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In vitro studies on the effects of plant-based bioinsecticide azadirachtin on the hemocyte-mediated immunity and various life history traits of *Plutella xylostella*

Syed Asad Ali BUKHARI[1], Zain UL ABDIN[1], Urooj Afzal CHUGHTAI[1], Hammad Ahmad KHAN[2], Waseem AKRAM[1], Muhammad ARSHAD[1], Hasooba HIRA[1], Shanza NAWAZ[1], Syeda Eisha Tu RAZIA[1]
[1]*Department of Entomology, University of Agriculture, Faisalabad, Pakistan*
[2]*Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan*

Abstract

The immune system of insects relies on cellular and humoral responses to defend against pathogens. Hemocytes, a crucial component of insects' innate immunity, play a role in various defence mechanisms such as phagocytosis, nodulation and encapsulation. In our initial study, we investigated how azadirachtin, an active ingredient found in botanical bioinsecticides, affects the hemocyte-mediated immunity of *Plutella xylostella* (L.). We followed a comprehensive approach that combined physiological and biochemical methods to assess multiple parameters, including hemocyte count, phagocytic activity, adult emergence rate, adult longevity and fecundity. An experimental study was conducted by applying three different concentrations of azadirachtin (125 ppm, 250 ppm, and 500 ppm) and using propanone-treated insect groups as a control. Hemocyte counts ranged from 13.186 to 8.87 cells × 10^6 per ml. Treated insects showed a higher percentage (57%) of hemocytes involved in phagocytosis than the control group (40.66%). The emergence of adult insects decreased from 85.33% to 43.67%. The longevity of males reduced from 12.6 days to 4.6 days, while that of females decreased from 15.33 days to 7 days. We also observed a reduction in fecundity from 211.6 eggs to 61.33 eggs in treated insects. This study sheds light on how azadirachtin impacts hemocyte-mediated immunity, highlighting the potential use of botanical bioinsecticides for sustainable and environmental friendly management of *P. xylostella* in vegetable crops.

Key words: hemocyte-mediated immunity, phagocytic activity, *Plutella xylostella*, azadirachtin, botanical insecticide.

Introduction

The diamondback moth *Plutella xylostella* (L.) (Lepidoptera Plutellidae), it is a widely distributed pest. It causes significant damage to cauliflower crops, resulting in a global crop loss of approximately 90% (Furlong *et al*., 2013). This pest significantly contributes to reduced agricultural yields, especially in regions where cruciferous plants are cultivated (Shelton, 2001; Sarfraz *et al*., 2006). It harms all stages of cabbage crops, leading to symptoms like defoliation, leaf distortion and inhibited growth. In cases of severe infestation by *P. xylostella*, crop losses can soar to as much as 100% (Arouiee and Karimzadeh, 2006).

Due to concerns about the environmental impact of synthetic pesticides, there is a growing interest in exploring alternative, eco-friendly methods for pest management. One promising approach involves the use of natural predators and biopesticides (Ahmad, 2012). Among these biopesticides, azadirachtin, a compound derived from neem tree (*Azadirachta indica* A. Juss.) seeds, has gained significant attention. Azadirachtin is effective against various agricultural, medical and veterinary pests while demonstrating minimal toxicity toward non-target organisms, including humans (Bempah *et al*., 2011).

Azadirachtin affects insects in multiple ways, including repelling them, reducing their feeding activity, inhibiting growth, disrupting egg laying and causing mortality and sterility (Mordue and Nisbet 2000; Alouani *et al*., 2009; Achio *et al*., 2012). Bioinsecticides derived from *A. indica* have also been shown to impact various physiological aspects of other insects, such as reducing the total hemocyte count in *Spodoptera litura* (F.) (Sharma *et al*., 2003), changing the differential hemocyte count in *Danaus chrysippus* (L.) (Pandey *et al*., 2008) and affecting phagocytic and prophenoloxidase activity in *Rhodnius prolixus* Stal (Figueiredo *et al*., 2006).

Insects lack adaptive immunity, which involves specific antigen receptors and immunological memory. However, they possess highly developed and efficient innate immunity (Jiravanichpaisal *et al*., 2006). Innate immunity in insects comprises two main responses: cellular innate immunity and humoral innate immunity (Hong *et al*., 2018; McLaughlin *et al*., 2019). Humoral immunity involves defence mechanisms that operate independently of hemocytes, including producing antimicrobial peptides and initiating enzymatic processes governing hemolymph melanisation and coagulation (Keshavarz *et al*., 2019). Cellular immunity relies on the functions of hemocytes, which can perform processes like nodulation, encapsulation and phagocytosis, playing a crucial role in protecting insects against pathogens, parasites and parasitoids (Lavine and Strand, 2002).

