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Effects of a Naturally Occurring and a Synthetic Synergist on Toxicity of Three Insecticides and a Phytochemical to Navel Orangeworm (Lepidoptera: Pyralidae)

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ABSTRACT The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is the most destructive lepidopteran pest of almonds [*Prunus dulcis* (Mill.) D.A.Webb] and pistachios (*Pistacia vera* L.) in California and is a serious problem in figs (*Ficus carica* L.) and walnuts (*Juglans* spp.). In addition to direct damage, larval feeding leaves nuts vulnerable to infection by *Aspergillus* spp., fungi that produce toxic aflatoxins. A potentially safe and sustainable approach for managing navel orangeworm in orchards may be to use natural essential oil synergists to interfere with this insect's ability to detoxify insecticides and phytochemicals. We tested the effects of a naturally occurring plant-derived chemical, myristicin, and a synthetic inhibitor of cytochrome P450 monooxygenases (P450s), piperonyl butoxide, on the toxicity of three insecticides (α-cypermethrin, τ-fluvalinate, and methoxyfenozide [Intrepid]) and a phytochemical (xanthotoxin) to *A. transitella*. Piperonyl butoxide significantly synergized α-cypermethrin and τ-fluvalinate, whereas myristicin synergized only α-cypermethrin. Piperonyl butoxide synergized the toxicity of xanthotoxin as early as 72 h after exposure, whereas myristicin synergized xanthotoxin after 120 h. In view of these findings and the limited availability of environmentally safe synthetic insecticides for sustainable management, particularly in organic orchards, myristicin is a potential field treatment in combination with insecticides to reduce both navel orangeworm survival and aflatoxin contamination of nuts. In addition, this study demonstrates that in *A. transitella* the insect growth regulator methoxyfenozide is not detoxified by P450s.

KEY WORDS *Amyelois transitella*, cytochrome P450 monooxygenase, synergist, myristicin, insecticide

The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is the most destructive lepidopteran pest of almonds [*Prunus dulcis* (Mill.) D.A.Webb] and pistachios (*Pistacia vera* L.) in California, as well as a serious pest of figs (*Ficus carica* L.) and walnuts (*Juglans* spp.) (Shelton et al. 1994, Connell 2001, Burks and Brandl 2004, Bentley et al. 2008). Neonates tunnel into the nut and successive instars consume the nutmeat, generating large quantities of frass and webbing (Bentley et al. 2008). The adults lay eggs in unharvested fruits (mummies) when new crop nuts are unavailable (Connell 2001, Molyneux et al. 2007), and the caterpillars have been reported as scavengers on mummies of at least 25 plant species (Shelton and Davis 1994). Control of this pest has been complicated by the rapid expansion of almond, pistachio, and walnut plantings over the past 7 yr, because of the proximity (can be <10 m between orchards) of multiple hosts for navel orangeworm (Higbee and Siegel 2009).

In addition to causing direct losses, larval feeding leaves almonds and pistachios vulnerable to infection by *Aspergillus* spp. that produce aflatoxins (Widstrom 1979; Schatzki and Ong 2000, 2001; Campbell et al. 2003). It is possible that the navel orangeworm has a long evolutionary association with these fungi because this insect tolerates very high levels of aflatoxin (>100 μg/g) in its diet (Niu et al. 2009) due to the activity of detoxification enzymes, including cytochrome P450 monooxygenases (P450s) (Lee and Campbell 2000, Niu et al. 2009). Aflatoxin contamination of nut crops is a major health concern for many countries, including those of the European Union, a major market for nut exports. Consequently, strict regulations have been applied to reduce aflatoxin levels in food (Williams et al. 2004, Wagacha and Muthomi 2008). The Food and Drug Administration set a maximum 20 ppb limit for total aflatoxins in food, including edible nuts, and a maximum 0.5 ppb in milk (Food and Drug Administration 2006), whereas the European Union set even lower limits: nuts can contain a maximum

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level of 8 ppb aflatoxin B1 and 10 ppb of total aflatoxins (http://gain.fas.usda.gov/Recent%20GAIN%20Publications/New%20EU%20Aflatoxin%20Levels%20and%20Sampling%20Plan_Brussels%20USEU_EU-27_3-9-2010.pdf).

