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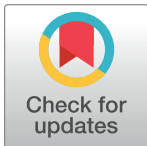
Nano-insecticides against the black cutworm *Agrotis ipsilon* (Lepidoptera: Noctuidae): Toxicity, development, enzyme activity, and DNA mutagenicity

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Abstract

Frequent applications of synthetic insecticides might cause environmental pollution due to the high residue. In addition, increasing insecticide resistance in many insect pests requires novel pest control methods. Nanotechnology could be a promising field of modern agriculture, and is receiving considerable attention in the development of novel nano-agrochemicals, such as nano-insecticides and nanofertilizers. This study assessed the effects of the lethal and sublethal concentrations of chlorantraniliprole, thiocyclam, and their nano-forms on the development, reproductive activity, oxidative stress enzyme activity, and DNA changes in the black cutworm, *Agrotis ipsilon*, at the molecular level. The results revealed that *A. ipsilon* larvae were more susceptible to the nano-forms than the regular forms of both nano-chlorine and sulfur within the chlorantraniliprole and thiocyclam insecticides, respectively, with higher toxicities than the regular forms (ca. 3.86, and ca. 2.06-fold, respectively). Significant differences in biological parameters, including developmental time and reproductive activity (fecundity and hatchability percent) were also observed. Correspondingly, increases in oxidative stress enzyme activities were observed, as were mutagenic effects on the genomic DNA of *A. ipsilon* after application of the LC₅₀ of the nano-forms of both insecticides compared to the control. These promising results could represent a crucial step toward developing efficient nano-insecticides for sustainable control of *A. ipsilon*.

Introduction

The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) is a major insect pest that can destroy many important crops worldwide [1]. Specifically, the insect larvae damage

many crop species, vegetables, and weeds [2]. *A. ipsilon* larvae can consume over 400 cm² of foliage during their development [3]. Chemical insecticides have been used to prevent crop loss by *A. ipsilon* [4].

The selection of highly effective insecticides and their appropriate application methods is a fundamental problem of integrated pest management strategies. Nanotechnology could provide new methods and agricultural products to counteract the fears related to the potential unwanted environmental impact of chemical insecticides through increased exposure and toxicity in non-target organisms [5]. Nanoparticle technology could develop through two distinct mechanisms: (a) supplying singular crop protection or (b) as carriers for existing pesticides [6]. Generally, nanopesticides are defined as any pesticide formulation of nano-sized, insecticide particles or small, engineered structures with pesticide properties [7,8]. Nano-pesticides have several advantages, including increased potency and durability, and a reduced amount of active ingredients [9]. They are also considered to be a promising solution for the reduction of the environmental footprint left by chemical pesticides [7]. The increasing interest in the use of nano-pesticides raises questions about their fate, toxicity, and biodegradation [10], as well as how to assess the environmental risk of these materials.

The introduction of nano-insecticides into the environment necessitates the careful identification of their potential. Generally, insecticide studies focus on evaluating the lethal and sub-lethal toxicity of chemical active substances on insect development and enzyme activities [11]. Insecticides are considered a stress factor that may upset the functional balance in insects [known as oxidative stress (OS)]. Insecticides are characterized by the enhanced production of reactive oxygen species (ROS) with the simultaneous impairment of their scavenging systems. Increased ROS concentrations result in oxidative damage to proteins, lipids, and nucleic acids, and thus cell function, organs, and the entire organism may be seriously disrupted, resulting in death [12]. To avoid, or at least reduce this effect, organisms have developed effective defense systems controlled by their nervous and endocrine glands. The oxidative stress enzymes are an essential group of enzymes that minimize the adverse effects of ROS on cells. Several defense mechanisms, including enzymatic and non-enzymatic components, have been developed by insects [13,14]. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione (L- γ -glutamyl-L-cysteinylglycine, GSH) are major antioxidant enzymes in insects that play a fundamental role in cell protection by removing oxidative stress [15]. SOD is an antioxidant enzyme, which converts superoxide into oxygen and hydrogen peroxide [16]. CAT is mainly a H₂O₂-scavenging enzyme that **principally** removes H₂O₂ generated from developmental or environmental stimuli into water and oxygen in all aerobic organisms [17]. CAT tends to reduce small peroxides, such as H₂O₂, but does not affect larger molecules, such as lipid hydroperoxides. POX utilizes either H₂O₂ or O₂ to oxidize a **wide** variety of molecules [18], and uses H₂O₂ to oxidize phenolic compounds. GSH is the heart of the essential cellular antioxidant system [19], and may serve as an electron donor (cofactor) for antioxidant enzymes like glutathione peroxidases and glutathione S-transferases [20].

Genetic molecular markers have become a central tool to determine the degree of genetic variability [21], molecular phylogenetics [22], genetic fidelity [23], and disease resistance [24] in organisms. Various kinds of molecular marker techniques have been identified in insect populations [25]. The inter-simple sequence repeats (ISSR) are a useful marker tool to detect genetic variation and differentiate closely-related individuals [26]. The high variability level of the ISSR marker has been indicated to be a common characteristic of Lepidoptera genomes and is applied as a fingerprint technique between groups of organisms [27].

Due to the lack of information on nano-insecticide toxicity, several studies are required to better understand their effects on the biological and physiological parameters of target insects [10]. There are currently no sufficient screening methods to assess whether nano-insecticides

are safe for field administration without significant side effects on human health. Thus, this study's main goal was to evaluate the potential effects of the nano-forms of two new insecticides, chlorantraniliprole and thiocyclam, on *A. ipsilon*. We also investigated the effects of the lethal and sublethal concentrations of both insecticides and their nano-forms on the development, reproductive activity, and oxidative stress enzyme activities, including; SOD, CAT, lipid peroxidase, and GR of *A. ipsilon*. This study examined both insecticides for nanoparticle-induced changes in *A. ipsilon* at the DNA molecular level.

Materials and methods

Insect rearing

Agrotis ipsilon was reared for 12 generations in the absence of insecticides in the laboratory in a rearing room at $26 \pm 1^\circ\text{C}$, $65 \pm 5\%$ relative humidity and a photoperiod of 16 L:8 D. The newly hatched larvae were kept in a clean glass jar (1 L) and provided a castor oil leaves daily until the third instar larvae emerged. Then they were transferred to larger, and clean glass jars (2 L) to prevent larval cannibalism. The bottom of each jar was covered with a thick layer of fine sawdust and the usual rearing techniques were performed along with the developing instars larvae until pupation occurred. After pupation, the developed pupae had been transferred to clean jar for the adult emergence. The adult moths (males and females in ratio 7:5) were transferred to a glass jar (5 L) which was supplied with hanged piece of cotton wool soaked in 10% sugar solution as dietary supplement [11].

Insecticides and chemicals

Chlorantraniliprole (Coragen[®] 20% SC, suspension concentrate, DuPont) and thiocyclam (Evisect-S[®] 75% SC) were tested. All chemicals used in the preparation of the nano-form of the insecticides were purchased from Sigma Chemical Co. (St. Louis, USA) without further purification. The oxidative stress enzymes kits were purchased from Biodiagnostic Company, Egypt.

Nanoinsecticides preparation and size measurements

Nano-chlorine (chlorantraniliprole) and nano-sulfur (thiocyclam) were prepared by De Oliveira et al. [28], Cota-Arriola et al. [29], and Xu et al. [30], with some modifications. Hydrochloric acid (HCl) and orthorhombic bravais were used as a chlorine source, while sodium thiosulphate and sulfuric acid were used as a sulfur source. The nano-chlorine was made from a sedimentary solution of HCl and MnO₂ v:v, where hydrochloric acid and manganese oxide were added slowly in a molar ratio (3:2) in the presence of the stabilizing agent PVA using forceful moving for 5 hours continuously. The obtained precipitation was filtered and washed thoroughly with deionized water, then 16 ml of NaCl 0.2 M aqueous solution was added to the suspension solution using forceful moving at a steady rate for 40 min. The reaction fusion was stirred for an additional 3 hours continuously at ambient temperature. The precipitate was mixed with orthorhombic brava by w:w (2:1) in the presence of HCl 90%, then centrifuged at 1500 rpm for 30 min. Then, the solution was cooled in an ice bath, subsequently exposed to 1.5 psi pressure continuously for 6 hours, and the final nano-suspension was ready for examination under transmission electron microscope.

