A Novel Approach to Controlling the Fall Armyworm (*Spodoptera frugiperda)* Using a Combination of *Bacillus thuringiensis* Concentrate and Azadirachtin

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Introduction

Spodoptera frugiperda, also known as the fall armyworm (FAW), has become a global corn pest. Outside of its natural habitat of North America, FAW has been recently discovered in multiple African countries in 2016, the Middle East and India in 2018, and Asia and mainland Australia in 2020 (Jin et al., 2021). Currently, FAW targets over eighty plant species across these continents. The corn strain of FAW, as its name suggests, primarily feeds on maize, corn, and sorghum, while the rice strain targets crops like rice and soybean (Boaventura et al., 2020). As one method of control for this invasion, chemical insecticides are sprayed on FAW target crops.

Some of the earliest pesticides used on FAW are pyrethroids and conventional chemical compounds such as permethrin, deltamethrin, and chlorantraniliprole (Amelia-Yap et al., 2018; Gutiérrez-Moreno et al., 2019). As previously mentioned, the main mode of actions for these insecticides is to apply them to the leaves of FAW target crops. However, FAW has shown evolved resistance to these synthetic insecticides, due to their frequent use on these pests. In light of this, biopesticides have been examined as an alternative to management of FAW. They have high specificity in their main modes of action, and they are environmentally friendly without long-term effects. Therefore, it has been a priority to develop a biopesticide effective in containing FAW.

One such solution was derived from *Bacillus thuringiensis* (Bt). Bt is a soil bacterium that produces δ-endotoxins, or Cry proteins. 3D-Cry toxins make up the biggest type of Cry protein, and they are used the most in terms of controlling the fall armyworm. The target crops of FAW are modified to express Cry proteins, and studies have shown the usefulness of these transgenic Bt crops in regulating FAW populations (Jin et al., 2021; Herrero et al., 2004). Over time, however, further evidence has shown that FAW has developed resistance to the Cry Bt toxins; research conducted by Botha et al. showed the presence of resistant alleles to Cry toxins, discovering that FAW can evolve decreased susceptibility to Bt in a short amount of time (2019). Because of this newfound resistance, it has become crucial to develop novel biological insecticides in order to achieve this same purpose. While Bt continued to be utilized over time, innovative biopesticides continued to be engineered to control the reach of FAW. For instance, azadirachtin is another widely used biopesticide on insects, and cyclosporin A, a new fungal metabolite has shown insecticidal activity on FAW (Lin et al., 2021; Sun et al., 2022).

Due to the inefficiency of individual insecticides, studies have been conducted assessing the impact of combined insecticidal activity on FAW. Researchers Huang et al. have introduced that combining Bt with chemical insecticides can lead to increased toxicity, finding that the toxicity of combined Bt, plant allelochemical flavone, and emamectin benzoate was additive (2023). FAW is a significant invasive pest in many regions, and because of its evolved resistance, multiple methods of resistance management must be employed to decrease its overall effects. Thus, the purpose of this research is to determine if Bt combined with novel insecticide azadirachtin can synergistically increase their overall toxicity to FAW larvae.

Literature Review

In early 2023, Ayilara et al. from the Department of Biological Sciences at Kings University in Nigeria examined the efficacy of various biological pesticides to improve their acceptability for use in insect pest management. In their study, they define biopesticides as insecticides containing biological matter such as plants, microbes, and biological nanoparticles. They also note the difference in action and effects of each type of biopesticides. For example, Ayilara et al. state that microbial insecticides consist of microorganisms such as bacteria, fungi, protozoa, viruses, and algae and use toxic metabolites to limit the spread of pests (2023). One such biopesticide is Bt. Yet, as stated earlier, the efficacy of Bt on FAW has depleted severely. Researchers Monnerat et al. discovered that FAW collected in Brazil was resistant to Cry1F Bt toxins (2015), somewhat discounting the validity of Ayilara et al.'s claim. Additionally, on the topic of phytopesticides, which are pesticides derived from plant extracts, Ayilara et al. report that they are able to destroy the eggs and larvae of pests and keep them from feeding on plants (2023). This is elaborated on by Lima et al., who concentrated on azadirachtin in their study. Extracted from *Azadirachta indica*, the Indian neem tree, azadirachtin is one the most widely used phytopesticides used for insect control (2015). Within the context of this research, Lin et al. of South China Agricultural University found over 50 percent mortality in FAW that was treated with different concentrations of azadirachtin over three days (2021). These instances further expand on the credibility of biopesticides as a worthy alternative to conventional insecticides.

