- Honey bee colonies are maintained at the Virginia Tech. 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 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 and varroa mites using oral, topical, or dip 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 bee (ethanol carrier solvent). Each acaricide was tested at 10% active ingredient on 10 bees per treatment. 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. Each acaricide was tested at six concentrations on 5 varroa mites per treatment. Each honey bee and varroa mite treatment was replicated three times
- 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.

GENERAL ESTERASE, GLUTATHIONE *S*-TRANSFERASE, CYTOCHROME P450 MONOOXYGENASE, and AChE 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 (AChE) 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).

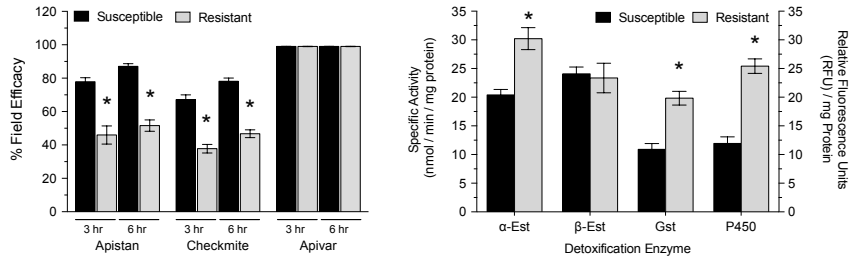

Figure 1. Field efficacy bioassays of *tau*-fluvalinate (Apistan®), coumaphos (CheckMite+™), and amitraz (Apivar®) and detoxification enzyme activities for acaricide-susceptible and –resistant varroa mite populations. Acaricide efficacy was assessed for the varroa mite populations after a 3- and 6-h 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 ($P < 0.05$, $n = 40$). General 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. Cytochrome P450 monooxygenase activity was measured using 7-ethoxycoumarin as a substrate. Enzyme activities are presented as the mean ± standard error ($P < 0.05$, $n = 20$).

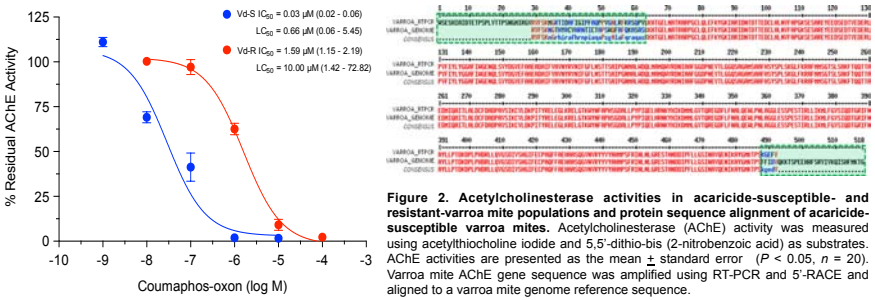

Figure 2. Acetylcholinesterase activities in acaricide-susceptible- and resistant-varroa mite populations and protein sequence alignment of acaricide-susceptible varroa mites. Acetylcholinesterase (AChE) activity was measured using acetylthiocholine iodide and 5,5'-dithio-bis (2-nitrobenzoic acid) as substrates. AChE activities are presented as the mean ± standard error ($P < 0.05$, $n = 20$). Varroa mite AChE gene sequence was amplified using RT-PCR and 5'-RACE and aligned to a varroa mite genome reference sequence.

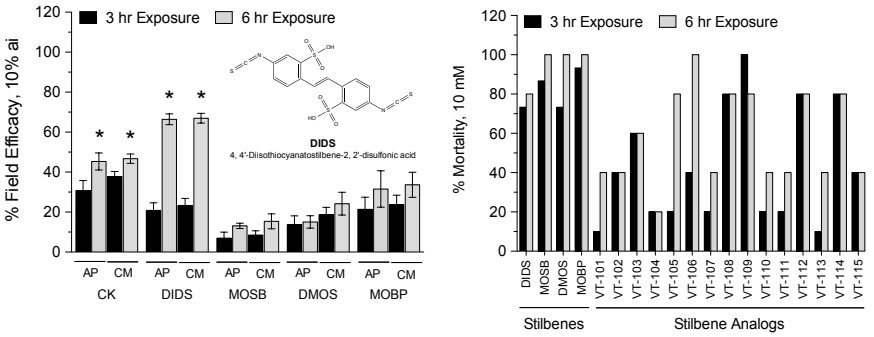

Figure 3. Field efficacy bioassays of DIDS and stilbene analogs against *tau*-fluvalinate- (Apistan®) and coumaphos- (CheckMite+™) resistant varroa mite populations. Stilbene (10.0% ai) efficacy was assessed for the *tau*-fluvalinate- (AP, Apistan®) and coumaphos- (CM, CheckMite+™) resistant varroa mite populations after a 3- and 6-h exposure period. Varroa mites remaining on the honey bees were removed after a 3-min ethanol wash. Stilbene efficacy < 60% suggests the possibility of tolerance 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 coumaphos (Fig. 1, $P < 0.05$, $n = 10$).

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% (Fig. 1).
- Acute toxicity of the standard-use acaricides *tau*-fluvalinate, coumaphos, and amitraz is greater than that of DIDS to acaricide-susceptible varroa mites (Table 1). Coumaphos-oxon has 15-fold less acute toxicity to coumaphos-resistant varroa mites compared to coumaphos-susceptible varrora mites (Fig. 2). Coumaphos-oxon had 53-fold less anticholinesterase activity to coumaphos-resistant varroa mites compared the coumaphos-susceptible varroa mites (Fig. 2).
- General esterases, glutathione *S*-transferases, and cytochrome P450 monooxygenases exhibit differential activities across the coumaphos-resistant varroa mite populations (Fig. 2); however, the differences in enzyme activities may not confer metabolic detoxification resistance in the varroa mite populations with reduced coumaphos field efficacy.
- Coumaphos-resistant varroa mite populations appear to be tolerant to the stilbenes 2-methoxystilbene (MOSB), 3,5-dimethoxystilbene (DMOS), and (E)-2-(4-methoxystyrl)phenol (MOBP) with field-efficacy percentages less than 60% (Fig. 3). However, the stilbene DIDS exhibited field efficacy higher than 60% against the coumaphos-resistant varroa mite populations compared to coumaphos (Fig. 3). These data suggest that DIDS might serve as a candidate chemistry for the development of alternative acaricides for coumaphos-resistant varroa mite populations (Fig. 3).
- Current research activities are focused on the acaricide-resistance monitoring and management, identification of metabolic detoxification and target-site resistance mechanisms, and discovery of alternative chemistries with acaricidal activity for the management of varroa mite populations and the protection of honey bee colony health.

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