Nano-sulfur was prepared from a sedimentary solution of sodium thiosulphate and sulfuric acid (1:1). Sodium polysulfide and hydrochloric acid solutions were incrementally mixed in a molar ratio of 3:2 under forceful moving for 8 hours discontinuously. The obtained precipitation was filtered and washed methodically with deionized water in a mixed water/toluene

system, then washed with ionized water for 3 hours continuously. The precipitation was mixed with oxalic acid 1 M and trimethylammoniumbromide compound solution in molar ratio 1:3 under slow stirring at 33°C for 6 hours discontinuously. Afterwards, drop benzene sulphonate in molar ratio 2:3 was added, and the obtained solution was kept at 1.5 psi pressure for 3 days discontinuously (7 hours per day). Finally, the solution was dried in an oven at 90°C for 3 days continuously. The final nano-suspension was prepared in deionized water and left on a shaker for 2 days continuously at 20°C. The final nano-suspension was ready for examination under transmission electron microscope.

Bioassays

The toxicity of chlorantraniliprole, thiocyclam, and their nano-forms on the second instar larvae were assessed using the leaf dipping technique [31], with some modifications. Briefly, six different concentrations were prepared for each insecticide, while water was used for the control. Castor oil leaves were dipped in each concentration for 20 s then left to air-dry. After drying, the leaves were transferred into a glass jar (0.25 L). Ten larvae were added to each jar with five replications and left to feed for 24 h. Afterwards, all larvae were offered untreated leaves, and the mortality% was recorded four days (96 hours) post-treatment [32] to calculate the lethal and sublethal concentrations for each insecticide form. The bioassay was repeated twice.

Effects on *Agrotis ipsilon* development

Lethal concentrations that kill 15 and 50% of the larvae exposed to each form of chlorantraniliprole and thiocyclam were applied on the second instar larvae using the method described above. Surviving insects were used to study the effect of each insecticide on larval and pupal development time, pupation%, and adult emergence. Seven days after exposure, the surviving larvae were transferred individually to a clean cup to record the development time of the larval and pupal stages and the pupation%. After pupation, each pupa was sexed, weighed, and kept individually in the same cup to record the emergence%.

Studies on fecundity and fertility

Groups of 5 females and 7 males in 3 replicates [33] were used to calculate the number of eggs and hatching% after the second instar larvae were treated with the LC₁₅ and LC₅₀ values of each insecticide and their nano-forms. Deposited eggs were collected and counted on days 2–6 in the mating jars. The eggs were transferred to a clean jar and kept for 5 days to record the hatching%.

Oxidative stress enzyme assays

Sample preparation. Seven days after LC₁₅ and LC₅₀ equivalent treatment of the second instar larvae, 100 mg fresh body weight of the surviving larvae were transferred to clean and sterile Eppendorf tubes (1.5 ml). The samples were stored immediately at –20°C until later analysis. Each treatment and control was replicated five times. The treated larvae were homogenized in a potassium phosphate buffer (50 mM, pH 7.0) at 30 µl buffer per 1 mg of body weight. The homogenate was centrifuged for 15 min at 7000 g at 4°C, and the supernatants were used for further analysis.

Enzymes measurement. SOD activity was determined according to Misra and Fridovich [34] at the absorbance of 560 nm. The CAT enzyme activity was estimated by measuring the rate of H₂O₂ consumption [35] via absorbance at 510 nm. The level of lipid peroxidase was assayed by monitoring the formation of malondialdehyde (MDA) at 534 nm [36]. GR activity

was estimated as the reduced glutathione (GSSG) in the presence of NADPH [37], which oxidizes to NADPH⁺ at 340 nm. The total protein concentration of all samples was measured spectrophotometrically based on the Biuret Method using Protein Biuret Kit (Biodiagnostic, Egypt).

Molecular analysis

DNA extraction. DNA was isolated from both treated and untreated second instar larvae using a G-spin™ total DNA extraction kit (INtRON Biotechnology) following manufacturer's instructions. The DNA was quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific Inc.). The DNA concentrations were measured and subsequently adjusted in all samples to 10 ng/μL for subsequent molecular analyses.

ISSR polymorphism analysis. For ISSR PCR amplification [38,39], a set of 15 ISSR primers were applied against the 9 treatments. The PCR was carried out in a total volume of 25 μL containing the following components: 25 ng genomic DNA; 1X PCR buffer; 1.5 mM MgCl₂; 0.25 mM of each dNTPs; 1 μM of each primer; 1 U Go-Taq Flexi polymerase (Promega).

Thermocycling amplification was performed with a GeneAmp PCR system 9700 (Applied Biosystem, Inc.). The amplification was programmed at 94°C for 5 min for the initial denaturation cycle, followed by 35 cycles with each cycle comprising 94°C for 1 min, 50°C for 1 min, then 72°C for 90 s; and a final extension at 72°C for 7 min. The produced PCR amplicons were electrophoresed using 1.5% agarose gel. A 100 bp plus DNA ladder and 1 kb were used as molecular size standards. PCR products were photographed using a Gel Doc™ XR+ System (Bio-Rad®).

Data analyses

Biological and biochemical data analyses. The statistical analysis program LDP line was used to determine the lethal and sublethal concentration values (LC₁₅, LC₅₀, and LC₉₀) for each insecticide and its nano-forms (with 95% confidence limits). All biological parameters and oxidative stress enzymes activity (SOD, CAT, lipid peroxidase, and GR) were performed using one way ANOVA in addition to Dunnett's multiple comparisons test with Graph Pad Prism 8 statistical analysis software. Moreover, a silhouette analysis was performed to evaluate the quality of the reproductive activity, enzymes, and developmental measurements by testing the cluster distances within and between each cluster [40]. Additionally, we performed a multi-dimensional preference analysis to disclose the interrelationships among parameters in addition to the similarity classification in terms of dependent and independent variables in different space dimensions [41]. Finally, hierarchical clustering based on the correlation analysis was conducted with two-dimensional heatmap plotting was constructed.

Molecular data analysis. For ISSR data analysis, the generated amplicons were scored visually. To generate a binary data set, the amplicons were scored as absent (0) and present (1). The polymorphism percentage was analyzed by dividing the number of amplified polymorphic bands by the total number of amplified bands separately for each primer [42]. A similarity matrix was built to estimate the genetic distances between all possible treatment pairs. The Jaccard coefficient was used for the pairwise comparisons [43]. The genetic similarity (GS) between each pair of treatments was calculated using $GS = a/(n-d)$, in which n is the total number of fragments; a is the number of positive coincidences; and d is the number of negative coincidences. The genetic distances (GD) between pairs of treatments were estimated using $GD = 1-GS$. The unweighted pair group method of arithmetic averages (UPGMA) was used to construct the dendrogram [44].

The efficiency of the ISSR primers was determined by calculating the following parameters: expected heterozygosity ($H = 1 - \sum p_i^2$ according to Liu [45]); polymorphism information content ($PIC = 1 - \sum p_i^2 - \sum p_i^2$) according to Botstein et al. [46]; effective multiplex ratio ($E = n \beta$) according to Powell et al. [47]; marker index ($MI = E \text{ Hav}$) according to Powell et al. [47]; mean heterozygosity ($\text{Hav} = \sum H_n/n_p$) according to Powell et al. [47]; discriminating power ($D = 1 - C$) according to Tessier et al. [48]; resolving power ($R = \sum I_b$) according to Prevost and Wilkinson [49].

Results

Characterization of nano-(chlorine) chlorantraniliprole and nano-(sulfur) thiocyclam

The nano-suspensions of chlorine (Fig 1) and sulfur (Fig 2) were achieved using top-down molecular chemical techniques. Briefly, a single drop of the nanoparticle solution was spread onto a carbon-coated copper grid, and then was posteriorly dried at room temperature for transmission electron microscope (TEM) analysis. The dimensions of the nanoparticles were established directly from the figure using Image-Pro Plus 4.5 software. The particles were irregular in shape, with dimensions of 3.99 nm for chlorine and 4.05 nm for sulfur (Table 1), as well as 98.5% purity for each element. The nanoparticles' shape and dimension were examined using a JEOL 1010 TEM at 80 kV (JEOL, Japan).