Ayilara et al.'s work also mentions that one crucial downside to microbial pesticides and phytopesticides is their rapid degradability, which translates to a short shelf life for these biopesticides. To counteract this, studies have been conducted to maximize the efficiency of these biopesticides by combining them. Researchers Zhang et al. evaluated the efficacy of combined emamectin benzoate, a biopesticide; chlorantraniliprole, a conventional insecticide; and an adjuvant. They observed that the toxicity of the mixed pesticides was additive and increased overall FAW mortality (2022). This conclusion was further supported by Huang et al. at Zhengzhou University in China. They tested the synergy between emamectin benzoate, Bt, and plant allelochemical flavone and noted that combining Bt toxins with chemical insecticides led to increased toxicity in FAW (2023). The fact that a significant amount of the literature exploring this topic has been published so recently highlights the novel nature of this proposed solution to resistance management of FAW.

This analysis of the current academic discussion in the field of FAW resistance management strongly establishes that there is a gap in the literature on the synergistic effects of combined novel biopesticides on FAW larvae. Thus, the goal of this study is to attempt to address that gap by analyzing FAW response in relation to exposure to treatments of Bt, azadirachtin, and both insecticides combined. Through this research, a toxicity bioassay will be used to gauge each treatment's effect on the environment of FAW, which will generate results to better address the limited amount of research done on the efficacy of combined biopesticides on FAW. It is expected that through this analysis, it will be shown that the natural environment of FAW larvae after being introduced to the combined Bt plus the new biopesticide azadirachtin will be toxic to FAW larvae and decrease its survivability.

Method

I. Method Justification

The study utilized three bioassays to investigate the toxicity of various biological insecticides on FAW natural environment —one to ascertain the toxicity of FAW environment in contact with Bt, one focused on contact with azadirachtin, and one to determine FAW toxicity to combined Bt and azadirachtin. The benefit of this approach is that is allowed for conclusions to be drawn about each individual objective, all of which contributed to accepting or refuting the overall hypothesis. The method utilized by Sims et. al was applied for the toxicity bioassays, with slight modifications. Soil samples were collected in order to assess azadirachtin and Bt's individual and combined impact on the natural environment of FAW. The samples were then subjected to varying concentrations of the biopesticides to determine which had the largest effect on the soil samples. The literature supports this procedure, with Javed et al. incorporating treating soil samples with a commercial azadirachtin product to test the biopesticide's persistence in the soil (2005). Additionally, it was also utilized in a more recent study conducting a laboratory bioassay testing the efficacy of biopesticides' persistence in soil and effects on a plant parasite, *Meloidogyne incognita* (d'Errico et al., 2023). Modifying the method in this way was particularly useful in putting the FAW natural environment into direct contact with the biopesticides and allowed the results to be clearly related to exposure to the insecticides.

For this method, the form of Bt used was Monterey® LG6332 Bacillus thuringiensis (B.t.) Worm and Caterpillar Killer Insecticide/Pesticide Concentrate. This brand consists of Bt subspecies *kurstaki* HD270, and research reports that it is widely used in laboratory and field studies due to its effectiveness against Lepidopteran agricultural pests. For instance, in a recent article by Xue et al., they employed Bt strain HD270 to determine its constructive collaboration with other insecticidal proteins, citing its efficacy against another Lepidopteran species, *Plutella xylostella* (2023). The brand of azadirachtin for this research was Azatin® O Biological Insecticide. Its active ingredient is azadirachtin, a widespread biopesticide used in the field and in a laboratory setting. The soil samples were exposed to individual Bt and azadirachtin concentrations of 0 mL, 1 mL, 2 mL, 5 mL, 10 mL, and 20 mL, as described by Lin et al. in their study (2021), and the combined Bt and azadirachtin solution was diluted were mixed at mass ratios of 1:9, 3:7, 5:5, 7:3, and 9:1, as described by Tabet et al. in their study (2023). To collect data, the pH of the soil samples after exposure to each treatment was determined with pH test strips and recorded. As first done in a study by Hardke et al., the pH of the soil after each treatment was recorded, where pH values of 1.0-6.0 were considered acidic, and values of 7.0-14.0 were considered basic (2011). This form of analysis was selected by Lin et al. in their study, which investigated the effects of azadirachtin on FAW environment, demonstrating its reliability and validity for this research.