Phagocytosis is a complex process used by insects to engulf small particles like yeast, apoptotic cells and bacteria (Jutras and Desjardins, 2005). In Lepidoptera, circulating plasmatocytes and granulocytes are the primary participants in phagocytosis (Zhu and Zhang, 2013). When there are only a few pathogens, phagocytosis is the preferred defence mechanism, but when the pathogen load is high, hemocytes aggregate and form nodules (Browne *et al*., 2013). In cases where the target is too large for phagocytosis, such as nematodes or parasitoids, insects employ encapsulation to eliminate the threat (Lavine and Strand, 2002).

In this study, we pursued with two primary objectives to comprehensively assess the impact of azadirachtin, a potent botanical insecticide, on the diamondback moth (*P. xylostella*). Firstly, we aimed to investigate azadirachtin's effectiveness by studying its influence on the total hemocyte count and the rate of hemocyte phagocytic activity. This allowed us to understand how azadirachtin affects the immune system of *P. xylostella*. Secondly, we assessed the toxicity of azadirachtin on the survival and reproductive capacity of the diamondback moth. By addressing these two objectives, our research aims to provide valuable insights into the potential use of azadirachtin as an insect control product. This evaluation is particularly relevant for managing diamondback moth infestations in cauliflower crops, offering a sustainable alternative to synthetic insecticides.

Materials and methods

Collection and rearing of experimental insects

Experimental culture of diamondback moths was established in a controlled environment at the Insect Molecular Biology Lab, Department of Entomology, University of Agriculture Faisalabad, Pakistan at 26 ± 5 °C and the relative humidity at 60%. The adults and fourth instar larvae were collected from nearby cauliflower fields and placed in plastic jars filled with cauliflower leaves. We provided cotton swabs in these jars soaked in a 10% sugar solution. Jars were covered with muslin cloth to ensure proper aeration and facilitate egg laying. Eggs were collected by using fine forceps and camel hair brushes in Petri dishes for further examination. Upon hatching, larvae were fed with fresh cabbage leaves daily to support their growth and development. Two separate colonies were maintained under identical conditions for our experimental treatments. One insect colony was reserved for studying immune responses and the second was used for investigating developmental processes. Both insect colonies were divided into four groups, mainly including a control group and three experimental groups, each subjected to different treatments.

Exposure of insects to azadirachtin

In this experiment, fresh cabbage leaves served as the primary dietary substrate. The treatment groups involved the incorporation of *A. indica* vegetable oil, a pure and non-emulsified natural extract derived from neem seeds with a concentration of 2,000 ppm of azadirachtin, into the larvae diet. This integration was carried out at varying concentrations (125, 250 and 500 ppm) with three replicates per treatment. The conversion factor was established at 1 ppm equivalent to 1 µl of oil per gram of diet. Emulsification of the oil was achieved using propanone at a ratio of 10 ml per 400 g of diet. The control group, on the other hand, received cabbage leaves treated exclusively with propanone. Individual containers with a volume of 50 ml were utilized to hold larvae weighing 2-3 mg and aged five days. The containers were filled with approximately 10 g of either a control diet or an oil-containing diet and the larvae were kept in a climate chamber until they reached the end of their fourth instars.

Bioassays for immune responses

Extraction of hemolymph

In each treatment, 4th instar larvae were subjected to sterilization using 70% ethanol. Following sterilization, the larvae underwent a thorough rinse with distilled water. Subsequently, a sterile pair of scissors was utilized to excise one of the abdominal prolegs. Hemolymph, measuring five µl, was carefully extracted using a glass capillary. To prevent hemocyte aggregation, the collected hemolymph was promptly mixed with 25 µl of anticoagulant buffer, composed of 41 mM citric acid, 17 mM Na2 EDTA, 98 mM NaOH and 186 mM NaCl, with a pH of 4.5, within a microfuge tube.

