

Simple Insecticide Bean-Dip Bioassay Shows Pyrethroid Susceptibility of *Helicoverpa zea* (Lepidoptera: Noctuidae) Populations in Virginia Varies across Locations and Years¹

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Abstract Corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), is a major insect pest of many agricultural crops in Virginia. Pyrethroids have been the major insecticide class used to control the pest for decades; however, *H. zea* resistance to pyrethroids has been widely documented in the southeastern and midwestern United States. To assess the pyrethroid susceptibility of *H. zea* in Virginia, we collected larval populations from the same five locations in 2021 and 2022 and used a quick end user–friendly bioassay to assess survival of larvae after feeding on insecticide-dipped edamame [*Glycine max* (L.) Merrill] pods. Pyrethroid efficacy varied between years and locations, with some 2021 *H. zea* populations showing reduced pyrethroid susceptibility, but not in 2022. This variability is most likely influenced by factors such as overwintering survivability in Virginia. Thus, the need for continued annual monitoring is important to track efficacy trends and grower recommendations. The simple bean-dip bioassay could be used as an efficient monitoring tool in the future, with pyrethroid susceptibility determined in 48 h from field collection.

Key Words corn earworm, edamame, pyrethroid, bioassay

Corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), is one of the most important insect pests of food and fiber crops in the United States (Swenson et al. 2013). In Virginia, *H. zea* can cause serious economic loss to soybean [*Glycine max* (L.) Merr.], cotton [*Gossypium hirsutum* (L.)], corn [*Zea mays* (L.)], sweet corn [*Zea mays* (L.) convar. *saccharata* var. *rugosa*], hemp [*Cannabis sativa* (L.)], tomato [*Solanum lycopersicum* Dunal (L.)], and edible bean crops (Britt et al. 2021; Dively et al. 2021; Fleisher et al. 2007; Herbert et al. 2009; Kuhar et al.

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2006, 2019). For many crops, especially those that do not have commercially available or viable transgenic varieties that contain *Bacillus thuringiensis* (Bt) Berliner proteins, pyrethroids (Insecticide Resistance Action Committee, IRAC Group 3) are the most widely used insecticide class to control corn earworm as they are effective, quick acting, relatively inexpensive, and control a wide range of other insect pests (Hutchison et al. 2007). Pyrethroid resistance has been identified in *H. zea* populations since the 1990s in the southern United States (Abd-Elghafar et al. 1993, Brown et al. 1998, Stadelbacher et al. 1990) and since the 2000s in the Midwest (Hutchison et al. 2007, Jacobson et al. 2009), posing a challenge for its management. Researchers have determined multiple mechanisms of pyrethroid resistance, including target site mutations (Head et al. 1998, Hopkins and Pietrantonio 2010a, Soderlund 2008), and metabolic processes, including the mixed-function oxidase system or cytochrome P450 enzymes (Sasabe et al. 2004, Yang et al. 2006).

Since the 1990s, monitoring for pyrethroid resistance in local *H. zea* populations has been an important part of pest management programs (Fleischer et al. 2007, Hutchison et al. 2007, Jacobson et al. 2009). One approach used for resistance monitoring is the adult vial test (AVT) method in which *H. zea* male moths are caught in pheromone traps and placed in 20-ml glass scintillation vials coated with 5 or 10 μg of cypermethrin (Plapp et al. 1990). Surviving moths, defined as those able to fly following 24 h of exposure, are classified as pyrethroid resistant. This method has been practical to document trends in pyrethroid susceptibility in mid-Atlantic U.S. populations (Fleischer et al. 2007); however, results may not correlate with efficacy of pyrethroids on nearby larval populations. In addition, cypermethrin was chosen for the original AVTs (Plapp et al. 1990) and is still used despite other, more advanced pyrethroids (e.g., lambda-cyhalothrin, bifenthrin, cyfluthrin, beta-cyfluthrin, zeta-cypermethrin) replacing cypermethrin use on most crops by the mid-1990s. Hopkins and Pietrantonio (2010b) showed that *H. zea* resistance ratios were different among different pyrethroids when screening a resistant population.

Other methods to monitor insecticide resistance in larvae include topical applications (Jacobson et al. 2009) and diet-overlay bioassays (Kaur 2018, Marçon et al. 1999). These methods are useful for calculating median lethal dose or median lethal concentration levels. However, they require technical grade insecticide formulations and a standardized larval instar and or weight, typically accomplished by rearing colonies to F_1 or F_2 generations in the laboratory (Jacobson et al. 2009).