Activities of chlorantraniliprole, thiocyclam, and their nano-forms

The toxicity of chlorantraniliprole, thiocyclam, and their nano-forms on the second instar larvae are presented in Table 2. The LC_{50} values were 0.058 and 9.20 mg/l for chlorantraniliprole

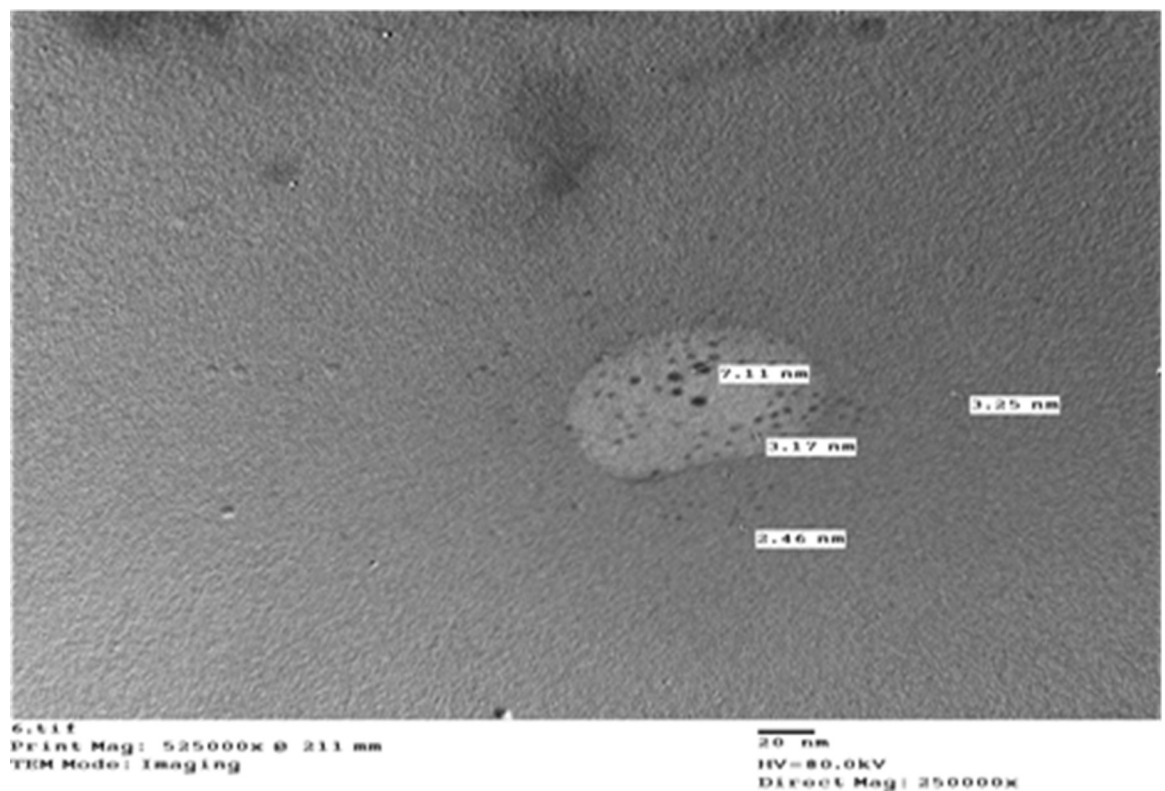


Fig 1. Nano-chlorine.

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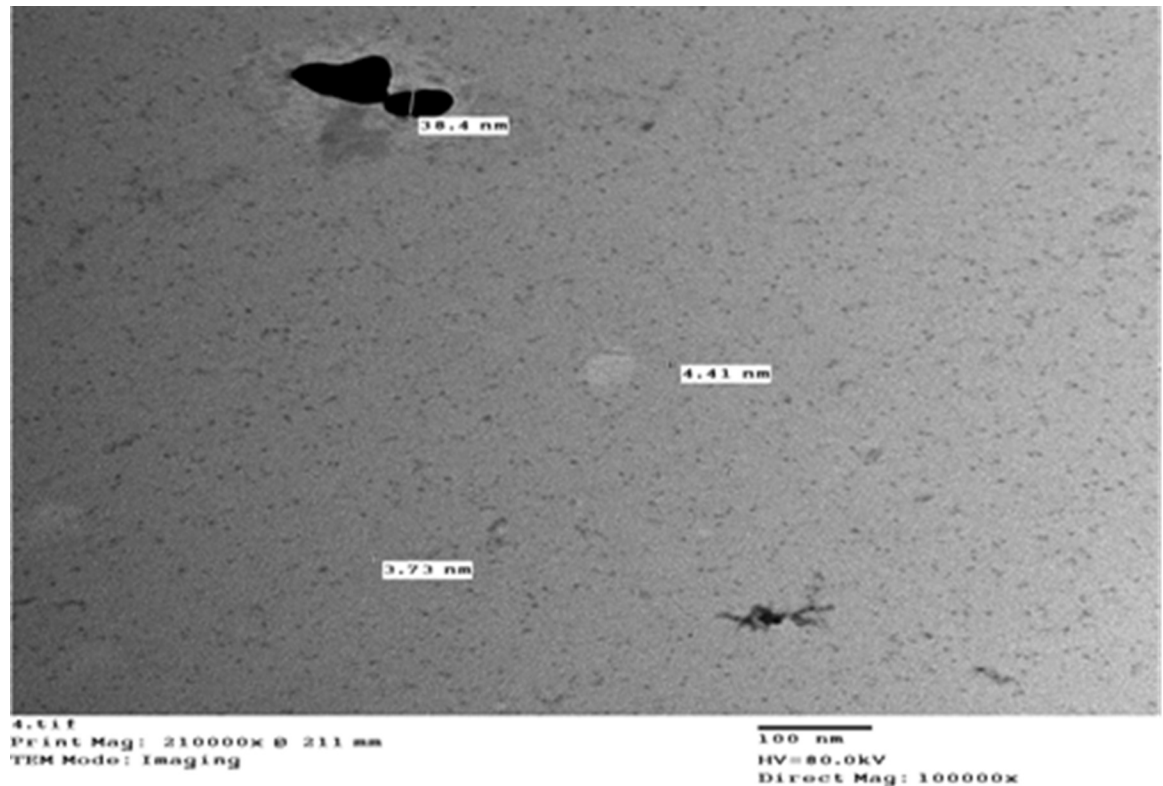


Fig 2. Nano-sulphur.

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and thiocyclam, respectively, 96 h post-treatment. In contrast, the nano-forms (nano-chlorantraniliprole and nano-thiocyclam) had significantly higher toxicities than their original compounds, with LC₅₀ values of 0.015 and 4.46 mg/l, respectively (Table 2).

Lethal and sublethal effects on the second instar larvae

Table 3 shows the latent effect of chlorantraniliprole, thiocyclam, and their nano-forms on *A. ipsilon* development (from second instar larvae until the emergence) due to LC₁₅ and LC₅₀ exposure. The results showed elongation of the larval developmental period under all LC₅₀ treatments, while pupal duration was significantly increased in larvae exposed to regular form of both insecticides. The pupation% decreased significantly after LC₁₅ and LC₅₀ treatment of nano-thiocyclam at $83.20 \pm 4.36\%$ and $85.88 \pm 0.56\%$, respectively (Table 3). No differences

Table 1. Particle size of nano-chlorine, and nano-sulphur.

Formulation	Sample number	Size (nm)	Average size (nm)
Nano-chlorine	1	3.25	3.99
	2	7.11	
	3	3.17	
	4	2.46	
Nano-sulphur	1	3.84	4.05
	2	4.41	
	3	3.73	
	4	4.22	

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Table 2. Lethal and sublethal activity of chlorantraniliprole, thiocyclam, and their nano-form in the 2nd instar larvae of *Agrotis ipsilon*.

Insecticides Treatments	^a LC ₁₅ (mg/L) (95% Confidence limit)	^b LC ₅₀ (mg/L) (95% Confidence limit)	^c LC ₉₀ (mg/L) (95% Confidence limit)	Slope ± SE	χ ²
Chlorantraniliprole	0.001 (0.000–0.003)	0.058 (0.010–0.159)	8.21 (2.17–141.89)	0.59 ± 0.12	4.10
Nano-Chlorantraniliprole	0.001 (0.000–0.003)	0.015 (0.006–0.038)	2.65 (0.57–48.27)	0.56 ± 0.09	3.96
Thiocyclam	0.052 (0.000–0.426)	9.209 (2.03–34.10)	1622.48 (233.63–38982.06)	0.57 ± 0.15	3.42
Nano-Thiocyclam	0.44 (0.11–0.89)	4.46 (2.87–6.78)	45.14 (23.29–154.65)	1.27 ± 0.21	4.44

^a LC₁₅: Concentration to cause mortality in 15% of individuals.

^b LC₅₀: Lethal concentration to cause mortality in 50% of individuals.

^c LC₉₀: Lethal concentration to cause mortality in 90% of individuals.