Currently, the insecticides used to control navel orangeworm are sprayed without synergists, and in orchards with heavy infestations insecticides are usually applied in a rotation bracketing hull split, when the kernel becomes exposed to larval feeding and consequently to infection by *Aspergillus* spp. The chemical insecticides registered for control include organophosphates, pyrethroids, diamides, diacyl hydrazines, avermectins, and spinosyns; of these insecticides, only spinosyns can be used in organic orchards. A possible strategy to improve navel orangeworm control in conventional and organic orchards may be disruption of the P450 enzymes responsible for insecticide metabolism, host phytochemical metabolism, and aflatoxin tolerance (Lee and Campbell 2000, Niu et al. 2009). Naturally occurring synergists that inactivate these enzymes should render navel orangeworm more sensitive not only to insecticides, allowing the use of lower concentrations, but also to phytochemicals and mycotoxins present in host plant tissues that in turn would increase larval mortality. This same strategy could decrease the prevalence of aflatoxin contamination of nuts, because constituents of herbs, spices, and other plants are known to inhibit the growth of aflatoxin-releasing fungi (Rusul and Marth 1988). Essential oils can suppress growth and aflatoxin formation by *Aspergillus* spp. (Belzile et al. 2000, Razzaghi-Abyaneh et al. 2008, Shukla et al. 2009, Singh et al. 2009, Nogueira et al. 2010) and myristicin, a compound found in the essential oils of several plant species, inhibits the biosynthesis of aflatoxin G1 in *Aspergillus parasiticus* (Razzaghi-Abyaneh et al. 2007, 2010).

Myristicin is a known synergist for synthetic insecticides (Lichtenstein and Casida 1963) and phytochemicals (Berenbaum and Neal 1985, Neal 1989, Neal and Berenbaum 1989). In this study, we compared myristicin activity with that of piperonyl butoxide, a commonly used synthetic inhibitor of P450 enzymes and synergist for pyrethroids (Ishaaya 1993) that has structural similarities to myristicin. We also set out to ascertain the ability of myristicin to synergize the toxicity of a host phytochemical to navel orangeworms. We report the results of feeding studies assessing the effect of myristicin and piperonyl butoxide on the toxicity of two pyrethroids, α-cypermethrin and τ-fluvalinate; a molting hormone agonist, methoxyfenozide (Intrepid), used in almond and pistachio orchards specifically for control of navel orangeworm; and the phytochemical xanthotoxin that occurs naturally in at least one host plant (*F. carica*) for navel orangeworm.

Materials and Methods

Chemicals. Piperonyl butoxide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Myristicin (78% purity), α-cypermethrin, τ-fluvalinate, and xanthotoxin were purchased from Sigma (St. Louis, MO). Methoxyfenozide was obtained from Helena Chemical Company (Memphis, TN). Dimethyl sulfoxide and methanol were obtained from Thermo Fisher Scientific (Waltham, MA). All of these chemicals were dissolved in methanol and stored at −20°C.

Insects. A laboratory colony of *A. transitella* designated as SPIRL-1966 (Siegel et al. 2010) was kept in an insectary at the University of Illinois Urbana-Champaign at 28 ± 4°C and photoperiod of 16:8 (L:D) h. Larvae were mass reared until pupation in 500-ml plastic containers containing 200 g of a wheat bran diet (Finney and Brinkmann 1967). Adults were transferred to a clean 900-ml (32-oz) cup with tissue paper placed inside the cup and also covering the top. One day later, the adults laid eggs on the tissue paper. For bioassays, tissue paper with eggs was placed into a clean 500-ml plastic cup and the newly eclosed larvae were collected after 48 h. Four larvae were gently transferred onto the artificial diet in each 30-ml (1-oz) plastic cup for the bioassays.