II. Method

A. Determining Toxicity to BT

Make sure all personal protective equipment is always worn in the lab. This includes latex gloves, eye goggles, apron, and a face mask.

1) Took out six equal-sized mason jars and stuck a piece of tape on each jar so it is visible and wrote the Bt insecticide volume that was used for that jar on each piece of tape.
2) Measured out equal amounts of soil into each jar. Measured out enough of the Bt insecticide concentrate into the soil samples as specified in subsection I.
3) Left the treatments for 24 hours, exposing the soil jars to a 16h:8h light/dark photoperiod.
4) After 24 hours, used pH strips to evaluate the acidity/basicity of the soil in the jars.

B. Determining Toxicity to Azadirachtin

1) Took out six equal-sized mason jars and stuck a piece of tape on each jar so it is visible and wrote the azadirachtin insecticide volume that was used for that jar on each piece of tape.
2) Measured out equal amounts of soil into each jar. Then, measured out enough of the azadirachtin insecticide concentrate into the soil samples as specified in subsection I.

3) Left the treatments for 24 hours, exposing the soil jars to a 16h:8h light/dark photoperiod.
4) After 24 hours, used pH strips to evaluate the acidity/basicity of the soil in the jars.

C. Determining Toxicity to Combined Bt and Azadirachtin

1) Took out six equal-sized mason jars and stuck a piece of tape on each jar so it is visible and wrote the combined Bt and azadirachtin volume that was used for that jar on each piece of tape.
2) Measured out equal amounts of soil into each jar. Measured out enough of the Bt insecticide concentrate and azadirachtin insecticide concentrate into the soil samples as specified in subsection I.
3) Left the treatments for 24 hours, exposing the soil jars to a 16h:8h light/dark photoperiod.
4) After 24 hours, used pH strips to evaluate the acidity/basicity of the soil in the jars.

III. Method Critiques

This mixed-method approach with the modifications made allowed for efficient testing of each objective for the overall research purpose. However, it also lent itself to a number of potential sources for error. For instance, the treatments used in this experiment were left alone under simulated conditions for 24 hours, rather than the standard x that was used in D'Errico et al.'s original experiment. It may not have allowed enough time for noticeable changes in the environment of FAW after the treatments. Because of this, it is possible that any recorded changes in soil pH may have been underestimated due to the shorter period of exposure to the insecticide treatments. In future research, it is crucial to examine how the period of time FAW's natural environment is exposed to the treatments can influence changes in it because of different insecticides.

In addition to this, there may have also been multiple sources of human error. For example, when measuring out the insecticides, there may have been inaccuracies in the solutions made with the original insecticide concentrations. This would understandably impact the outcome of the overall experiment, either over- or underestimating the true effects of various dilutions of the insecticides utilized. In addition, the pH test strips used to determine the pH of the soil before and after treatment tended to present the colors darker after 1-2 minutes. Therefore, analyzing the strips immediately after testing or after waiting 1-2 minutes may have impacted the true soil pH observed. For future studies, it is recommended that a more valid strategy be employed to ensure the uniformity of the controlled variables in these experiments, such as accuracy in concentrations and materials used in the experiment.

Data and Analysis

In this experiment, three sets of data were collected: pH of FAW exposed to Bt, mortality rate of FAW exposed to azadirachtin, and that of FAW to combined Bt and azadirachtin. The results correlating to the three research objectives are presented below.