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were found in the female and male pupal weight, sex ratio, or emergence% for all treatments (Table 3).

Fecundity and fertility

Chlorantraniliprole, thiocyclam, and their nano-forms significantly decreased the hatchability percent under LC₁₅ and LC₅₀ compared to the control (Table 4). After chlorantraniliprole and nano-chlorantraniliprole treatment, the hatchability percentages were 84.60, and 81.29% at LC₁₅, and 73.83, and 73.08% at LC₅₀, respectively. Under thiocyclam and nano-thiocyclam treatment, the hatchability percentages were 82.64, and 78.32% at LC₁₅, and 78.79, and 71.99% at LC₅₀. In contrast, the number of eggs laid by one female showed no significant differences between treated and untreated larvae (Table 4).

Activity of oxidative stress enzymes

Exposure to both insecticides and their nano-forms caused a significant increase in SOD activity after treatment with the LC₅₀ of thiocyclam (40.09 U/g of protein) and nano-thiocyclam

Table 3. Effects of chlorantraniliprole, thiocyclam, and their nano-form in the developmental stages of *A. ipsilon*.

Treatments		^a Larval duration	Pupation%	^b Pupal duration	Pupal weight (mg)		Sex ratio%		^c Emergence%
					Female	Male	Female	Male	
Control		20.22±0.15	93.79±3.10	17.00±0.15	0.43±0.01	0.39±0.01	43.44±3.79	56.56±3.79	98.61±1.39
Chlorantraniliprole	LC ₁₅	21.04 ^{ns} ±0.29	95.84 ^{ns} ±0.31	17.91 ^{**} ±0.16	0.40 ^{ns} ±0.01	0.39 ^{ns} ±0.01	49.55 ^{ns} ±7.58	50.45 ^{ns} ±7.58	98.61 ^{ns} ±1.39
	LC ₅₀	24.13 ^{****} ±0.46	90.16 ^{ns} ±0.15	18.48 ^{****} ±0.28	0.39 ^{ns} ±0.01	0.35 ^{ns} ±0.01	42.85 ^{ns} ±2.28	57.15 ^{ns} ±2.28	98.15 ^{ns} ±1.85
Nano-Chlorantraniliprole	LC ₁₅	22.25 ^{***} ±0.31	93.92 ^{ns} ±1.31	17.73 ^{ns} ±0.24	0.39 ^{ns} ±0.01	0.36 ^{ns} ±0.01	49.38 ^{ns} ±9.97	50.62 ^{ns} ±9.97	96.74 ^{ns} ±1.63
	LC ₅₀	22.30 ^{****} ±0.26	91.07 ^{ns} ±2.25	17.46 ^{ns} ±0.20	0.42 ^{ns} ±0.01	0.40 ^{ns} ±0.01	51.78 ^{ns} ±10.26	48.22 ^{ns} ±10.25	90.54 ^{ns} ±2.32
Thiocyclam	LC ₁₅	21.66 [*] ±0.34	92.60 ^{ns} ±1.23	17.77 [*] ±0.19	0.39 ^{ns} ±0.02	0.38 ^{ns} ±0.01	42.15 ^{ns} ±5.32	57.85 ^{ns} ±5.32	98.24 ^{ns} ±1.75
	LC ₅₀	23.30 ^{****} ±0.39	93.82 ^{ns} ±1.91	17.46 [*] ±0.16	0.41 ^{ns} ±0.01	0.37 ^{ns} ±0.01	46.40 ^{ns} ±1.32	53.60 ^{ns} ±1.32	98.61 ^{ns} ±1.39
Nano-Thiocyclam	LC ₁₅	22.52 ^{****} ±0.28	86.54 ^{ns} ±1.73	17.54 ^{ns} ±0.19	0.36 [*] ±0.008	0.35 ^{ns} ±0.01	42.67 ^{ns} ±4.66	57.33 ^{ns} ±4.66	96.63 ^{ns} ±1.71
	LC ₅₀	22.75 ^{****} ±0.35	82.55 [*] ±3.79	17.27 ^{ns} ±0.12	0.39 ^{ns} ±0.01	0.39 ^{ns} ±0.01	49.59 ^{ns} ±12.14	50.41 ^{ns} ±12.14	88.85 [*] ±3.38

ns is no significant different at P > 0.05

* Significant different at P ≤ 0.05

** significant different at P ≤ 0.01

*** significant different at P ≤ 0.001

**** significant different at P ≤ 0.0001.

^anumber of days from 2nd instar larvae till pupation.

^bnumber of days from the pupation till the emergence.

^cEmergence % = Number of moths/Total number of pupae × 100.

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Table 4. Mean fecundity and hatchability % (\pm SE) of *A. ipsilon* females after treating the 2nd instar larvae with LC₁₅ and LC₅₀ values of chlorantraniliprole, thiocyclam, and their nano-form.

Treatments		^a Fecundity	^b Hatchability %
Control		539.10 \pm 13.71	90.78 \pm 0.31
Chlorantraniliprole	LC ₁₅	412.93 ^{ns} \pm 10.88	84.60 ^{ns} \pm 3.65
	LC ₅₀	282.00 ^{**} \pm 58.29	73.84 ^{**} \pm 5.53
Nano-Chlorantraniliprole	LC ₁₅	386.70 ^{ns} \pm 39.50	81.29 ^{ns} \pm 4.60
	LC ₅₀	286.3 ^{**} \pm 32.92	73.08 ^{**} \pm 0.93
Thiocyclam	LC ₁₅	446.60 ^{ns} \pm 37.99	82.64 ^{ns} \pm 3.31
	LC ₅₀	365.00 ^{ns} \pm 63.60	78.79 ^{ns} \pm 2.23
Nano-Thiocyclam	LC ₁₅	276.70 ^{**} \pm 15.38	78.32 ^{ns} \pm 2.22
	LC ₅₀	265.00 ^{**} \pm 77.12	71.99 ^{**} \pm 1.78

ns is no significant different at $P > 0.05$

* Significant different at $P \leq 0.05$

** significant different at $P \leq 0.01$

*** significant different at $P \leq 0.001$

**** significant different at $P \leq 0.0001$.

^aFecundity was estimated by counting the eggs from the first day till the sixth day (total number of eggs laid by one female).

^bFertility was calculated by counting of the emerged larvae from collected eggs batch.

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(43.0 U/g of protein) (Table 5). The SOD activity after treatment with the LC₅₀ of chlorantraniliprole and that of nano-chlorantraniliprole was highly significant compared to the control treatment with 45.38 and 53.96 U/g of protein (ca. 5.44, and 6.47 fold), respectively. No significant changes relative to the control were recorded in any other treatments.

The LC₁₅ and LC₅₀ of both insecticides and their nano-forms stimulated CAT activity compared to the control (Table 5). A significant increase in CAT activity was observed at the LC₁₅ of chlorantraniliprole and nano-chlorantraniliprole, the LC₁₅ of thiocyclam, and both the LC₁₅, and LC₅₀ of nano-thiocyclam (80.22, 87.76, 76.03, 80.28 and 86.43 U/mg of protein,

Table 5. Mean (\pm SE) of oxidative stress enzymes (SOD, CAT, glutathione reductase, and lipid peroxidase) activities of *A. ipsilon* after exposure of 2nd instar larvae to LC₁₅ and LC₅₀ values of chlorantraniliprole, thiocyclam, and their nano-forms.

Treatments		Mean \pm SE			
		SOD U/g of protein	CAT U/g of protein	Glutathione reductase U/g of protein	Lipid peroxidase nmol/g of protein
Control		8.33 \pm 2.4	17.95 \pm 4.74	1.42 \pm 0.10	0.23 \pm 0.07
Chlorantraniliprole	LC ₁₅	33.68 ^{ns} \pm 2.66	80.22 [*] \pm 4.71	11.04 [*] \pm 1.57	1.60 ^{ns} \pm 0.41
	LC ₅₀	45.38 ^{**} \pm 11.78	99.78 ^{**} \pm 25.06	11.76 [*] \pm 3.79	2.24 [*] \pm 0.58
Nano-Chlorantraniliprole	LC ₁₅	35.41 ^{ns} \pm 2.05	87.76 [*] \pm 3.95	12.56 ^{**} \pm 2.65	1.68 ^{ns} \pm 0.07
	LC ₅₀	53.96 ^{**} \pm 10.53	99.60 ^{**} \pm 12.6	14.33 ^{**} \pm 2.06	3.75 ^{****} \pm 0.57
Thiocyclam	LC ₁₅	24.01 ^{ns} \pm 1.31	76.03 [*] \pm 8.34	3.73 ^{ns} \pm 0.06	1.25 ^{ns} \pm 0.14
	LC ₅₀	40.09 [*] \pm 8.45	89.90 ^{**} \pm 21.81	6.41 ^{ns} \pm 1.63	2.02 [*] \pm 0.26
Nano-Thiocyclam	LC ₁₅	34.59 ^{ns} \pm 4.67	80.28 [*] \pm 10.40	12.67 ^{**} \pm 2.00	1.54 ^{ns} \pm 0.22
	LC ₅₀	43.0 [*] \pm 6.61	86.43 [*] \pm 12.55	14.42 ^{**} \pm 2.20	2.26 [*] \pm 0.68

ns is no significant different at $P > 0.05$

* Significant different at $P \leq 0.05$

** significant different at $P \leq 0.01$

*** significant different at $P \leq 0.001$

**** significant different at $P \leq 0.0001$.

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respectively). The highest significance was observed for the LC_{50} of chlorantraniliprole and nano-chlorantraniliprole, and the LC_{50} of thiocyclam (ca. 5.56, 5.54, and 5.0-fold, respectively).