Hemocyte count

A hemocytometer under a light microscope was used to count hemocytes and the total hemocyte count per millilitre was estimated by using the formula:

$$N = (Q1 + Q2 + Q3 + Q4)4 \times DF \times 10000$$

Where, N represents the estimated number of cells per millilitre (cells/ml), Q (1-4) refers to the count of cells within the external quadrants of the Neubauer chamber and DF stands for the dilution factor used in the cell counting process.

Phagocytic activity

A solution was prepared, comprising fluorescent red latex beads constructed from carboxylate-modified polystyrene, with an average diameter of 0.5 µm. This solution was formulated by combining the beads with 1× phosphate-buffered saline (PBS) at a ratio of 1:10 (Ajamhassani *et al*., 2013). The pH of the PBS was adjusted to 7.4. Three 4th instar larvae from each treatment were immobilized. Using an aseptic needle, 5 µl of the solution was injected into the fourth and fifth abdominal segments without puncturing the insect's gut. After 6 hours, hemolymph was collected, diluted and counted to determine the total number of hemocytes and the number of hemocytes that phagocytized the latex beads.

Bioassays for the developmental process

Effects on emergence

The emergence rate (Re) was determined using the following formula:

$$Re = (NAe / NLs) \times 100$$

Where, NAe represents the number of emerged adults and NLs stands for the number of surviving larvae after 24 hours of exposure to both treated and untreated leaves.

Longevity and fecundity of emerged adults

Three male-female pairs were allocated to Styrofoam containers (500 mL) in each treatment group. A 10% sugar solution served as their food source, while cabbage-treated aluminium foil sheets were provided for egg laying. Egg sheets were collected at 2, 4 and 6-day intervals to assess reproductive success. The lifespan of moths was recorded during the experiment.

Data analysis

Data analysis was conducted to assess multiple parameters of *P. xylostella* adults. The variables under exami-

nation encompassed hemocyte count, phagocytized hemocytes, rate of adult emergence, longevity and fecundity. To accommodate the randomized distribution of subjects across different treatments, a completely randomized design was employed. Subsequently, to analyse the variation and determine the significance of differences among the measured variables, ANOVA was applied. Additionally, pairwise comparisons between means were performed using Tukey's test at a significance level (α) of 0.05 to discern statistically significant distinctions. For all analyses, IBM SPSS version 16.0 software served as the tool of choice, ensuring the accuracy and reliability of the results.

Results

Hemocyte count and phagocytosis activity

The total estimated count of hemocytes in insects that developed on a diet containing oil was notably lower than in those on the control diet ($F = 56.188$; $df = 3$; $P = 0.000$; figure 1). Specifically, a significant reduction in hemocyte count was observed at the highest concentration, where the count decreased from 13.186 to 8.87 cells × 10^6 per ml (figure 1). Larvae that developed on diets containing different concentrations of *A. indica* vegetable oil showed a stimulatory effect in the phagocytosis activity ($F = 37.54$; $df = 3$; $P = 0.000$; figure 2). The highest treatment group exhibited 57% of hemocytes that phagocytosed latex beads while the control group exhibited 40.66% (figure 2).

Effect on adult emergence, longevity and fecundity

Larvae that were fed diets with different concentrations of azadirachtin, exhibited a significant reduction in adult emergence rate, longevity and fecundity compared to the control group. Among the azadirachtin doses tested, the concentration of 500 ppm had a pronounced effect. Adult emergence was reduced from 85.33 to 43.67% ($F = 9.475$; $df = 3$; $P = 0.005$; figure 3). Male longevity was reduced from 12.6 days to 4.6 days ($F = 23.167$; $df = 3$; $P = 0.000$) while female longevity decreased from 15.33 days to 7 days ($F = 19.819$; $df = 3$; $P = 0.000$) represented in figure 4. Fecundity of *P. xylostella* decreased from 211.6 eggs to 61.33 eggs ($F = 53.844$; $df = 3$; $P = 0.000$; figure 5).

Discussion and conclusions

This study is the first to investigate the impact of sublethal concentrations of *A. indica* oil on the hemocyte-mediated immunity of *P. xylostella*. The decrease in the total hemocyte count observed in this study, has also been observed in other insects such as *S. litura*, *D. chrysippus* and *G. mellonella* when exposed to different azadirachtin concentrations, (Sharma *et al*., 2003; Pandey *et al*., 2008; Er *et al*., 2017).