Herein, we evaluated a simple and quick bean-dip bioassay that is end user oriented (scouts, consultants, extension agents, growers, and others) for determining susceptibility of field-collected *H. zea* larvae to pyrethroids applied at field application rates. We assessed *H. zea* larvae collected from five locations in Virginia over 2 yr. In addition, each pyrethroid was screened alone and with the inclusion of a synergist, piperonyl butoxide (PBO), to determine whether the resistance mechanism, when detected, was of metabolic origin.

Materials and Methods

***Helicoverpa zea* collections.** In 2020 and 2021, second to fourth instar corn earworm larvae were collected from non-Bt sweet and field corn that was not treated with broadcast insecticides across five locations in Virginia to be used in

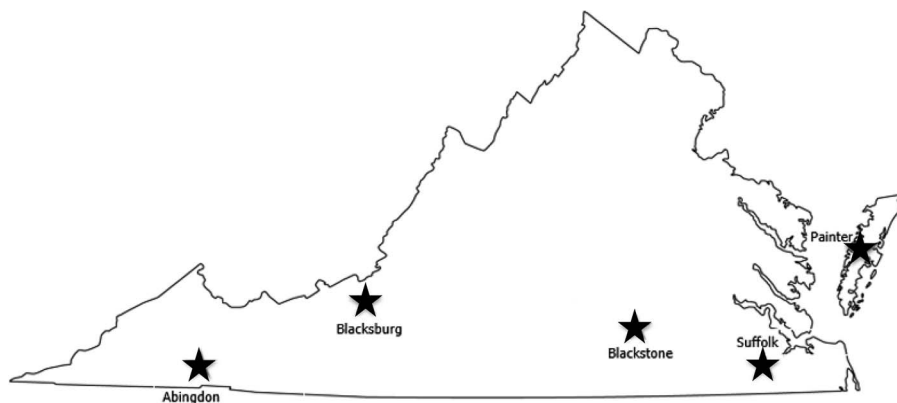


Fig. 1. Map of *Helicoverpa zea* collection sites for 2020 and 2021.

laboratory bioassays. Instars were estimated visually according to Capinera (2000). Larvae collections were initiated when sufficient populations were available (i.e., 200 or more could be collected at once). Collection sites included Blacksburg (7 August 2020 and 3, 7, 9, and 13 August 2021), Blackstone (16 September 2020 and 28 July 2021), Abingdon (1 September 2020 and 12 August 2021), Suffolk (5 August 2020 and 12 August 2021), and Painter (31 August 2020 and 14 September 2021) (Fig. 1). Collecting third to fourth instar larvae represented the majority of field instar size distributions available. Blacksburg populations in 2021 were lower than in 2020, and all four replications for the bioassay were collected on different days. All larvae were collected during or within 2 wk of peak moth flights in July or August, which are typical timings in Virginia (Herbert et al. 2009). In addition to field-collected populations, a laboratory colony from North Carolina State University was included for comparison. The laboratory colony had been reared from a field collection from Louisiana since 2016. Assuming the fitness cost of resistance that has been demonstrated in *H. zea* (Pietranonio et al. 2006) and the lack of insecticide selection pressure, we believed the colony to be susceptible to pyrethroids after years of rearing. High mortality from pyrethroids in our bioassays confirmed this hypothesis. Field-collected larvae were placed individually into 30-ml clear plastic souffle cups (Solo Cup Company, Lake Forest, IL) with fresh sweet corn silks as a temporary food source. Larvae were transported to the laboratory and placed into a Percival Scientific growth chamber set to $23.0 \pm 2.0^\circ\text{C}$ and 50% relative humidity until they were used for bioassays, usually within 1–2 d of collection.

Bioassays. A bean-dip bioassay was used following Kuhar et al. (2012). There were five insecticide treatments and an untreated control, each with 10 larvae (mix of third and fourth instars), replicated four times, for a total of 240 larvae per location. Replications were conducted on different days to avoid pseudo-replication. Treatments included (1) a tap water control, (2) lambda-cyhalothrin (Lambda-Cy EC, 11.4% active ingredient, UPL, Inc., King of Prussia, PA) at 0.12 g active ingredient [ai]/L, (3) pyrethrins (PyGanic Crop Protection EC 5.0 II, 5.0% active ingredient, Valent U.S.A. LLC, San Ramon, CA) at 0.054 g ai/L, (4) lambda-cyhalothrin at 0.12 g ai/L combined with PBO (Exponent insecticide synergist, 91.3% piperonyl

Table 1. Results of mixed model ANOVA of factors influencing mortality of corn earworm, *Helicoverpa zea*, larvae at 48 h after being placed on dipped edamame pod sections in Virginia 2021 and 2022.