An increase in lipid peroxidase activity was observed under exposure to the LC_{50} of nano-chlorantraniliprole with 3.75 nmol/g of protein (ca. 16.30-fold). Meanwhile, the lipid peroxidase activity after treatment with the LC_{50} of chlorantraniliprole, thiocyclam, and nano-thiocyclam was slightly significant at 2.24, 2.02, and 2.26 nmol/g of protein, respectively. No significant differences in lipid peroxidase activities were observed in any other treatments (Table 5).

Exposure to all investigated insecticides and their nano-forms resulted in significant stimulation of GR activity in the LC_{15} and LC_{50} of chlorantraniliprole (11.4 and 11.76 U/g of protein) (Table 5). GR activity was significantly high under the LC_{15} and LC_{50} nano-chlorantraniliprole treatments at 8.84 and 10.09-fold, respectively and under the LC_{15} and LC_{50} nano-thiocyclam treatments at 8.92, and 10.15-fold, respectively. No significant changes in enzyme activity was observed for the LC_{15} and LC_{50} thiocyclam treatments (3.73 and 6.41 U/g of protein) compared to the control.

Correlation between development, reproductive, and enzyme activity

The plots for silhouette analysis were calculated based on the Euclidean distance metric to assess the cluster quality of the treatments based on reproductive activity, enzymes, and developmental measurements via cluster distance tests within and between each cluster (Fig 3). The results revealed that all parameters, except GR, SOD, and CAT enzymes, exhibited negative values, thus indicating that the clusters were mostly similar, and that the cluster configuration

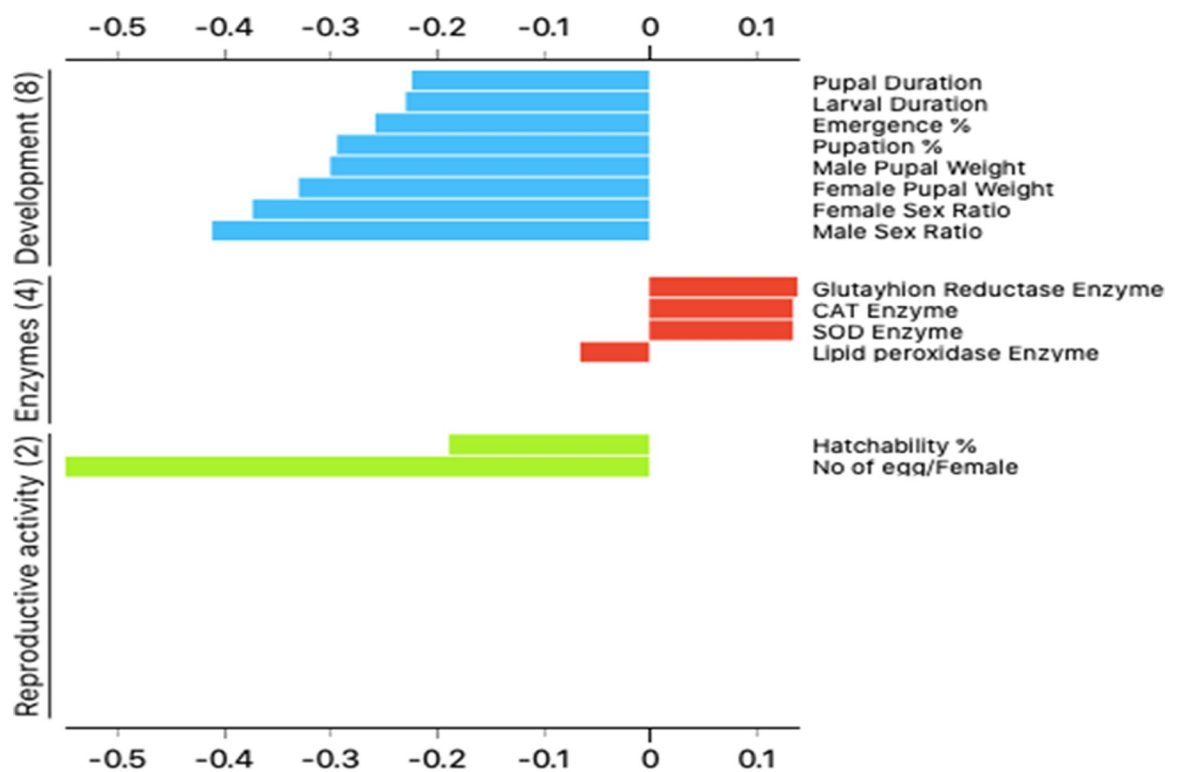


Fig 3. Plot of Silhouette analysis values for clustering of reproductive activity, enzymes and developmental variables. On the y-axis each cluster are ordered by decreasing silhouette value. The silhouette value can range between -1 and 1.

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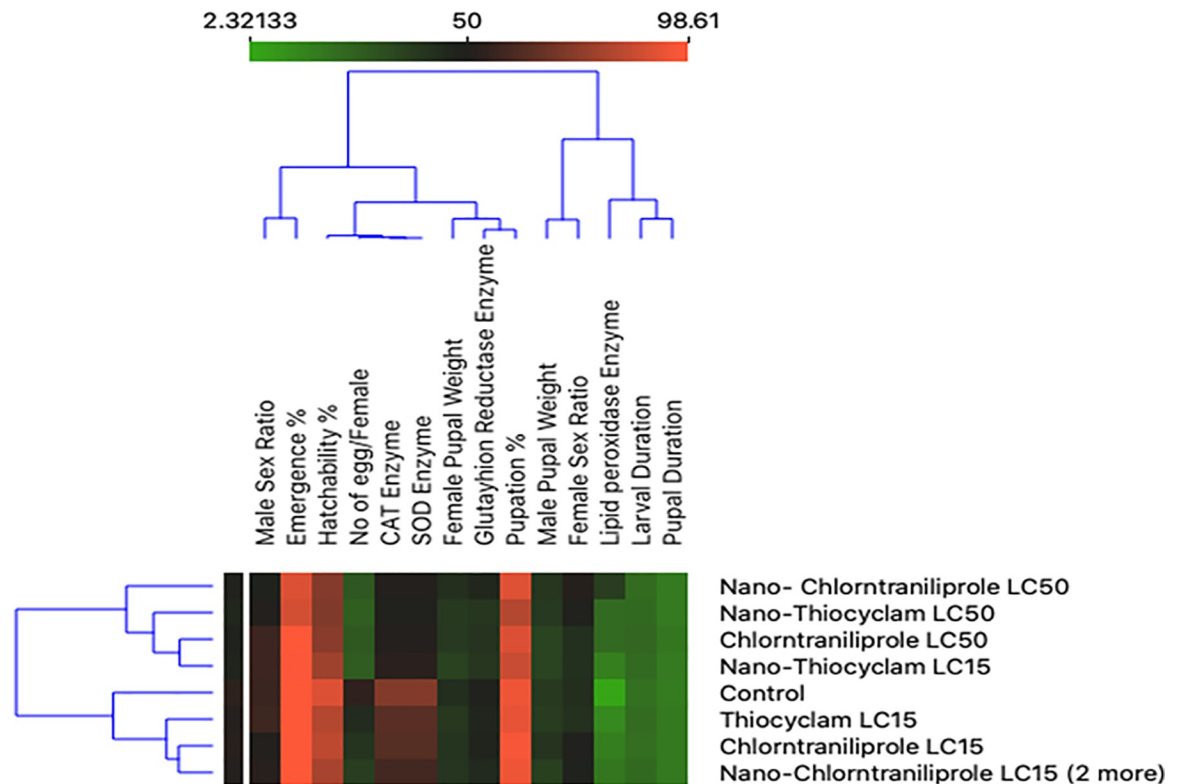


Fig 4. Two-dimensional heatmap visualization shows the interaction between the treatments and (A) the eight developmental parameters (B) the two reproductive activity parameters (C) the four enzymes parameters.