The findings of this study demonstrate a significant decrease in the mean hemocyte count with increasing doses of azadirachtin. Several factors could contribute to this decrease, including the accumulation of hemocytes in specific regions within the hemocoel and the inhibitory

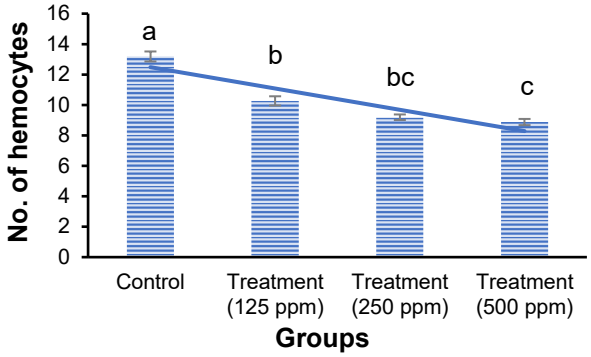

Figure 1. Mean hemocyte count (± SE) (cells × 10^6 /ml) in 4th instar *P. xylostella* larvae exposed to different concentrations of *A. indica* oil and the control group (propanone only). Statistically significant differences denoted by distinct letters (a-c) ($P < 0.05$; Tukey's HSD test), based on 3 replicates per treatment.

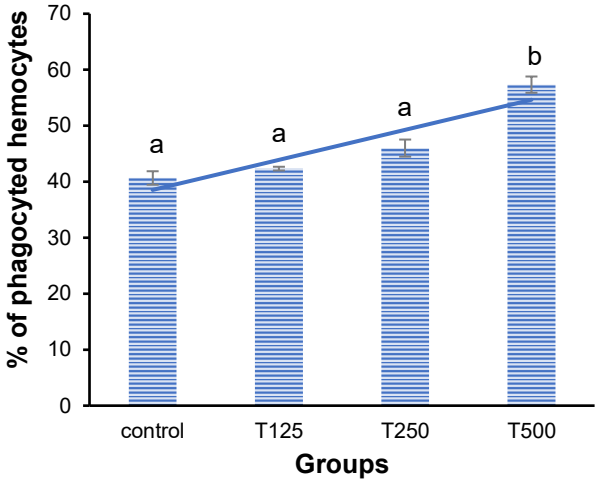

Figure 2. Mean percentage (± SE) of hemocytes engaging in latex bead phagocytosis in larvae fed with *A. indica* oil at various concentrations and the control group (propanone only). Statistically significant differences denoted by distinct letters (a-b) ($P < 0.05$; Tukey's HSD test), based on 3 replicates per treatment.

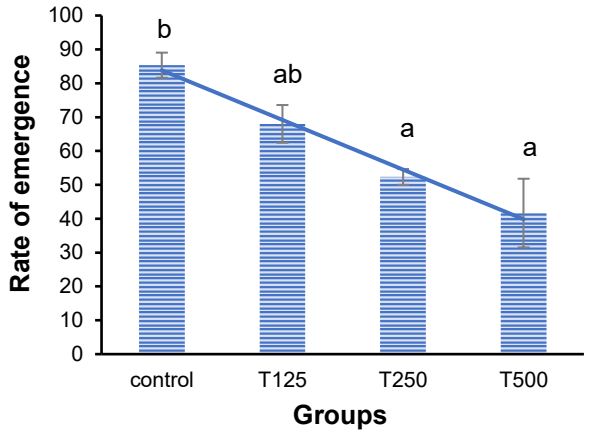

Figure 3. Rate of adult emergence (%) of *P. xylostella* in response to azadirachtin doses. Statistically significant differences denoted by distinct letters (a-b) ($P < 0.05$; Tukey's HSD test), based on 3 replicates per treatment.

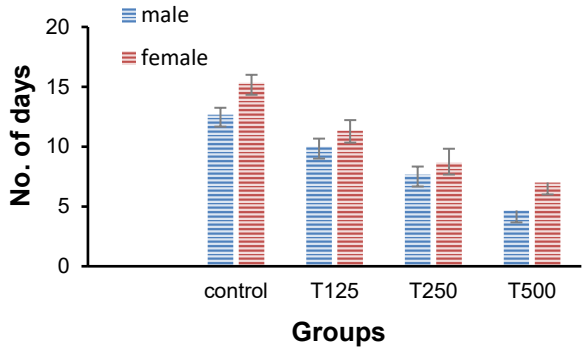

Figure 4. Longevity of male and female *P. xylostella* adults following exposure to *A. indica* oil across varied concentrations. Each treatment involved 3 replicates of both male and female individuals.