Source	df	Sum of Squares	F Ratio	Prob > F
Year	1	1.045402	46.7480	<0.0001*
Location	5	0.727455	6.5060	<0.0001*
Treatment	5	17.600710	157.4128	<0.0001*
Year × Location × Treatment	25	1.211314	2.1667	0.0018*
Year × Location	5	0.304544	2.7237	0.0209*
Year × Treatment	5	9.971716	89.1825	<0.0001*
Location × Treatment	25	1.443327	2.5817	0.0001*

* Denotes significance at $P < 0.05$.

butoxide, Valent U.S.A. LLC) at 0.944 g ai/L, (5) pyrethrins at 0.054 g ai/L combined with PBO at 0.944 g ai/L, and (6) PBO alone at 0.944 g ai/L.

When possible, fresh untreated edamame [*Glycine max* (L.) Merr.] pods were collected from Virginia Tech's Kentland Farm in Whitethorne and used for bioassay reps. Frozen organic edamame was thawed and used instead when fresh pods were past their ideal time for human consumption. Frozen pods were rinsed before use. Edamame pods were cut into halves or thirds (~2.5 cm square) so that each piece had one seed inside the pod. Using forceps, each bean piece was dipped into a beaker with the mixed treatments for 2 s, was allowed time for excess moisture to dry off, and then was placed into an empty 30-ml clear plastic souffle cup (Solo Cup Company). There were 10 cups per treatment per rep. A single larva was placed in each cup and a lid with holes placed on top, securing the larvae inside with the treated bean piece. Mortality was recorded every 24 h for 96 h after placement. Larvae were recorded as dead if they did not respond to stimuli such as prodding with tweezers.

Data analysis. Proportion of corn earworm mortality data were analyzed using a standard least squares model in JMP, Version 2016 statistical software (JMP Pro 2021). Model factors included year, location, treatment, and the interactions among these effects. If significant interactions were found in the effect tests report, data were partitioned into separate two-way analyses of variance (ANOVAs) by main factors (such as year and location). Means were separated using Tukey's honestly significant difference (HSD) at the $\alpha < 0.05$ level of significance and are reported \pm SEM.

Results

Mortality typically did not change from 48 to 72 h (data not shown); thus, we used 48-h mortality data for all analyses. The mixed model was significant ($F = 21.59$; $df = 71$; $P < 0.0001$) for year, location, and treatment and all interactions of these terms (Table 1). Because of the significant interactions, data were summarized separately by treatment, year, and location.

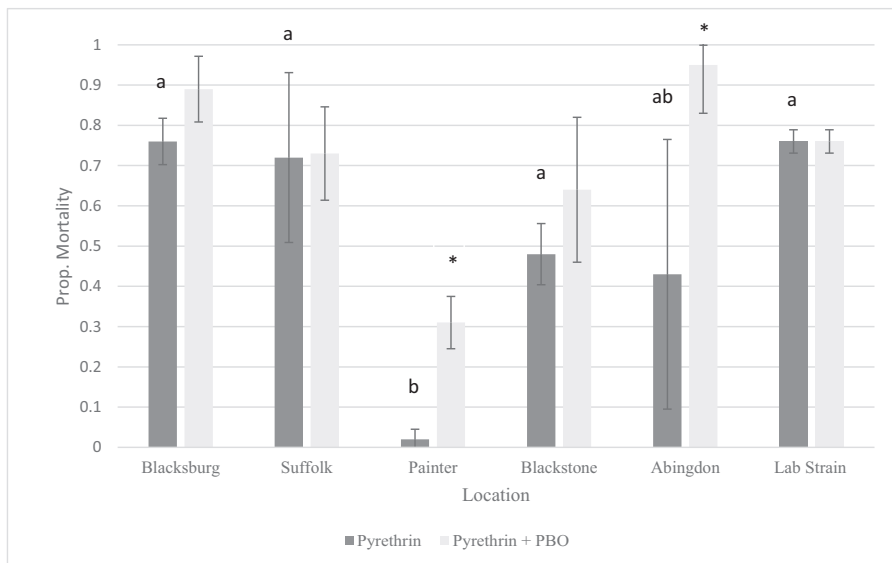


Fig. 2. Proportion mortality (mean \pm SE) of field-collected populations of *Helicoverpa zea* (third to fourth instar) 48 h after exposure to edamame beans dipped in pyrethrins with or without PBO in 2020. Pyrethrin bars with a letter in common are not significantly different, and an asterisk denotes significant differences between pyrethrin and pyrethrin + PBO mortality at a single location according to Tukey's HSD at the $P < 0.05$ level.