Insecticide Preparation. All three insecticides evaluated are stomach poisons, and in these assays the insecticides were incorporated into artificial diets and fed to neonate larvae. Solutions of each compound in methanol were prepared at a range of concentrations (α-cypermethrin: 10, 20, 50, 100, 300, and 500 ng/g; τ-fluvalinate: 0.2, 0.5, 1, 3, and 5 μg/g; methoxyfenozide: 20, 50, 100, 300, and 500 ng/g), pipetted into 30-ml plastic cups containing still-liquid diet and then incorporated by stirring for 2 min. An equal amount of methanol was added into diet for the controls. For each bioassay series, 20 newly eclosed larvae (collected between 0 and 12 h after hatching) were reared on each concentration of insecticide and mortality was recorded at 24 and 48 h. The larvae that did not move when touched with a soft brush were scored as dead.

Synergist Preparation. For the synergist bioassays, the three insecticides (α-cypermethrin, τ-fluvalinate, and methoxyfenozide) and xanthotoxin were supplemented with myristicin and piperonyl butoxide to determine the synergistic effects on the larvae. The final concentrations of the synergists myristicin (25 μg/g) and piperonyl butoxide (200 μg/g) were determined by preliminary tests to be nonlethal for first-instar larvae. A 2-mg/g dose of xanthotoxin was selected because it caused at least 20% mortality to first-instar larvae at 2 d in range-finding assays. This concentration is well within the concentrations known to occur in plants, including *F. carica* (Zaynoun et al. 1984, Duke 1992). The concentrations selected for both the insecticides and xanthotoxin caused >10% mortality to the newly hatched larvae after 36 h.

Bioassays. For the insecticide assays, the diets containing 25 ng/g α-cypermethrin, 2 μg/g τ-fluvalinate, or 50 ng/g methoxyfenozide in the presence or absence of myristicin (25 μg/g) or piperonyl butoxide (200 μg/g) were fed to the larvae. When high concentrations of xanthotoxin stock were dissolved in methanol, the xanthotoxin crystallized immediately after being added to the artificial diets and made ho-

Table 1. LC_{50} values for first-instar *A. transitella* exposed to pyrethroid and diacyl hydrazine insecticides (mortality was recorded at 24 and 48 h after exposure)

Insecticide	LC_{50} at 24 h (95% CL), μg/g	LC_{50} at 48 h (95% CL), μg/g
τ-Fluvalinate	1.900 (1.200–3.000)	0.730 (0.450–1.200)
α-Cypermethrin	0.072 (0.045–0.110)	0.013 (0.007–0.023)
Methoxyfenozide	—[a]	0.076 (0.043–0.130)

[a] No apparent toxicity until larvae molt.

mogeneous incorporation difficult. Therefore, xanthotoxin powder was mixed with the sucrose component of the diet, ground with a mortar and pestle until it was homogeneous, and then added directly to the diets to a final concentration of 2 mg/g xanthotoxin. The controls for the xanthotoxin assays were diet, diet with methanol, and diet supplemented with 25 μg/g myristicin or 200 μg/g piperonyl butoxide. Mortality was recorded daily until >80% of the treated insects were dead. Each bioassay included 20 first instars subjected to a single concentration of these chemicals, and all bioassays were repeated at least three times. Synergism in this study was defined as mortality exceeding the combined baseline mortality of the toxicant and the synergist.

Statistical Analysis. The Probit Analysis function in SPSS version 17 software (SPSS Inc., Chicago, IL) was used to calculate the median lethal concentration (LC_{50}) at 24 and 48 h in the rangefinder studies for the three insecticides. In the synergism assays, separate analyses were calculated for each toxicant by using multiple regression with dummy coding (Cohen and Cohen 1983) and JMP version 5.1 (SAS Institute, Cary, NC); separate analyses were conducted for each time point. There were at least three replicates for each treatment, for a total of 660 larvae assessed per toxicant at each point in time. Initially, differences in survival were assessed for the diet and carrier controls, and these data were pooled to establish baseline mortality when there were no significant differences. In the next stage of the analysis, the mortality from the toxicant alone was set as a baseline, and synergism occurred when the mortality of the toxicant + synergist was significantly higher ($P < 0.05$) than the mortality of the toxicant alone. Differences among synergists at the final observation period were assessed using multiple regression with orthogonal polynomial analysis (Cohen and Cohen 1983) when applicable.

