Enhanced Insecticidal Activity of Thiamethoxam by Zinc Oxide Nanoparticles: A Novel Nanotechnology Approach for Pest Control

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ABSTRACT: Indiscriminate and unregulated application of pesticides produces deleterious effect in various groups of organisms including humans and the environment. To solve these issues, it has been reported that the residue-free green nanocomposite synergistically enhances the pesticide efficacy. In this study, ZnO nanoparticles (NPs) with a thiamethoxam nanocomposite were synthesized and we investigated their synergistic effect on 4th instar larvae of Spodoptera litura (Lepidoptera: Noctuidae). These larvae were allowed to feed on the composite of ZnO NPs with thiamethoxam (10–90 mg/L) and thiamethoxam-impregnated castor leaves. Observations showed an increased larval mortality (27% increased mortality), a malformation in pupae and adults, overdrive emergence, and reduced fecundity and fertility. A significant dose-dependent variation in the biochemical parameters such as superoxide dismutase (SOD), glutathione-S-transferase (GST), and thiobarbituric acid-reactive substances (TBARS) in the treated larvae was also observed. A decline of 72.42 and 33.82% in SOD and GST activity, respectively, was observed at higher concentration as compared to the control. On the contrary, it enhanced the TBARS level up to 56.7%. The synthesized nanocomposite was characterized by different biophysical techniques such as X-ray diffraction (average crystalline size 34 nm), scanning electron microscopy, transmission electron microscopy (average particle size 30 nm), and Fourier transform infrared spectroscopy (Zn=O stretching peaks at 432 cm⁻¹ and 503 cm⁻¹). The observation of the present study suggests that ZnO NPs pave the way for developing cost-effective, eco-friendly, and capable nanomaterial for its applications in the field of biological sciences.

1. INTRODUCTION

The population of the world is increasing in an exponential manner day by day. In order to feed the increasing population, the need of the present situation is to produce more and more food.¹ The crops which provide us food were attacked by different groups of organisms including insects, causing severe damage to plants.² To control these pests, a variety of synthetic insecticides were frequently used in an indiscriminate manner, which leads to many serious concerns in various groups of organisms including humans.³⁻⁴

Fortunately, the rapid development of the nanotechnology along with alternative strategies aimed to produce residue-free nanocomposites with increased insecticidal activity and least environmental persistence, along with little damaging effect to human health and the environment. Besides, the judicious application of pesticide usage can delay the resistance development against the insecticides.⁵ In recent years, different nanoparticles (NPs) have been developed, namely, Ag, CuO, MgO, and ZnO, with confirmed efficient insecticidal activity either alone or in the combined form with different drugs against the insects of different orders.⁶⁻⁸ Interestingly, to the best of our knowledge, a few research papers have been shown to study the combined effects of NPs with organic pesticides to control the damage caused to plants by these insect pests.⁹⁻¹¹

Moreover, semiconductor NPs provide a feasible solution to remove the residues of pesticides through photocatalytic activity.¹² and also encourage us to lay down a novel green nanotechnology to control insect pests with a synergistic approach along with successive degradation through the photocatalytic activity and hence are environmentally friendly. Because of these properties of NPs, the ZnO NPs were chosen in the present study as they are cheap, stable, and sensitive to pathogens. Considering it, we predict that thiamethoxam might bind with the Zn atoms at the ZnO crystal surface to form a composite structure and consequently cause more damaging effects.¹³ This study is devoted to investigate the relative effect

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of ZnO NPs with thiamethoxam and thiamethoxam alone on *Spodoptera litura* and evaluate their enhanced insecticidal activity and low persistence in the environment.

To accomplish this, we have focused on the biological and biochemical parameters of *S. litura* such as superoxide dismutase (SOD), glutathione-S-transferase (GST), thiobarbituric acid reactive substances (TBARS), and “the enzymatic antioxidants”, formed against the reactive oxygen species (ROS) and free radicals developed in insects and utilized scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy for the characterization of ZnO NPs.

2. MATERIALS AND METHODS

2.1. Chemicals. Thiamethoxam was purchased from the local market (25% Wettable Granules) of the commercial grade with the brand name “Thioxam” manufactured by Kalyani Industries, Maharashtra, India, and all other chemicals used in the present study were procured from the Sigma-Aldrich and were of the analytical grade.