Pyrethrins. In 2020, mortality from pyrethrins varied by location ($F = 6.26$; $df = 11$; $P \leq 0.0001$) with high mortality ($>70\%$) occurring in Blacksburg and Suffolk populations similar to the susceptible laboratory strain ($76.0 \pm 0.03\%$; Fig. 2). Mortality from pyrethrins was lower in Painter ($2.0 \pm 0.03\%$), Blackstone ($48.0 \pm 4.4\%$), and Abingdon ($43.0 \pm 33.9\%$). However, the only location that was significantly different than the susceptible colony was Painter ($F = 7.94$; $df = 5$; $P \leq 0.0001$). The addition of PBO to pyrethrins increased proportion mortality in Painter (from $2.0 \pm 0.03\%$ to $31.0 \pm 6.9\%$) and Abingdon (from $43.0 \pm 33.9\%$ to $95 \pm 5.0\%$; $F = 10.05$; $df = 1$; $P < 0.0037$).

In 2021, mortality from pyrethrins varied by location ($F = 2.83$; $df = 11$; $P \leq 0.0097$), with the Abingdon population having less mortality ($48.0 \pm 23.4\%$) than the susceptible colony ($96.0 \pm 4.7\%$; $P \leq 0.0083$; Fig. 3). The addition of PBO to pyrethrins did not increase the proportion mortality at any location (Fig. 3).

Lambda-cyhalothrin. In 2020, lambda-cyhalothrin mortality was similar among all populations (63.0 ± 1.9 – $82.0 \pm 7.4\%$) and the susceptible colony ($80.0 \pm 0.0\%$) ($F = 0.92$; $df = 11$; $P = 0.53$). The addition of PBO to lambda-cyhalothrin did not increase mortality at any location (Fig. 4).

In 2021, lambda-cyhalothrin mortality was similar among locations and compared with the susceptible colony ($F = 0.99$; $df = 11$; $P = 0.47$). The addition of PBO did not increase the proportion mortality at any locations (Fig. 5).

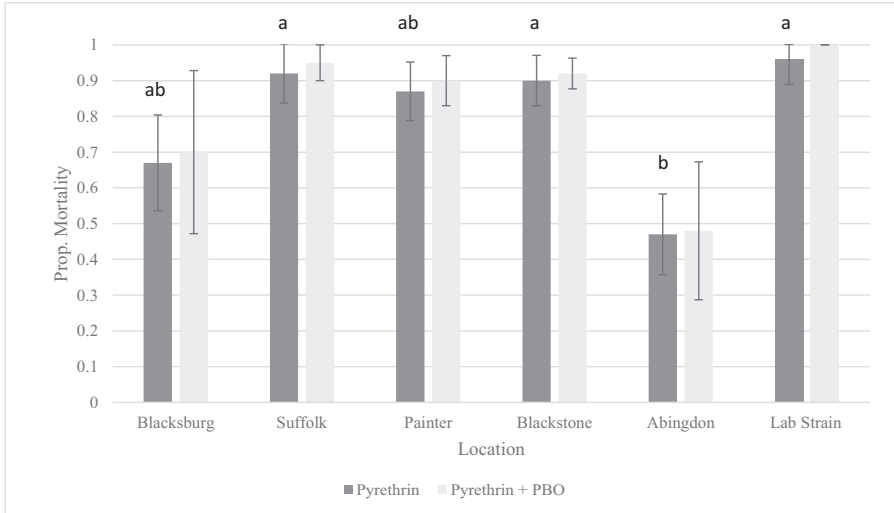


Fig. 3. Proportion mortality (mean \pm SE) of field-collected populations of *Helicoverpa zea* (third to fourth instar) 48 h after exposure to edamame beans dipped in pyrethrins with or without PBO in 2021. Pyrethrin bars with a letter in common are not significantly different according to Tukey's HSD at the $P < 0.05$ level.

PBO. PBO mortality by itself did not differ from the water control in any location or year except for the susceptible laboratory strain in 2020, which had $47 \pm 0.07\%$ mortality compared with the control at $10 \pm 0.11\%$ at 48 h. The susceptible laboratory strain in 2021 had $31 \pm 13.45\%$ mortality compared with the control at $0 \pm 0.00\%$.