Results

Insecticide Comparison. The insecticides exhibited differential toxicity to the navel orangeworm. Among pyrethroids, α-cypermethrin, with an LC_{50} of 0.072 μg/g at 24 h and an LC_{50} of 0.013 μg/g at 48 h, was 26- and 56-fold more toxic than τ-fluvalinate. The ecdysteroid agonist methoxyfenozide did not cause mortality until 36 h, when the larvae started molting. The toxicity of α-cypermethrin at 48 h was 5.8-fold greater than methoxyfenozide (Table 1).

Synergism of α-Cypermethrin. There were no differences among the diet and carrier controls, and these data were combined to establish the baseline mortality (1.7–7.7%). Piperonyl butoxide was synergistic beginning at 12 h ($F = 44.3$; df = 3, 659; $P < 0.0001$), increasing α-cypermethrin mortality by 34.4% ($P < 0.0001$). Myristicin had no effect until 36 h ($F = 29.7$; df = 3, 659; $P < 0.0001$) when it increased mortality by 7.8% ($P = 0.035$), although the magnitude of its effect increased thereafter (Fig. 1). At 144 h, the addition of piperonyl butoxide still caused higher mortality than the addition of myristicin ($F = 342.7$; df = 3, 659; $P < 0.0001$ for all comparison, $P = 0.041$ for piperonyl versus myristicin).

Synergism of τ-Fluvalinate. There were no differences among the diet and carrier controls and these data were combined to establish the baseline mortality (3.1–15%). τ-Fluvalinate did not cause mortality until 36 h, and at this point piperonyl butoxide was synergistic, increasing mortality by 22.0% ($F = 22.2$; df = 3, 659; $P < 0.0001$). Myristicin did not synergize τ-fluvalinate at any time point assessed (Fig. 2).

Synergism of Methoxyfenozide. There were no differences among the diet and carrier controls, and these data were combined to establish the baseline mortality. Methoxyfenozide did not show an effect until 36 h, and mortality from all treatments was 100% at 120 h. Neither piperonyl butoxide nor myristicin synergized methoxyfenozide (data not shown; Fig. 3).

Synergism of Xanthotoxin. There were no differences among the diet and carrier controls, and these data were combined to establish the baseline mortality (8–18.4%). Xanthotoxin did not increase mortality above the baseline value until 72 h. At this point in time, piperonyl butoxide was synergistic, increasing mortality by 17.8% ($F = 10.9$; df = 3, 659; $P < 0.0001$). Myristicin had no effect until 120 h ($F = 38.3$; df = 3, 659; $P < 0.0001$) when it increased mortality by 12.6% ($P = 0.018$) and remained at this level for the remainder of the assay (Fig. 4).

Discussion

Currently, there are numerous insecticides registered for use in California nut crops to control lepidopteran and hemipteran pests. The pyrethroids bifenthrin, β-cyfluthrin, λ-cyhalothrin, fenpropathrin, permethrin, and esfenvalerate are registered for use in almonds and pistachios. Some pyrethroids have been combined (bifenthrin and α-cypermethrin) for use in vegetable crops and cotton (*Gosssypium hirsutum* L.), which may be adjacent to almond and pistachio orchards, and pyrethroids also are combined with two new classes of insecticides, the diamides (λ-cyhalothrin + rynaxypyr) and diacyl hydrazines (λ-cyhalothrin + methoxyfenozide) as tank mixes for use in almonds and pistachios. In pistachios, neonicotinoids used to control *Ferrisia gilli* Gullan also have activity against navel orangeworm and are applied when navel orangeworm is present. In almonds, some insecticides used to control peach twig borer (*Anarsia lineatella* Zeller) are also toxic to navel orangeworm.