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may have too few clusters. Meanwhile, two-dimensional heatmap plotting based on all parameters clustered the LC₁₅ of thiocyclam, chlorantraniliprole, and nano-chlorantraniliprole as the most similar to the control (1st cluster), whereas the LC₅₀ of nano-chlorantraniliprole, nano-thiocyclam, chlorantraniliprole, and the LC₁₅ of nano-thiocyclam were grouped in the second cluster as less-similar relative to the control (Fig 4). Moreover, multidimensional preference analysis was performed to summarize the interrelationships of all treatments, parameters, and classes (Fig 5). The plot shows that the LC₅₀ of nano-thiocyclam, chlorantraniliprole, and the LC₁₅ of nano-thiocyclam deviated the most compared to the control. GR, SOD, and CAT enzymes revealed almost the same pattern.

ISSR analysis

To determine the mutagenic levels of the chlorantraniliprole, thiocyclam, and their nano-forms on the DNA of the second instar larvae, the ISSR marker system was used. The DNA of the untreated second instar larvae and all other treatments were amplified using 15ISSR primers (Fig 6). The 15 ISSR primers yielded a total of 252 scorable amplicons with an average of 16.8 bands/primer (Table 6). The number of amplified DNA fragments per primer ranged from 11 bands (primer ISSR-18) to 21 bands (primers ISSR-10 and ISSR-12). The number of polymorphic bands per primer ranged from 7 bands (primer ISSR-1) to 19 bands (primer ISSR-5). A narrow range of the expected heterozygosity values was observed between 0.37 to 0.50. Notably, 13 out of the 15 ISSR primers showed values near 0.50. The polymorphism information content almost revealed the same pattern for all primers, with values ranging from 0.30 to 0.37. The effective multiplex ratio values were almost all high, with values ranging

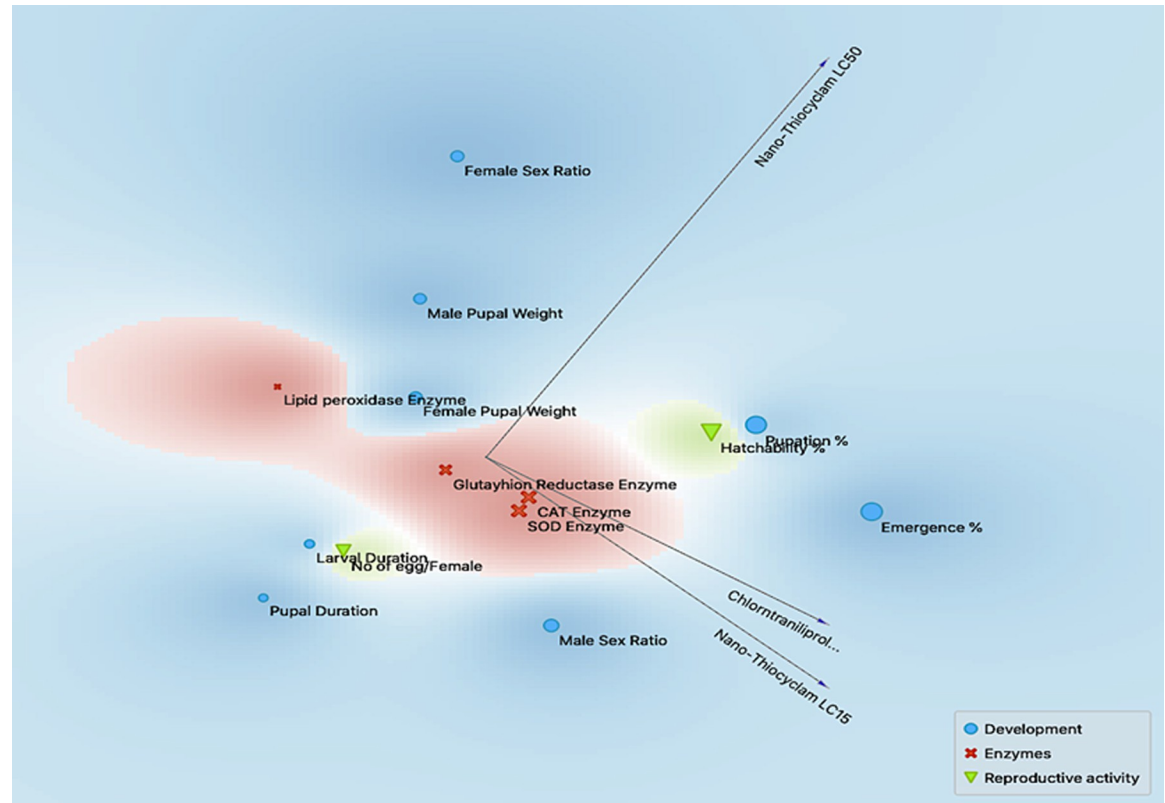


Fig 5. Multidimensional preference analysis plot summarizing the interrelationships amongst treatments, parameters, and classes.

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from 6.00 to 12.67. In contrast, the marker index values were shallow (near to 0.01). The discriminating power values ranged from 0.44 to 0.86. The resolving power values ranged between 4 to 10. The primers ISSR-2 and ISSR-5 showed the highest-resolving power value (10.44) among all the ISSR primers (Table 6). The highest GS was observed between the LC₅₀ of chlorantraniliprole and the LC₁₅ of thiocyclam. The lowest GS was determined to be between the LC₅₀ of nano-chlorantraniliprole, and nano-thiocyclam (Table 7).

Analysis of molecular phylogeny

A dendrogram based on the UPGMA cluster analyses of the ISSR data was constructed for the nine treatments (Fig 7). The dendrogram was comprised of two main clusters: the first cluster included only the LC₅₀ of chlorantraniliprole, while the second cluster comprised two sub-clusters. The first sub-cluster consisted of only the LC₅₀ of nano-thiocyclam, and the second sub-cluster involved two major groups. The first major group included the LC₁₅ treatments of chlorantraniliprole, and nano-thiocyclam. The second major group consisted of the control, the LC₁₅ treatments of chlorantraniliprole, nano-chlorantraniliprole, and thiocyclam, and the LC₅₀ of thiocyclam. Furthermore, the PCA analysis of the ISSR data revealed highly similar results to the cluster analysis. The PCA results indicated that the LC₁₅ of nano-chlorantraniliprole and the LC₅₀ of thiocyclam were most similar to the control (Fig 8).

Discussion

Insecticide efficacy depends on the mode of action, insect species, developmental stage, application methods, and the number of days post-treatment [50]. Thus, pesticide nano-