Discussion

Pyrethroid resistance in *H. zea* has been a major concern for >30 yr in the United States (Fleischer et al. 2007, Hutchison et al. 2007, Jacobson et al. 2009, Plapp et al. 1990). In some regions, such as the Mid-South, pyrethroids are no longer recommended to control *H. zea* on agronomic crops (Crow et al. 2022), or combinations with other insecticide mode of actions are suggested (Stewart 2019). Based on our monitoring, there was some evidence that suggested pyrethroid resistance (based on location), although most populations were susceptible to lambda-cyhalothrin or pyrethrins similar to that of a susceptible colony strain. Painter (2020) and Abingdon (2021) populations had lower mortality than the susceptible lab strain. Thus, some reduced susceptibility was present in Virginia, but it was not widespread or consistent across years.

Lambda-cyhalothrin resulted in high mortality from all locations in 2020 (63–82%) and 2021 (85–100%). This was unexpected as we believed that pyrethroid efficacy on *H. zea* had declined in the Mid-Atlantic United States, as documented in other states (Fleischer et al. 2007, Jacobson et al. 2009, Owens et al. 2022). However, our findings support the need for resistance monitoring as population genetics of *H. zea* is

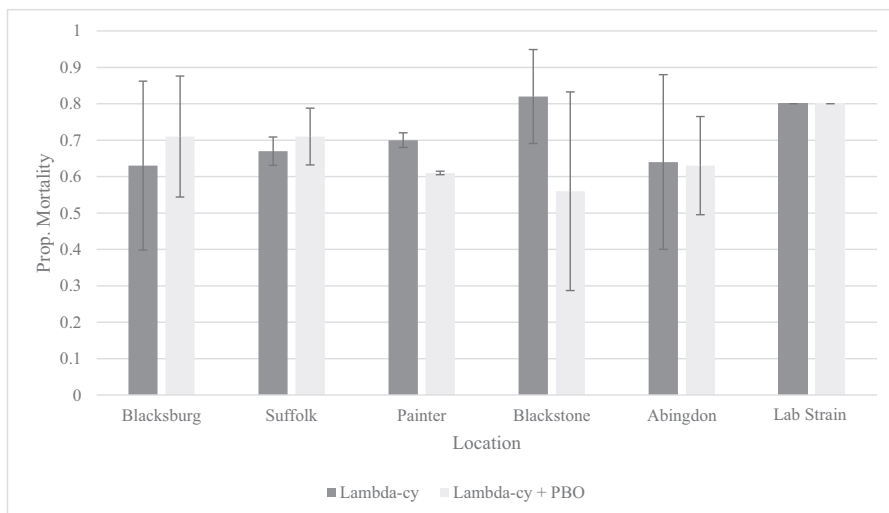


Fig. 4. Proportion mortality (mean \pm SE) of field-collected populations of *Helicoverpa zea* (third to fourth instar) 48 h after exposure to edamame beans dipped in lambda-cyhalothrin with or without PBO in 2020. There was no significant difference among locations or from the addition of PBO in mortality according to ANOVA, $P < 0.05$.

complicated and influenced by many factors including overwintering survival (Hardwick 1965), variable patterns in temperature and storm activity influencing migration routes (Fleisher et al. 2007, Hutchison et al. 2007, Lawton et al. 2022), and changes in insecticide use patterns by growers reducing selection pressure. Many growers have stopped using pyrethroids for *H. zea* control in the southern United States.

Mortality from pyrethrins varied among locations and years and, in general, *H. zea* were less susceptible to pyrethrins than the synthetic pyrethroid lambda-cyhalothrin. In 2020, pyrethrins resulted in less than 50% mortality in three locations (Painter, Blackstone, and Abingdon). In 2021, only one location (Abingdon) had less than 50% mortality. In our study, *H. zea* from Blacksburg had 76 and 86% mortality from pyrethrins in 2020 and 2021, respectively. In another experiment conducted on hemp, Britt and Kuhar (2020) also showed high susceptibility to pyrethrins in *H. zea* larvae collected from Blacksburg.

Pyrethrins are toxins derived from the chrysanthemum flower that may be registered for organic use, including the product used in our experiment. First-generation pyrethroids were developed to slow degradation from heat and light, and second-generation pyrethroids were developed to increase insecticidal properties. Lambda-cyhalothrin is a type II pyrethroid. Pyrethroids and pyrethrins prolong the opening of sodium channels; however, type II pyrethroids, like lambda-cyhalothrin, slow the deactivation of the sodium channels considerably more than type I pyrethroids (Nasuti et al. 2003, Ray 2001). Pyrethrins are widely used in organic growing operations for *H. zea* control.