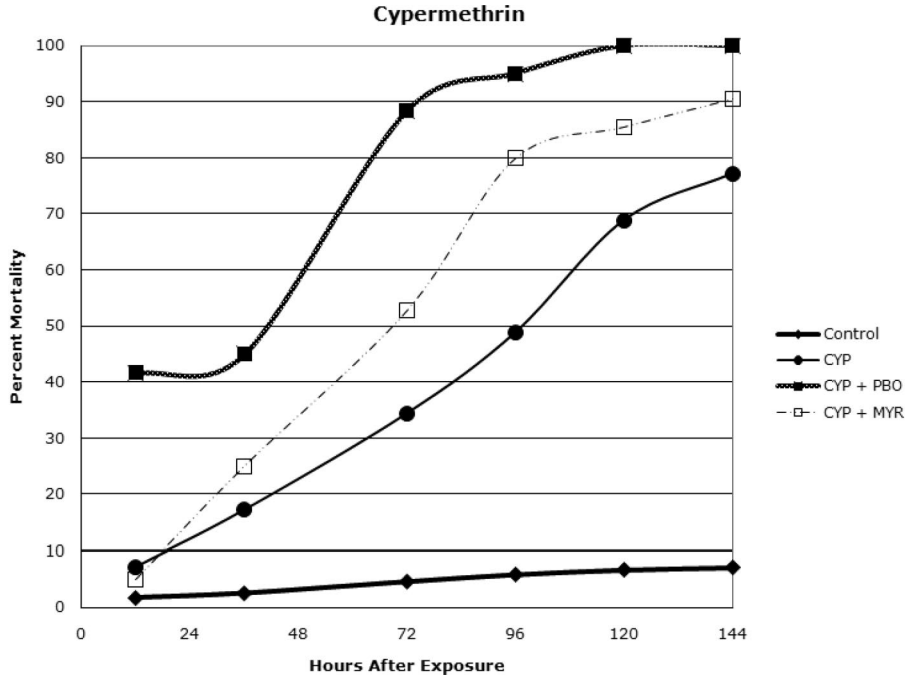

Fig. 1. Synergistic effects of piperonyl butoxide (PBO) and myristicin (MYR) on α-cypermethrin (CYP). Navel orangeworm larvae were exposed to artificial diets containing 25 ng/g CYP supplemented with or without 25 μg/g MYR or 200 μg/g PBO. Plain diet and diets containing the synergists alone or an equal amount of methanol were used as controls. PBO was synergistic beginning at 12 h ($F = 44.3$; df = 3, 659; $P < 0.0001$), increasing CYP mortality by 34.4% ($P < 0.0001$). MYR had no effect until 36 h ($F = 29.7$; df = 3, 659; $P < 0.0001$) when it increased mortality by 7.8% ($P = 0.035$), although the magnitude of its effect increased thereafter. At 144 h, the addition of PBO still caused higher mortality than the addition of MYR ($F = 342.7$; df = 3, 659; $P < 0.0001$ for all comparisons, $P = 0.041$ for piperonyl butoxide versus MYR).

Although the mainstay organophosphate insecticide azinphos-methyl has been phased out of almonds and pistachios, other organophosphates such as phosmet and chlorpyrifos are still used occasionally (Connell 2001, Food and Drug Administration 2006, Bentley et al. 2008).

Insecticides for navel orangeworm control are applied in rotation bracketing hull split and hull slip (e.g., diacyl hydrazine, pyrethroid; diacyl hydrazine + pyrethroid, pyrethroid, pyrethroid; diamide, pyrethroid) but, as stated, organophosphates, neonicotinoids and pyrethroids may have been previously applied for control of other insects. It is possible for navel orangeworm to be exposed to as many as four classes of insecticide in a single season. Applicators assume that rotating insecticides with different modes of action will delay resistance. However, very little is known about the susceptibility of navel orangeworm to insecticides that target other pest species in the orchard or nearby row crops, the pressure these insecticides place on navel orangeworm populations, or the enzymes used by navel orangeworm to detoxify these different classes of insecticides. If insecticide pressure is constant and several classes of insecticide share a common mode of detoxification, multiple resistance may arise. To optimize control and delay insecticide resistance, it is important to understand the relative susceptibility of navel orangeworm to the insecticides used in the orchards and neighboring row crops, as well as the mechanism of detoxification for these different classes of toxicant.