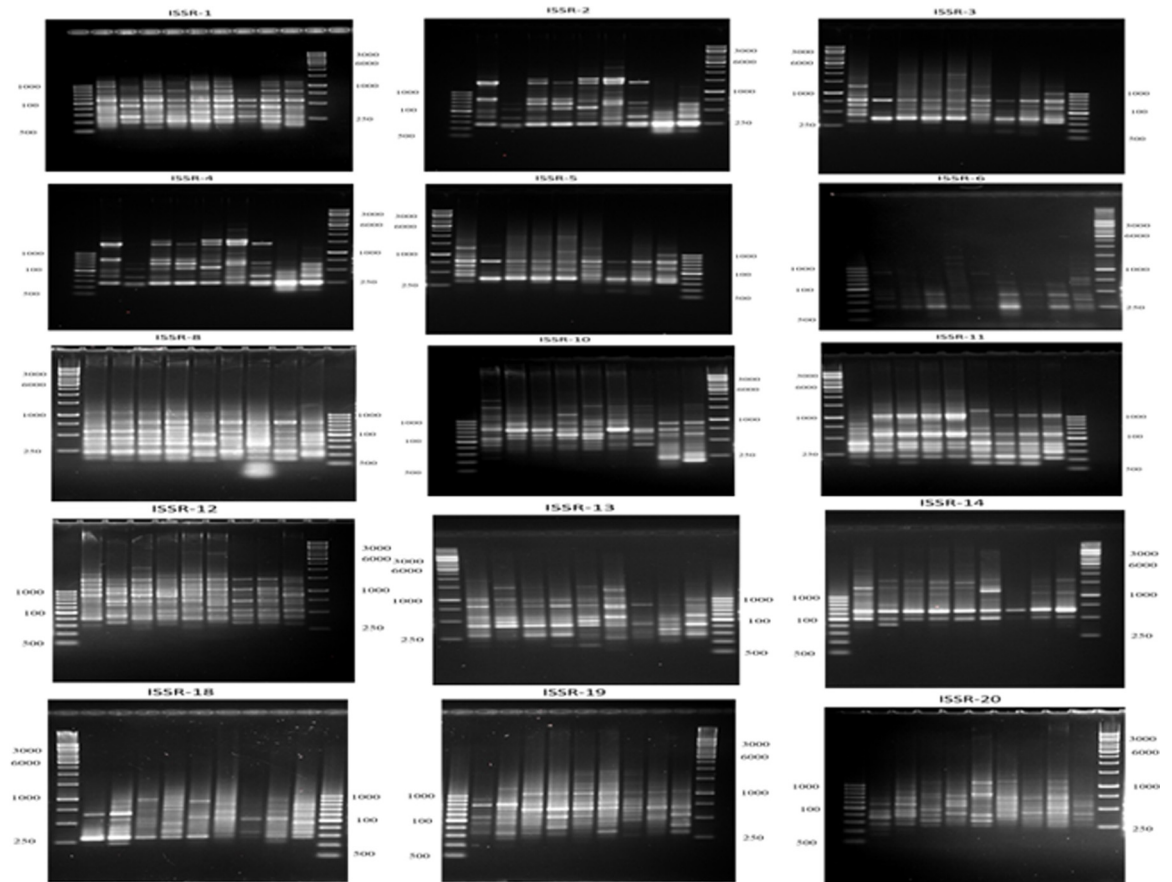


Fig 6. A representative agarose gel where PCR products of the 15 ISSR primers for the nine treatments.

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Table 6. Primer names, number of total bands, polymorphic bands, percentage of polymorphism and markers efficiency parameters of ISSR primers.

Primer Name	No. of Polymorphic Bands	No. of Monomorphic Bands	Total No. of bands	% of polymorphism	H	PIC	E	H.av	MI	D	R
ISSR-1	7	6	13	53.8	0.37	0.30	9.78	0.00	0.03	0.44	4.00
ISSR-2	15	2	17	88.2	0.50	0.37	8.56	0.00	0.03	0.75	10.44
ISSR-3	18	1	19	94.7	0.50	0.37	9.11	0.00	0.03	0.77	10.22
ISSR-4	15	1	16	93.8	0.50	0.37	7.44	0.00	0.03	0.79	9.56
ISSR-5	19	1	20	95.0	0.50	0.37	9.89	0.00	0.03	0.76	10.44
ISSR-6	16	2	18	88.9	0.47	0.36	6.67	0.00	0.02	0.86	7.56
ISSR-8	13	2	15	86.7	0.41	0.33	10.67	0.00	0.03	0.50	5.56
ISSR-10	18	3	21	85.7	0.49	0.37	11.89	0.00	0.03	0.68	8.22
ISSR-11	16	4	20	80.0	0.46	0.36	12.67	0.00	0.03	0.60	7.11
ISSR-12	18	3	21	85.7	0.49	0.37	12.00	0.00	0.03	0.67	8.00
ISSR-13	13	2	15	86.7	0.49	0.37	8.67	0.00	0.03	0.67	5.33
ISSR-14	10	2	12	83.3	0.49	0.37	6.67	0.00	0.03	0.69	4.67
ISSR-18	10	1	11	90.9	0.50	0.37	6.00	0.01	0.03	0.71	7.33
ISSR-19	15	2	17	88.2	0.47	0.36	10.44	0.00	0.03	0.62	7.78
ISSR-20	17	0	17	100.0	0.50	0.37	7.78	0.00	0.03	0.79	9.33
Total	220	32	252	252							
Average	14.66	2.13	16.8								

<https://doi.org/10.1371/journal.pone.0254285.t006>

Table 7. Genetic similarities between the nine treatments based on Jaccard's similarity coefficient based on ISSR primers data.

	CONTROL	C15	C50	T15	T50	Cn15	Cn50	Tn15	Tn50
CONTROL	100%	--	--	--	--	--	--	--	--
C15	49%	100%	--	--	--	--	--	--	--
C50	56%	50%	100%	--	--	--	--	--	--
T15	54%	51%	66%	100%	--	--	--	--	--
T50	54%	46%	65%	62%	100%	--	--	--	--
Cn15	58%	51%	65%	56%	60%	100%	--	--	--
Cn50	41%	47%	41%	44%	42%	52%	100%	--	--
Tn15	50%	54%	51%	50%	47%	55%	48%	100%	--
Tn50	46%	43%	52%	58%	44%	47%	37%	50%	100%

Symbols: C; Control, C15; chlorantraniliprole LC₁₅, C50; chlorantraniliprole LC₅₀, Cn15; nano-chlorantraniliprole LC₁₅, Cn50; Nano-chlorantraniliprole LC₅₀, T15; thiocyclam LC₁₅, T50; thiocyclam LC₅₀, Tn15; nano-thiocyclam LC₁₅ and Tn50; nano-thiocyclam LC₅₀.

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formulations may change the toxicity or residue activity of insecticides, and reducing its application frequency, according to the Environmental Protection Agency's recommendation [51]. In the present study, we investigated the effects of the lethal and sublethal concentrations of

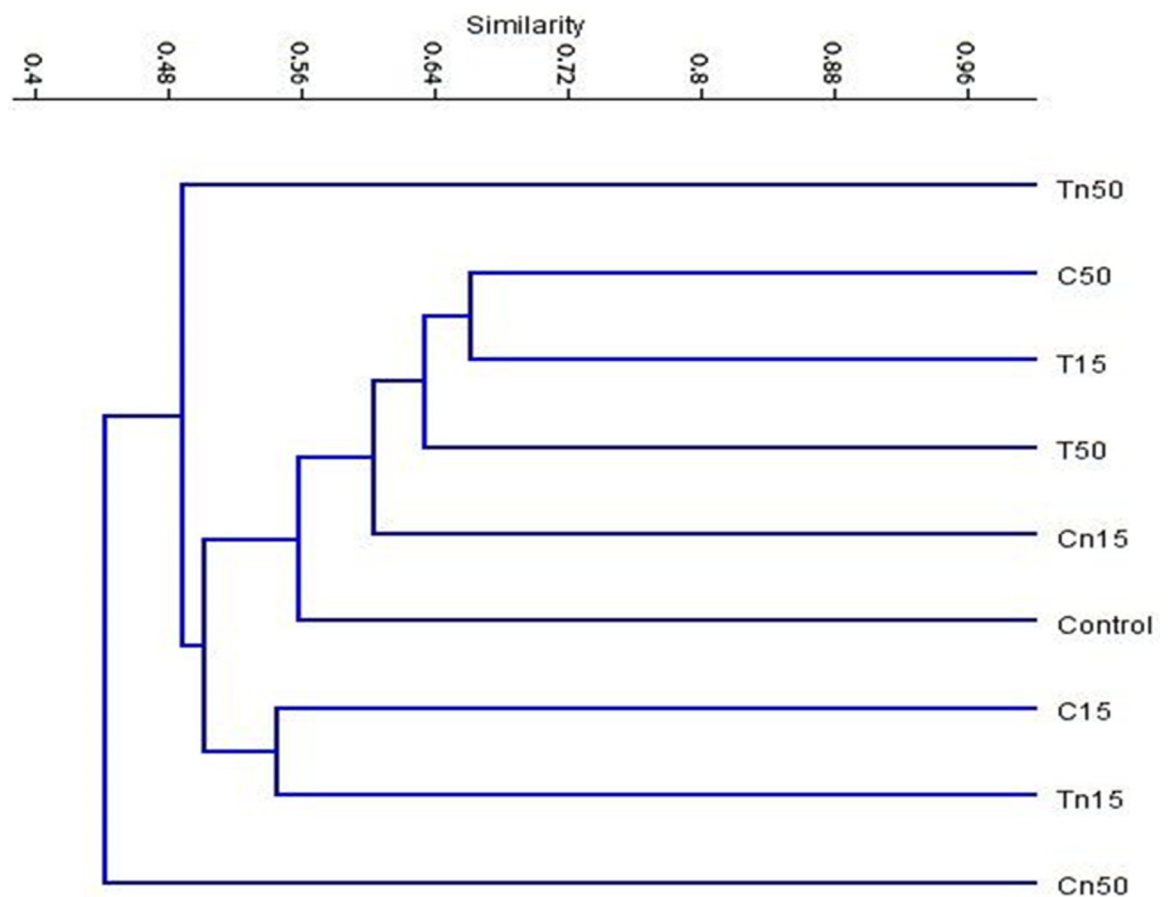


Fig 7. UPGMA cluster analysis based on Jaccard's similarity coefficient of ISSR analysis of the nine treatments: C; Control, C15; chlorantraniliprole LC₁₅, C50; chlorantraniliprole LC₅₀, Cn15; nano-chlorantraniliprole LC₁₅, Cn50; nano-chlorantraniliprole LC₅₀, T15; thiocyclam LC₁₅, T50; thiocyclam LC₅₀, Tn15; nano-thiocyclam LC₁₅ and Tn50; nano-thiocyclam LC₅₀.