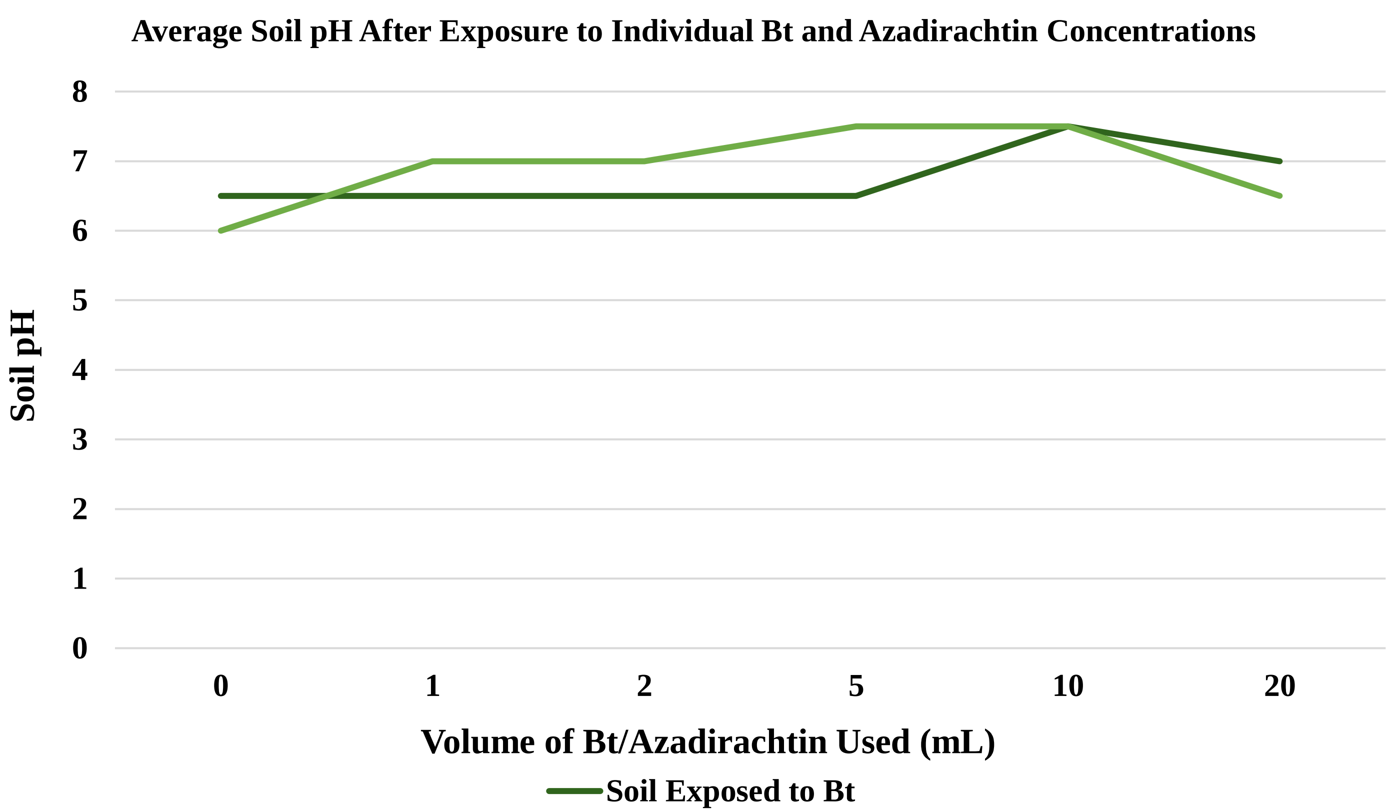

Figure 1. The bar graph shows the average soil acidity or basicity after exposure to individual *Bacillus thuringiensis* and azadirachtin concentrations. Soil samples were treated with either 0, 1, 2, 5, 10, or 20 mL of Bt or azadirachtin. Measurements were taken in pH.