In this study, ingestion of a pyrethroid used for row crops, α-cypermethrin, and a pyrethroid used to control aphids (Hemiptera: Aphididae) and varroa mites (*Varroa destructor* Anderson & Trueman), τ-fluvalinate (Johnson et al. 2006), killed navel orangeworm larvae. Pyrethroid insecticides are metabolized by P450 enzymes and esterases in several insect species (Ishaaya 1993, Pilling et al. 1995, Johnson et al. 2006), and in this study piperonyl butoxide, a well-known synergist that inhibits many (but not all) insect P450 enzymes (Feyereisen 1999), synergized the toxicity of both pyrethroids. Myristicin, a phytochemical previously demonstrated to be capable of synergizing the toxicity of insecticides by inhibiting P450-mediated detoxification, also increased the toxicity of α-cypermethrin. These data reaffirm that P450 enzymes are involved in the metabolism of two pyrethroid insecticides and that inhibition of P450 enzymes by the proper synergist can be a management strategy to increase insecticide efficacy for navel orangeworm. Because myristicin also can inhibit the growth of *A. flavus* and decrease formation of aflatoxins (Razzaghi-Abyaneh et al. 2007), this compound may be a useful candidate for chemical control on several fronts, because it is relatively nontoxic to humans (http://www.accessdata.fda.gov/

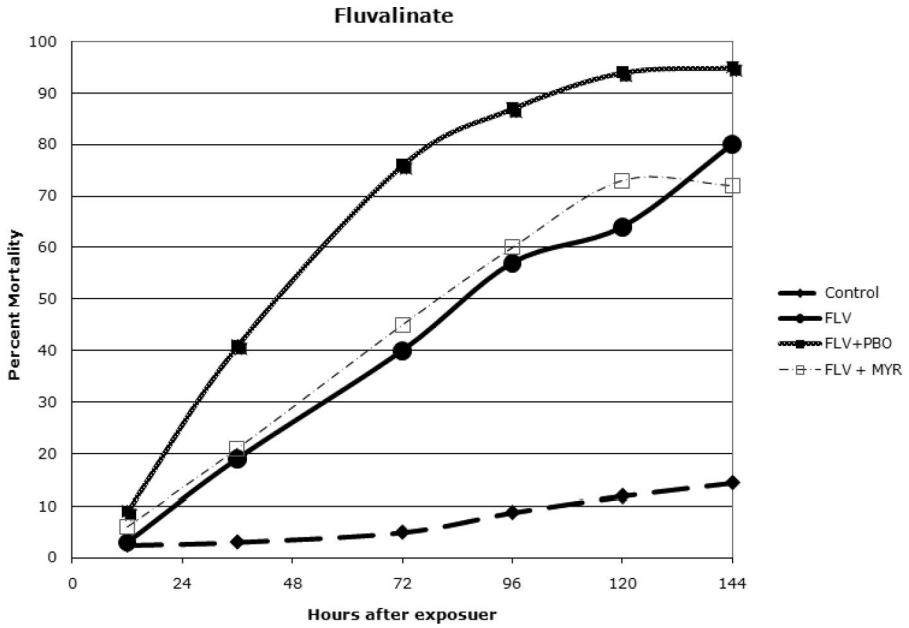

Fig. 2. Synergistic effects of piperonyl butoxide (PBO) and myristicin (MYR) on τ-fluvalinate (FLV). Navel orangeworm larvae were exposed to artificial diets containing 2 μg/g FLV supplemented with or without 25 μg/g MYR or 200 μg/g PBO. Plain diet and diets containing the synergists alone or an equal amount of methanol were used as controls. FLV did not cause mortality until 36 h, and at this point PBO was synergistic, increasing mortality by 22.0% ($F = 22.2$; df = 3, 659; $P < 0.0001$). MYR did not synergize FLV at any time point assessed.

scripts/fcn/fcnDetailNavigation.cfm?rpt=scogsListing&id=223), synergizes a pyrethroid, and it can help reduce aflatoxin contamination in orchards.