2.2. Synthesis of TZnO NCs. ZnO NPs were synthesized according to the work of Shoeb et al.\(^\text{14}\) (detailed description is provided in the Supporting Information). Thiamethoxam was added to ZnO NP (5:1 ratio) water suspension under mild magnetic stirring at room temperature (27 °C) and the stirring is continued for 30 min followed by aging at 27 °C for 1 h. After aging, the reaction mixture was heated to 50 °C and it was maintained at this temperature for 60 min. A white color precipitate formed is washed twice with double distilled water and dried at 50°C for 2 h in a hot air oven.

2.3. Characterization of TZnO NCs. XRD analysis of the as-synthesized TZnO NCs was performed in the 2θ range of 20–80° (Rigaku Miniflex II) with Cu Kα radiation (λ = 1.5406 Å) operating at a voltage of 30 kV and a current of 15 mA. For surface morphology, as well as the size of TZnO NCs, SEM was performed (JSM67500F, JEOL model).\(^\text{16}\) The microscope was operated at an accelerating voltage of 200 kV. For the FTIR spectroscopic measurements, the TZnO NC powder was mixed with spectroscopic-grade potassium bromide (KBr) in the ratio of 1:100 and the spectra were recorded in the range of wave numbers of 400–4000 cm\(^{-1}\) on a PerkinElmer FTIR Spectrum BX (PerkinElmer Life and Analytical Sciences, CT, USA) in the diffuse reflectance mode at a resolution of 4 cm\(^{-1}\) in KBr pellets.\(^\text{18}\)

2.4. Rearing of *S. litura*. The test insect *S. litura* adults were captured from in and around the agricultural fields of Faculty of Agriculture, AMU, Aligarh, India. They were kept in the rearing jar of size (20 × 15 cm) under the laboratory conditions of 26 ± 2 °C temperature, 65–70% relative humidity, and a photoperiod of 14 h light:10 h dark in a B.O.D incubator. The adults were fed on the 10% glucose solution soaked in the cotton. For laying eggs, the jars were provided with tissue paper. The collected eggs were kept in other jars for hatching. Throughout the larval period, fresh castor leaves were given as larval food. From the stock culture, fourth instar larvae of *S. litura* were selected for the present study.

2.5. Preparation of Different Concentrations of the Insecticide. After calculating the LC\(_{50}\) value, different sublethal concentrations were prepared by dissolving it in distilled water, viz., 10, 30, 60, and 90 mg/L (T1–T4 & ZT1–ZT4). Distilled water alone was considered as the control.

2.6. Mode of Application of Different Sublethal Concentrations. The castor leaves were dipped in the different sublethal concentrations of the insecticide and insecticide-containing NPs for 10 min. After that, the leaves were dried at room temperature. Thirty individuals of fourth instar larvae were chosen for each sublethal concentration and were allowed to feed upon the treated leaves. There were three replicates for each sub lethal concentration along with the untreated control.

2.7. Assay of GST. GST activity was measured according to the method of Habig et al.\(^\text{19}\) The reaction mixture containing 10 mM GSH and 1 mM CDNB (1-chloro-2,4-dinitrobenzene) was used as the substrate. After adding 50 μL of the protein sample prepared in 0.1 M phosphate buffer (pH 7.4), the reaction initiated. The activity of the enzyme was measured as the CNDB conjugate formed in nmol/min/mg protein.

2.8. Analysis of SOD Activity. To measure the SOD activity, we followed the method of Marklund and Marklund (1974). The reaction mixture (3 mL) contained 2.85 mL of 50 mM Tris-cacodylate buffer (pH 8.5) and 50 μL of the sample. To initiate the reaction, we added 100 μL of 0.13 mM pyrogallol. The absorbance was noted at 420 nm for the period of 3 min. SOD activity of one unit is defined as the amount of the enzyme involved in 50% inhibition of autoxidation of pyrogallol. SOD activity was expressed in units per mg of protein.