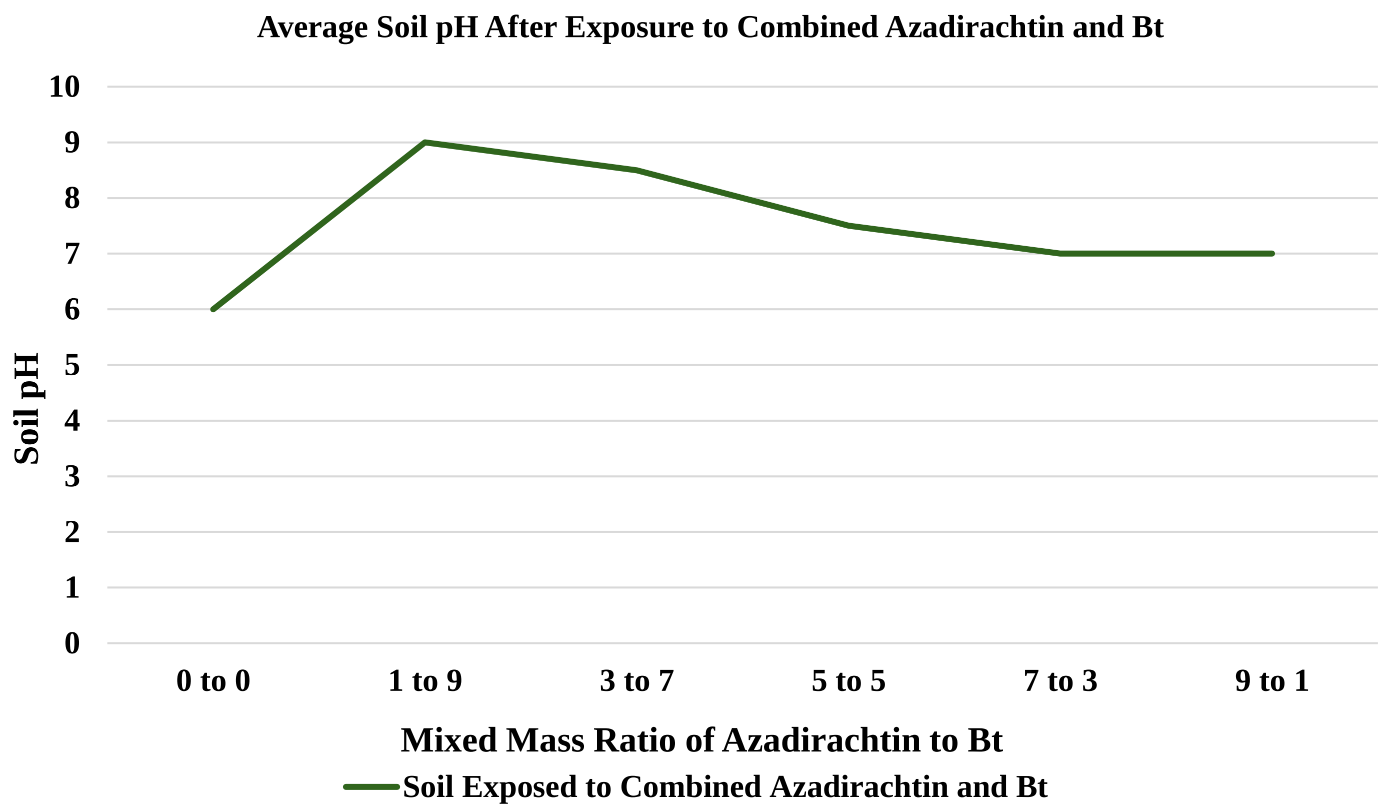

Figure 2. The bar graph shows the average soil acidity or basicity after exposure to combined azadirachtin and Bt. Soil samples were treated with azadirachtin to Bt ratios of 1:9, 3:7, 5:5, 7:3, or 9:1. Measurements were taken in pH

In Figure 1, mortality rate is expressed in pH. It is clearly shown that the average increase in soil basicity is greater when subjected to azadirachtin than Bt. However, what is also apparent is the low toxicity of both biopesticides. Indeed, both groups exposed with individual Bt and azadirachtin saw a peak at a pH of 7.5 in the 5-10 mL treatments, before decreasing in pH in the 20 mL treatment.

In Figure 2, the toxicity of FAW to Bt/Azadirachtin concentrations is expressed as a relationship between Bt/azadirachtin mixed mass ratios and soil acidity in pH. Most of the average soil pH values presented on the line graph are significantly higher than those recorded while evaluating the individual biopesticides, with a sharp peak of a pH of 9.0 when the soil samples were exposed to a ratio of one part azadirachtin to 9 parts Bt. These factors imply a stronger link between and combined Bt and azadirachtin toxicity and FAW natural environment than that with the singular biopesticides, as well as introduce the notion that varying mixed ratios of combined insecticides can produce different results.