Other enzyme systems play an integral role in detoxifying insecticides in navel orangeworm. Although Mosallanejad and Smagghe (2009) reported that pip-

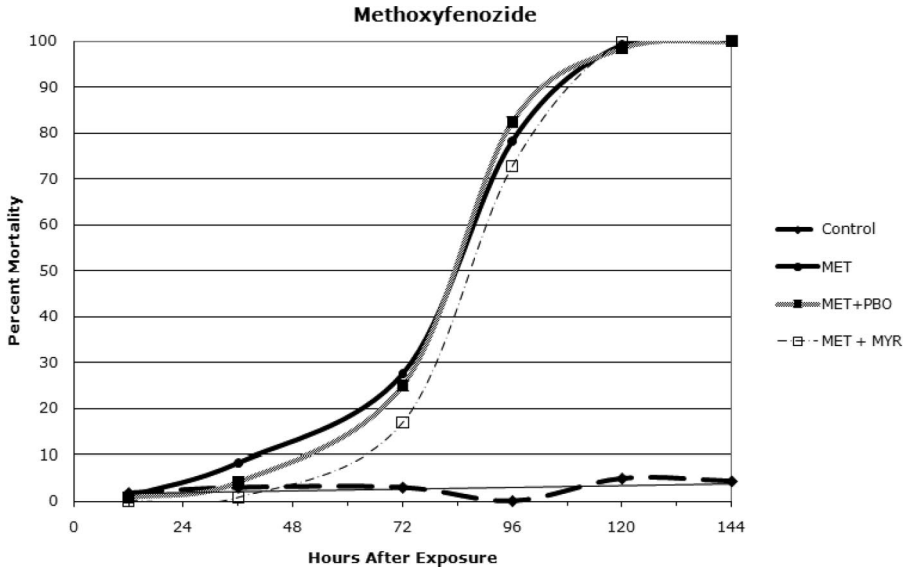

Fig. 3. Synergistic effects of piperonyl butoxide (PBO) and myristicin (MYR) on methoxyfenozide (MET). Navel orangeworm larvae were exposed to artificial diets containing 50 ng/g MET supplemented with or without 25 μg/g MYR or 200 μg/g PBO. Plain diet and diets containing the synergists alone or an equal amount of methanol were used as controls. MET did not show an effect until 36 h, and mortality from all treatments was 100% at 120 h. Neither PBO nor MYR synergized MET.