2.9. LPO Assay. The MDA level as marker lipid peroxidation (LPO) was performed by the method of Buege and Aust (1978). The gut homogenate was prepared in chilled 0.1 M potassium chloride solution. The reaction mixture contained 0.250 mL of the gut homogenate, 1 mL of TBA, and 3 mL of OPA. After vortexing, the reaction mixture was kept for incubation at 90°C for 45 min. Different aliquots were prepared, and the absorbance was calculated at 535 nm. The rate of LPO was expressed as nanomoles of MDA formed using a molar extinction coefficient of 1.56 × 10^5 M\(^{-1}\) cm\(^{-1}\) for the MDA–TBA colored complex.

2.10. Statistical Analysis. Data of the three independent experiments were presented as mean ± SEM. Data obtained related to oxidative stress were analyzed by two-way analysis of variance (ANOVA) followed by the Bonferroni’s post-hoc test using statistical software Graph Pad Prism 5.01 (CA, USA). Data obtained related to larval mortality, fecundity, fertility, and longevity were analyzed by one-way ANOVA followed by the Tukey’s test. The significant level is set at p < 0.05.

3. RESULTS AND DISCUSSION

3.1. Characterization of TZnO NPs. XRD characterized the crystal structure of TZnO NCs with Cu Kα radiation (λ = 0.15418 nm). The data revealed ten well-resolved XRD peaks at 2θ = 31.89, 34.65, 36.13, 47.52, 56.70, 62.89, 66.72, 68.09, 69.45, and 77.10°, which correspond to the crystal planes \([100]\), \([002]\), \([101]\), \([102]\), \([110]\), \([103]\), \([200]\), \([112]\), \([201]\), and \([202]\) of the polycrystalline wurtzite structure (zincite, \(\text{JCPDS} \ 0-0644\), respectively (Figure 1). They relate XRD peaks, at 8.60, to carbon (a different allotrope of carbon) in thiamethoxam and the occurrence of sulphur at 16.40, 23.70, and 202.
25.70, 27.30, 32.90, and 58.70 in thiamethoxam. The XRD data of TZN O NPs showed the successful functionalization of thiamethoxam on ZnO nanoparticles. We found the average crystallite size to be 34 nm using the Debye−Scherrer formula.

Figure 2A−C shows SEM images of TZN O NCs, whose sizes ranged between 5 and 0.5 μm with relatively round shapes and smooth surfaces of ZnO NPs around the thiamethoxam insecticide. Introduction of ZnO NPs to the thiamethoxam insecticide suspensions triggered ZnO NP adsorption on the surface as shown in Figure 2A,B. The sizes of the functionalized ZnO NP insecticide ranged between 5 and 0.5 μm on the scale, suggesting multilayer functionalization of the ZnO NPs. Figure 2D reveals EDX (elemental composition) mapping which confirms the successful functionalization of ZnO NPs over the thiamethoxam insecticide and confirmed the presence of elements such as zinc (Zn), oxygen (O), carbon (C), sulphur (S), and chlorine (Cl). TEM (Figure 2E,F) analyzed the effect of the thiamethoxam insecticide on ZnO NP surface functionalization. Figure 3E,F shows two distinct phases on the scale of 100 and 20 nm. TEM images revealed two distinct phases (i) the light phase and (ii) the dark phase. It shows that the light phase of the TEM image belongs to the organic phase (thiamethoxam insecticide) and the dark phase belongs to the inorganic phase of the NPs (ZnO NPs). In addition, it shows TEM images of interaction between these two phases. Therefore, we can see the successful functionalization between ZnO NPs and the thiamethoxam insecticide.

3.2. SOD Activity. Following the treatment with different concentrations of thiamethoxam (T1−T4) and the ZnO−thiamethoxam composite (ZT1−ZT4), SOD activities vary in the dose- and time-dependent manner (Figure 4a,b). The SOD activities during early exposure and at the lower concentration increase significantly showing a preliminary stimulatory response. The SOD activity increases during 48 h of exposure and started decreasing at 72 h of exposure. In the highest concentrations (ZT4 and T4), the SOD activity decreases significantly, indicating the toxicity of the pesticide. The groups treated with ZnO−thiamethoxam showed the higher SOD activity than the groups treated with thiamethoxam alone (Figure 4b). The enhanced SOD activities in the groups treated
with ZnO–thiamethoxam as compared to those treated with thiamethoxam alone clearly show the synergistic effects of ZnO NPs.