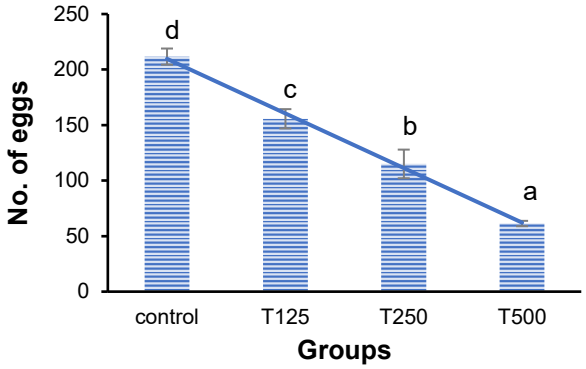

Figure 5. Fecundity of *P. xylostella* in response to different azadirachtin values. Statistically significant differences denoted by distinct letters (a-d) ($P < 0.05$; Tukey's HSD test), based on 3 replicates per treatment.

effects of neem on endocrine glands (Pandey *et al*., 2008). Azadirachtin has been shown to disrupt ecdysteroid synthesis and juvenile hormone levels, which are critical for hemocyte regulation (Mordue and Nisbet, 2000). Additionally, azadirachtin can impact cell division and protein formation in some insects such as *R. prolixus* (Jones, 1967).

Granulocytes and plasmatocytes together make up over 70% of the hemocyte types in Lepidoptera (Gardiner and Strand, 2000) and they have pivotal functions in processes such as encapsulation, nodulation and phagocytosis (Strand and Pech, 1995). While the study did not evaluate the specific hemocyte profile, it suggests that the presence of oil did not significantly influence the proportion of phagocytic cells. However, the observed decrease in hemocyte count is likely associated with prohemocytes, which are generated in Lepidoptera during the larval phase within the hematopoietic organs (Nakahara *et al*., 2010). Azadirachtin may disrupt the production of new prohemocytes through its toxic effects on hematopoietic organ cells, as previously investigated in *Sarcophaga argyrostoma* (Robineau-Desvoidy) larvae (Diptera Sarcophagidae) (Dorrah *et al*., 2019).

Our research highlights the insecticidal effects of azadirachtin on the emergence rate, longevity and fecundity of *P. xylostella* adults. Even though we used different concentrations in our study, azadirachtin showed consistent effects with prior research conducted on various insect species including *Helicoverpa armigera* (Hubner) (Ahmad *et al*., 2015), *Anopheles gambiae* Giles (Okumu *et al*., 2007), *Spodoptera eridania* (Stoll) (Shannag *et al.,* 2013), *Bactrocera cucurbitae* (Cocquillett) and *Bactrocera dorsalis* (Hendel) (Singh *et al*., 2003). Azadirachtin's impact on insect reproduction could be attributed to its effects on reproductive organs, gametogenesis and hormone-mediated signaling pathways. By disrupting these critical biological processes, azadirachtin may lead to reduced emergence rates, shortened longevity and diminished fecundity in adult insects.

Notably, this study focuses specifically on the impact of azadirachtin on the immunity and development of *P. xylostella*, distinguishing itself from previous studies that examined azadirachtin's effects on insect development in general. Furthermore, this study's range of doses provides a comprehensive understanding of how different concentrations of azadirachtin affect the immunity, development and reproduction of *P. xylostella.*

In conclusion, the preliminary findings of our research recommend the use of azadirachtin, a biopesticide, at 500 ppm formulation in cauliflower fields for the management of *P. xylostella*. The research will play a role in ensuring food security.

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Authors' addresses: Zain UL ABDIN (corresponding author, zainentomology@uaf.edu.pk), Syed Asad Ali BUKHARI (22asad.bukhari@gmail.com), Urooj Afzal CHUGHTAI (uroojchughtai2@gmail.com), Waseem AKRAM (areeba14@yahoo.com), Muhammad ARSHAD (arshaduaf@gmail.com), Hasooba HIRA (hasoobahira@gmail.com), Shanza NAWAZ (shanza.nawaz7002@gmail.com), Syeda Eisha Tu RAZIA, Department of Entomology, University of Agriculture, Faisalabad-38040, Pakistan; Hammad Ahmad KHAN (druafhammad@yahoo.com), Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan.

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