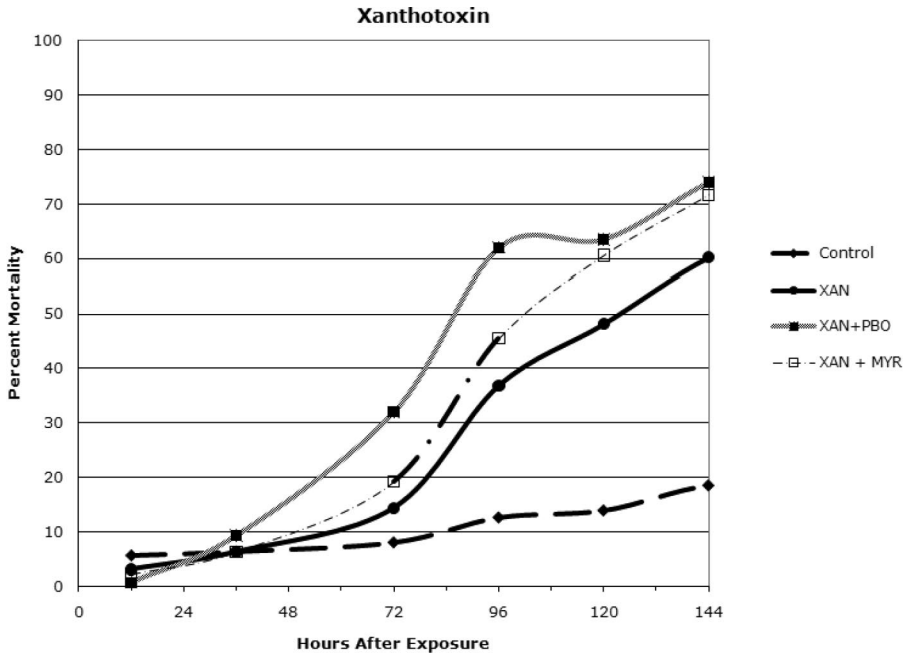

Fig. 4. Synergistic effects of piperonyl butoxide (PBO) and myristicin (MYR) on xanthotoxin (XAN). Navel orangeworm larvae were exposed to artificial diets containing 2 mg/g XAN supplemented with or without 25 μg/g MYR or 200 μg/g PBO. Plain diet and diets containing the synergists alone or an equal amount of methanol were used as controls. XAN did not increase mortality above the baseline value until 72 h. At this point in time PBO was synergistic, increasing mortality by 17.8% ($F = 10.9$; df = 3, 659; $P < 0.0001$). MYR had no effect until 120 h ($F = 38.3$; df = 3, 659; $P < 0.0001$) when it increased mortality by 12.6% ($P = 0.018$) and remained at this level for the remainder of the assay.

eronyl butoxide significantly synergized methoxyfenozide toxicity to the resistant strain of *Spodoptera littoralis* (Boisduval), possibly by inhibition of methoxyfenozide oxidation, it had no effect in navel orangeworm. It is possible that P450 enzymes are not the main detoxification system for methoxyfenozide in this insect, and if P450 enzymes are involved in detoxification, the specific P450 enzymes mediating this process are resistant to inhibition by piperonyl butoxide or that higher concentrations of inhibitors are needed to effect synergism. Future research should examine inhibitors of other detoxification enzymes, such as glutathione *S*-transferases and hydrolases, to determine whether these pathways are involved in methoxyfenozide detoxification in navel orangeworm.

Both piperonyl butoxide and myristicin synergized xanthotoxin toxicity, although piperonyl butoxide was more effective. The mechanisms by which navel orangeworm tolerate xanthotoxin are not yet known, but furanocoumarins in general are metabolized by P450 enzymes in three different families of Lepidoptera (Oecophoridae, Noctuidae, and Papilionidae). Moreover, the enzyme CYP6AB11 from navel orangeworm, expressed heterologously in a baculovirus system, is capable in vitro of metabolizing imperatorin, a furanocoumarin related to xanthotoxin that is found in some species in the Rutaceae, a family that contains some of the reported hosts of this species (Niu et al. 2011). Myristicin also synergizes furanocoumarin toxicity in several other lepidopteran species (Berenbaum and Neal 1985, Neal 1989, Neal and Berenbaum 1989, Yu and Hsu 1993, Mao et al. 2008). There are few approved insecticides for sustainable management of navel orangeworm in organic orchards, and myristicin may be of potential utility as a field treatment to increase the toxicity of naturally occurring phytochemicals to navel orangeworm as well as reduce aflatoxin contamination in the orchard.

In conclusion, we have demonstrated the toxicity to navel orangeworm of two pyrethroids used primarily for control of other pests; when used in proximity to almond and pistachio orchards, they may put additional selection pressure on navel orangeworm for pyrethroid resistance. α-Cypermethrin was synergized by both piperonyl butoxide and myristicin, and τ-fluvalinate was synergized by piperonyl butoxide. This variability in response makes it impossible to predict a priori whether myristicin can synergize the activity of the six most commonly used pyrethroids for control of navel orangeworm (beta cyfluthrin, bifenthrin, esfenvalerate, fenpropathrin, λ-cyhalothrin, and permethrin). Given the economic importance of this pest and the widespread use of these insecticides, further research is necessary to determine whether myristicin can synergize the activity of these chemicals. Both piperonyl butoxide and myristicin synergized the activity of xanthotoxin, indicating that it may be possible to alter navel orangeworm sensitivity to host phytochemicals in the field. This is a novel control strategy and may be worth pursuing for organic production of almonds, because the current choice of insecticides is limited. We believe that future research to determine the

detoxification pathways of navel orangeworm is essential to optimize control and slow insecticide resistance because this insect is exposed to almost every category of insecticide currently registered for orchard crops. Finally, this study demonstrated that methoxyfenozide and pyrethroids have different routes of detoxification, and this result may affect the combined use of these insecticides in orchards.

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