3.3. GST Activity. Following the treatment with different concentrations of thiamethoxam (T1–T4) and the ZnO–thiamethoxam composite (ZT1–ZT4), the activity of the detoxifying enzyme, glutathione-S-transferase (GST), in the gut of S. litura larvae is presented in Figure 4c,d. We observed a dose- and time-dependent increase in the GST activity up to 48 h of exposure both in thiamethoxam (T1–T4) and the ZnO–thiamethoxam composite. However, during 72 h of exposure, the GST activity in the highest concentration (ZT4) decreases significantly showing the phase of transition from active antioxidant defense to the exhausted state. In the ZnO–thiamethoxam composite, GST activity decreases significantly as compared to the thiamethoxam-treated groups.

3.4. LPO Assay. After treating with different concentrations of thiamethoxam (T1–T4) and ZnO–thiamethoxam (ZT1–ZT4), the level of malondialdehyde (MDA) formed as an end product of LPO is presented in Figure 4e,f. We observed a dose- and time-dependent significant (p < 0.05) increase in the level of MDA in all test concentrations of thiamethoxam and ZnO–thiamethoxam. In the highest concentration (ZT4), we observed a significant increased level of MDA as compared to the highest concentration (T4) of thiamethoxam.

3.5. Effect on Larval Mortality. Larval mortality after treating with different concentrations of ZnO–thiamethoxam and thiamethoxam increases in the dose-dependent manner (Figure 5a,b). We observed the enhanced larval mortality in all test concentrations of ZnO–thiamethoxam as compared to the groups treated with thiamethoxam alone. In the highest concentration (ZT4), we observed 27% enhanced mortality than the groups treated with thiamethoxam alone. These

Figure 4. Graphs showing the effects of thiamethoxam on SOD activity (a) thiamethoxam–ZnO NCs on SOD activity, (b) thiamethoxam on glutathione-S-transferase, (c) thiamethoxam–ZnO NCs glutathione-S-transferase, (d) thiamethoxam on LPO, and (e) thiamethoxam–ZnO NCs on LPO. (f) Values are mean ± SEM. (n = 3). Data showed a significant increase at *p < 0.05 vs control and a significant decrease at #p < 0.05 vs control.
apparent differences in the larval mortality showed the synergistic effect of the ZnO NPs.

3.6. Effect on the Adult Emergence and Malformation. Following the treatments with the different concentrations of thiamethoxam alone and ZnO–thiamethoxam, inhibition of adult emergence is presented (Figure 5c,d). We observed a significant \( (p < 0.05) \) decline in the emergence of adults in all test concentrations of thiamethoxam alone and ZnO–thiamethoxam. Most pronounced effects are seen in the groups treated with the different concentrations of ZnO–thiamethoxam as compared to the groups treated with thiamethoxam alone. In the highest concentration (ZT4) of ZnO–thiamethoxam (percent), inhibition of adult emergence was recorded. The post molting effects on the pupae and adults were also seen in different concentrations (Chart 1). In the groups treated with ZnO–thiamethoxam, the rate of malformations was higher than that of the thiamethoxam-treated groups.

3.7. Effect on Fecundity and Fertility. Effect of ZnO NPs and thiamethoxam on the fecundity and fertility of the females was presented in Figure 6a–d. We observed a significant \( (p < 0.05) \) dose-dependent decrease in the fecundity and fertility in all the test concentrations. We observed a significant \( (p < 0.05) \) difference in the highest concentration (ZT4 and T4) of ZnO–thiamethoxam and thiamethoxam. In the highest concentration of ZnO–thiamethoxam (ZT4), a significant decline (26.2 & 20.34%) in the fecundity and fertility clearly showed the synergistic effects of ZnO NPs.