Two one-way analysis of variance tests with post-hoc Tukey honestly significant difference tests was used to further analyze the results. The first was to compare individual Bt and azadirachtin concentrations' impact on FAW environment. The null hypothesis for this test was there was no significant difference in the effects of Bt or azadirachtin on FAW natural environment. After running the test, an f-statistic of 2.4 was obtained, which was then used to obtain a p-value of 0.1583. This value is greater than common significance levels of 0,01, 0.05, and 0.10, and the null hypothesis could not be rejected—signaling that there was in fact no significant difference in the effects of Bt or azadirachtin on FAW natural environment.

The results of the second variance analysis test to compare combined Bt and azadirachtin concentrations' impact on FAW environment contrasted greatly. For this test, the null hypothesis was that combined Bt and azadirachtin concentrations would not have a significant impact on FAW environment. After running the test an f-statistic of 29.8 was obtained, and a p-value of 0.0004 was generated. The p-value for combined Bt and azadirachtin concentrations' impact on FAW environment is less than $\alpha = 0.01$. The null hypothesis is rejected at the 99% confidence level, proving that the data is statistically significant. From this, it can be concluded that combining Bt and azadirachtin does affect the natural environment of FAW and that There is convincing evidence of a relationship between the biopesticides and additive toxicity, effectively supporting the initial hypothesis.

Conclusion and Contributions to Literature

Research has introduced the notion that combining Bt toxins with chemical insecticides can lead to increased toxicity, with studies showing that the effects of *Bacillus thuringiensis* toxins and other insecticides are additive. The preliminary assumption was that the natural environment of FAW larvae after being introduced to the combined Bt plus the new biopesticide azadirachtin would decrease its survivability. To support this, Bt and azadirachtin were assessed on soil to predict its effect on FAW natural environment. This study showed that combined Bt and azadirachtin showed an increase in soil basicity, with the azadirachtin to Bt ratio of 1:9 showing the highest increase. The data proved to be statistically significant, which suggested that the toxicity of the azadirachtin/Bt mixtures were additive and negatively impacted each soil sample. This research effectively supports the original hypothesis that the natural environment of FAW larvae after being introduced to the combined Bt plus the new biopesticide azadirachtin will be toxic to FAW larvae and decrease its survivability, demonstrating that a solution of Bt and azadirachtin containing a greater concentration of Bt could impact FAW survivability in its natural environment. This research lends credibility to the theory of using combined chemical insecticides and biopesticides to increase their toxicity to insect pests. The original conclusion reached through an analysis of the data presented was that combining biological insecticides such as Bt and azadirachtin could have significant role in the control of FAW. This analysis, along with the data shown in Figure 2, introduce the notion that not only is there a link between combined Bt and azadirachtin use and toxicity to FAW, but the combined biopesticides are more effective than when utilized individually.

Future Implications and Limitations

Regarding this research, we must consider its future implications and limitations. One way to do so is to further explore the additive effects of *Bacillus thuringiensis* and other biopesticides and how they impact mortality of invasive insect species. In this study, the environment of FAW was tested rather than the actual species. Thus, further studies should test the impact of combined azadirachtin and Bt directly on FAW larvae to support the overall claim of combined toxicity. Additionally, researchers could test other solutions of concentrations of different chemical insecticides and biopesticides and how they work together to increase insect pests' susceptibility to chemicals to which they are otherwise resistant. Bt and azadirachtin are only two biopesticides being used in the control of FAW. Any other chemicals existing or currently being developed, when combined, may prove to help control FAW.

This study can provide valuable insight into the conclusion initially posted by Huang et al. in their work. By introducing the FAW larvae environment to Bt in addition to azadirachtin, another biopesticide effective against this invasive species, it further supports the concept of mixing biopesticides to increase their toxicity. Additionally, this investigation addresses a problem highlighted by Botha et al. Despite the resistance alleles of FAW to Bt, by combining it with various biopesticides and conventional chemical insecticides, the impact of Bt on FAW and its natural environment has the potential to increase. Finally, laboratory experiments have shown promising results, and the next logical steps for further research would be to implement novel chemical insecticide and biopesticide solutions into actual agricultural crop fields and test wide-spread FAW reactions to the new form of pest control. In turn, similar research can be conducted to contain other invasive species. Based on the new conclusion stemming from this research, it would be applicable to study the effects of Bt with other biopesticides on different *Spodoptera* and other invasive moth species.

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