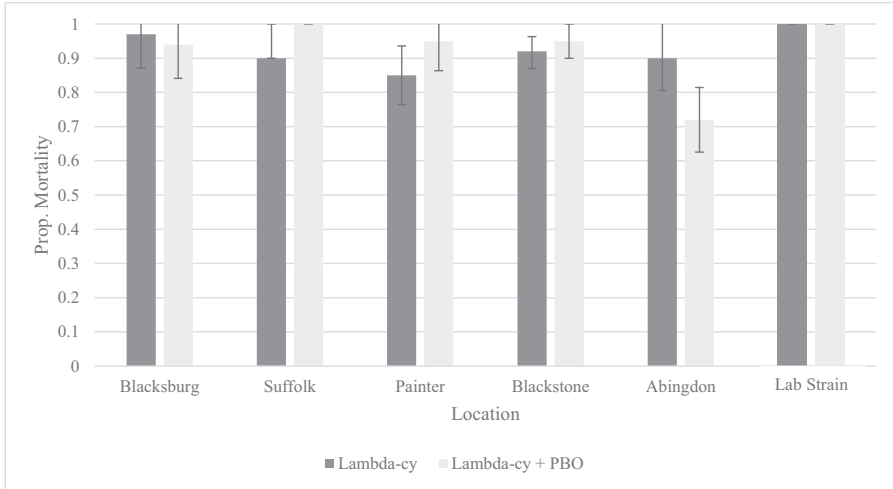


Fig. 5. Proportion mortality (mean \pm SE) of field-collected populations of *Helicoverpa zea* (third to fourth instar) 48 h after exposure to edamame beans dipped in lambda-cyhalothrin with or without PBO in 2021. There was no significant difference among locations or from the addition of PBO in mortality according to ANOVA, $P < 0.05$.

Although the potential mechanism of pyrethroid resistance was not a focus of our research, previous studies have identified both target site mutations (Head et al. 1998, Hopkins and Pietrantonio 2010a, Soderlund 2008) and metabolic, or enzymatic, processes (Sasabe et al. 2004, Yang et al. 2006). PBO is a neutral antagonist of G-coupled CB1 receptors that inhibit the cytochrome P450 enzymes (Dhopeswarkar et al. 2011, Feyereisen 1999). The addition of PBO significantly increased the efficacy of pyrethrins over pyrethrins alone in two of our populations (Painter and Abingdon) in 2020, suggesting some metabolic resistance was involved.

PBO has no pesticidal activity of its own (Conney et al. 1972), and our data showed no significant difference in mortality between PBO alone and the water control. However, PBO caused some mortality (47 and 31%) in the susceptible laboratory strain in 2020 and 2021. Laboratory-reared *H. zea* may not have the ability to tolerate inhibition of the cytochrome P450 enzyme system without negative health effects. PBO coupled with the transition from artificial diet (Sheikh et al. 1990) to edamame may have contributed to mortality, due to limiting the direct access to nutrients.

Our research showed that edamame bean-dip bioassays can be a quick and effective way to monitor insecticide efficacy in field populations, providing pyrethroid efficacy information within 48 h. Field data support this finding, with several pyrethroids lacking efficacy in a sweet corn insecticide experiment conducted at the same location in 2020 (Kuhar et al. 2021). Bean-dip bioassays showed high mortality with pyrethrins on larvae collected from Blacksburg, and all pyrethroids performed well in the field experiments conducted on sweet corn (Kuhar et al. 2021). Although these examples are anecdotal evidence, they suggest that the

bean-dip bioassay may prove useful as an assessment tool. However, the conclusions that are drawn from these assays may be limited by differences in application coverage between overhead foliar broadcast applications and complete submersion of the edamame pod. Although there was evidence of similar efficacy between bioassays and field trials at some locations, that was not always the case. It is important to note that efficacy between lab and field experiments may be variable. Because of the variability, more evidence is needed to validate our hypotheses.

In conclusion, this study indicated that bean-dip insecticide bioassays could be a promising tool for end users to test for insecticide efficacy against *H. zea* in a timely manner. It also added to our current knowledge of the pyrethroid susceptibility in Virginia and provided evidence that suggested metabolic resistance in some Virginia populations. Continued surveillance will be needed to determine how resistance levels in this area evolve over time.

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