3.8. Effects on the Longevity. After treating with the different concentrations (ZT4 and T4) of thiamethoxam and ZnO–thiamethoxam, we observed their effects on the larval...
longevity (fifth and sixth) and adult longevity (male and female). A significant (p < 0.05) dose-dependent increase in the longevity of the fifth and sixth instar larvae was observed. In the highest concentration (ZT4) of ZnO–thiamethoxam and thiamethoxam alone, longevity of the fifth and sixth instar larvae was enhanced by 56.28 and 52.6%, respectively, as compared to the control (Figure 7a–d). Interestingly, we also observed enhanced longevity of males and females in ZnO–thiamethoxam-treated groups (Figure 8a–d), indicating the synergistic effects of ZnO NPs.

The problem of resistance toward the different groups of insecticides due to their extensive use is reported in different parts of the world.20 As a consequence, there is need to develop an alternative approaches. Zinc oxide (ZnO) NPs derived from the metal that has strong inclination toward the production of ROS and finally resulted in development of oxidative stress in different groups of the organisms including insects.21 Apart from the resistance problem in different groups of insects, the indiscriminate use of insecticides/pesticides leads to adverse effects on the ecosystem in general and on the humans in particular.22 Therefore, it is the requisite of the present situation to develop alternative methods to minimize the use of insecticides/pesticides. The synergistic effect (ability to enhance the effects of insecticides/pesticides) of ZnO NPs has been studied to overcome the aforementioned problem. The current study is the first effort to explore synergistic

Figure 6. Graphs showing the effects of thiamethoxam on fecundity: (a) thiamethoxam–ZnO NCs on fecundity, (b) thiamethoxam on fertility, and (c) thiamethoxam–ZnO NCs on fertility. (d) Values are mean ± SEM. (n = 3). Data showed a significant increase at *p < 0.05 vs control.

Figure 7. Graphs showing the effects of thiamethoxam on longevity of the fifth instar larvae: (a) thiamethoxam–ZnO NCs on the fifth instar larvae, (b) thiamethoxam on longevity of sixth instar larvae, and (c) thiamethoxam–ZnO NCs on longevity of sixth instar larvae. (d) Values are mean ± SEM. (n = 3). Data showed a significant increase at *p < 0.05 vs control.
behavior of ZnO NPs in insects in general and *S. litura* in particular. To the best of our information, this is the first time to explore the synergistic insecticidal ability of ZnO NPs with thiamethoxam. According to the FTIR analysis (Figure 3), we demonstrated the synergistic effects because of weak interaction between thiamethoxam and ZnO NPs. As a result, thiamethoxam concentration increased at the sites where thiamethoxam has more contact with the larvae of *S. litura* and hence causes more destruction as compared to thiamethoxam used alone.

The infiltration of ZnO NPs might cause more ZnO-stimulated oxidative damage within the cell. However, after formation of the ZnO–thiamethoxam composite, ZnO NPs possibly will help thiamethoxam to enter inside the body of the larvae and endorse to exert the thiamethoxam toxicity.

Following the treatments with different concentrations of ZnO–thiamethoxam, effects are recorded in terms of oxidative stress. In the lower concentrations, the oxidative stress is probably developed as a result of ROS production in the insect gut. The production of ROS in an excess amount causes undesirable oxidative stress that leads to cell damage. Most of the cells can tolerate the small elevated level of ROS, through increasing scavenging activity of antioxidant enzymes such as SOD and GST. However, when ROS are produced in large quantity, this defense system becomes weak and inactive.

The antioxidant enzyme SOD causes dismutation of $\text{O}_2^-$ to $\text{H}_2\text{O}_2$, whereas GST induces a reaction in which xenobiotics are converted to (GSH) reduced glutathione. Both SOD and GST with the help of other antioxidants play an important protective role against the attack of ROS. However, when the larvae of *S. litura* were treated with the highest concentration of ZnO–thiamethoxam, this protective system of SOD and GST appeared to be inactivated and we recorded a significant decline in the activity possibly because of high toxicity of thiamethoxam. The activity of SOD and GST in the ZnO–thiamethoxam at the highest concentration and at 72 h declined significantly. This decline in SOD and GST activity might be due to the synergistic effects of ZnO NPs, which lead to increased efficiency of the pesticide thiamethoxam. The studies of other workers also confirmed the synergistic behaviors of ZnO NPs.

The oxidative stress induced due to NPs also targets the lipids and instigates LPO. The highest concentration of ZnO–thiamethoxam and thiamethoxam resulted in sufficient oxidative stress that causes LPO. The increased levels of ROS within the cells, after exposure to the NPs perhaps not only influence the membrane potential and permeability but also influence the mitochondrial function as well. Such changes inhibit the ATP production by disrupting the electron transport system, ultimately causing death of the insects. We observed the increased level of MDA after exposure to the highest concentration of the ZnO–thiamethoxam composite as compared to the same concentration of thiamethoxam alone. This increased MDA level might be due to the synergistic effect of ZnO NPs, which enhanced the toxicity of thiamethoxam.

The effects of thiamethoxam alone and the ZnO–thiamethoxam composite were also evaluated on the different biological parameters, such as mortality, fecundity, fertility, malformation, and longevity. We observed a dose-dependent significant increase in the abovementioned parameters. In the integrated pest management program, insect pest mortality has been considered as an important parameter. Maximum mortality followed by minimum use of pesticides is considered
to be an appropriate approach for reducing pollution in an ecosystem.\textsuperscript{33} ZnO–thiamethoxam at its higher concentration caused the highest mortality as compared to when thiamethoxam is used alone. This might be due to synergistic effect of the NPs.

Fecundity and fertility of the insect pest determined the population level in a particular area.\textsuperscript{34} It is considered as an indirect method of controlling the population of insect pests after exposure to different groups of insecticides, instead of killing the adult insects. It would be greatly beneficial from the ecological point of view to control the insect pest population through reducing the egg-laying capacity of females and its emergence.\textsuperscript{4} In the present study, maximum reduction of fecundity and fertility was observed in the ZnO–thiamethoxam-treated groups as compared to when thiamethoxam was used alone.

Longevity is considered as an important aspect in the life cycle of insect pests. They maintain their population size through maintaining longevity of different stages. After exposure to insecticides, the longevities of larvae and adults get disturbed, which leads to reduced population of insect pests.\textsuperscript{35} In the present study, the longevity of the larvae and adults increases significantly in the dose-dependent manner. The maximum increased longevity was observed in the ZnO–thiamethoxam groups as compared to when thiamethoxam was used alone possibly because of the synergistic behavior of NPs used in the present study. Inducing malformation in the insect pest population in the integrated pest management program is also considered as one of the important aspects in controlling pest population below the economic injury level.\textsuperscript{36,37} In the present study, the malformation was observed to increase in the dose-dependent manner. However, at the highest concentration in the ZnO–thiamethoxam group, the highest number of malformed adults was found as compared to when thiamethoxam was used alone. This observed effect might be due the synergistic effect of the ZnO NPs used in the present study. In the highest concentration of ZnO–thiamethoxam groups, we observed the enhanced effects as compared to the groups treated with thiamethoxam alone. These enhanced effects might be due to synergistic effects of ZnO NPs.

4. CONCLUSIONS

The findings of the present study suggested that ZnO–thiamethoxam NPs were used to control the population of \textit{S. litura}. We speculated that the formation of the ZnO–thiamethoxam composite insecticide system, electrostatic adsorption of ZnO–thiamethoxam groups to insect cells, and the cellular internalization ZnO NPs played important roles in synergistic insecticidal activities which facilitate the interaction of ZnO NPs, thiamethoxam, and their combination with different components of cells of \textit{S. litura} leading to enormous damage.

As a result, the antioxidant competency of \textit{S. litura} was debilitated and led to more destruction in ZnO-induced oxidative stress; finally, the synergistic insecticidal activity was attained against \textit{S. litura}. Owing to the synergistic insecticidal activity, this nanotechnology first offers the prospect to moderate thiamethoxam usage in the presence of ZnO NPs devoid of compromise in insect control. Hence, we have observed increased alterations in biological parameters and oxidative stress markers in thiamethoxam-containing zinc oxide NPs as compared to groups treated with thiamethoxam alone. The present study demonstrated prominent effects of nano-technology; however, further work still needs to be done for further deeper understanding the synergistic mechanism and extending the application range with other groups of pesticides.

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Notes
The authors declare no competing financial interest.

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