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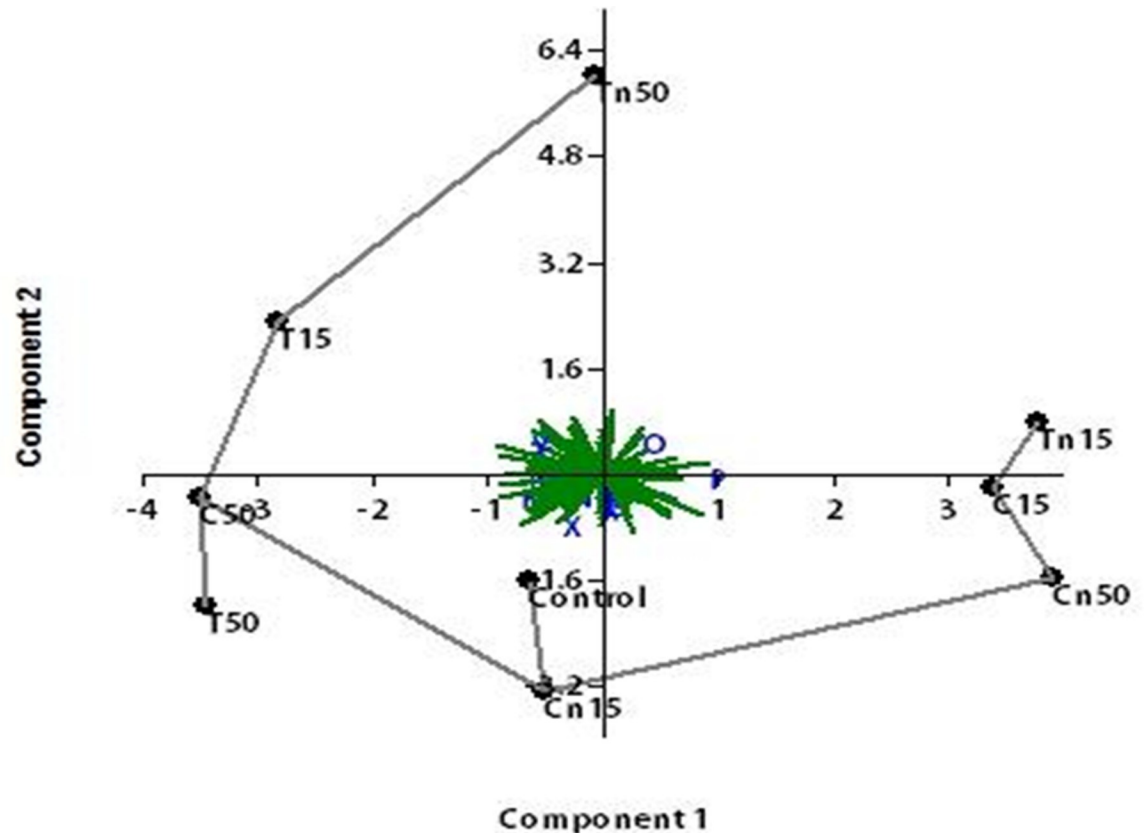


Fig 8. A representative agarose gel where PCR products of the 15 ISSR primers for the nine treatments.

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thiocyclam, chlorantraniliprole, and their nano-forms on *A. ipsilon* larval and pupal duration, larval mortality, adult emergence, reproductive activity, sex ratio, and oxidative stress enzymes with particular emphasis on DNA mutagenicity.

Chlorantraniliprole has a novel mode of action [52] that activates insect ryanodine receptors (RyRs), leading to paralysis and mortality in sensitive species [53]. Thiocyclam is a broad-spectrum nereistoxin antagonist that blocks the transmission of cholinergics [52], resulting in insect death. Our results revealed that *A. ipsilon* larvae were more susceptible to the nano-forms than the regular forms for both insecticides (Table 2). This might be due to (1) the nano-chlorine within chlorantraniliprole, since chlorine is a non-selective oxidant with a number of effects on the living biota (e.g., reacts with a variety of cellular components, deactivates enzymatic active sites, decreases the biological functions of proteins, and produces deleterious effects on DNA [54]. In some cases, different layers of protein present in insects, larvae or even spores provide protection against chemical attacks, including chlorine, but we speculate that nano-chlorine has a greater effect on the cytoplasmic membrane permeability, causing the loss of refractivity, separating the spore coats from the cortex, extensively discharging Ca^{+} , dipicolinic acid, and DNA, and finally causing lysis to occur, which can lead to cell death and growth inhibition [55]. The increased susceptibility to the nano-forms may also be due to (2) the nano-sulfur within thiocyclam, since sulfur destroys an insect's normal energy-producing bodily functions [56]. Normal pesticides containing sulfur have some caveats, because sulfur can damage plants during hot, dry weather and is incompatible with some other pesticides. In addition, sulfur should not be used on plants that have been sprayed with horticultural oils, as

the induced reaction can damage foliage. Sulfur is non-toxic to humans and animals, unless ingested [57].

Similarly, thiocyclam is a highly effective insecticide against the field strain [58,59] of *Tuta absoluta* (Meyrick) (9.98 mg/L). In contrast, *A. ipsilon* larvae were more susceptible to chlorantraniliprole and its nano-form than thiocyclam (Table 1). Likewise other lepidopteran pests, including *Helicoverpa armigera* (Hübner), *Spodoptera exigua* (Hübner), and *Spodoptera littoralis* (Boisd.) [31,60,61] are also susceptible to this insecticide. The chlorantraniliprole showed harmful activity toward the third instar larvae of *A. ipsilon* [62], whereas its LC₅₀ was 0.187 µg/g 72 h post-treatment. This variation in susceptibility to chemical insecticides could be due to nutrition type, size, and physiological status of the host [63].

Sublethal effects of insecticides could be considered a common toxicological phenomenon and represent a secondary effect of insecticide application [62,64]. This phenomenon may occur in insects due to exposure to degraded insecticide after its initial application on crops [31]. In our results, the developmental rates of the larvae and pupae were substantially extended (Table 2) after treating the 2nd instar larvae of *A. ipsilon* with the original form of both insecticides compared to their nano-forms. These results agree with He et al. [62], Lutz et al. [65], El-Dewy [66], and Han et al. [67], whom found that the development of *A. ipsilon*, *Plutella xylostella* (Linnaeus), *S. littoralis*, and *Spodoptera cosmioides* (Walker) larvae was prolonged by low concentrations of chlorantraniliprole. In this study, both insecticides and their nano-forms did not show any significant differences in other biological parameters except for the LC₅₀ of nano-thiocyclam, which exhibits a significant difference in pupation% and emergence%. Additionally, the nano-form of both insecticides at LC₅₀ showed a significant difference in the number of eggs laid per female and fertility.

In the last decade, pesticide-induced oxidative stress has been a focus of toxicological research as a possible toxicity mechanism that causes a final manifestation of a pro-oxidant and antioxidant defense mechanism imbalance [68]. Pesticide intoxication causes a derangement of different antioxidant mechanisms in various tissues [68]; however, exposure to sublethal insecticide concentrations can induce oxidative stress enzymes to increase (e.g., SOD, CAT, and GR) [69]. Our results indicate that the CAT activity was significantly increased in both the insecticides and their nano-forms. Significantly increased SOD activity was observed in the LC₅₀ of both insecticides and their nano-forms. Also, significantly higher GR activities were observed in nano-chlorantraniliprole and nano-thiocyclam. The antioxidant enzyme systems, such as CAT or SOD, play an essential role in detoxifying harmful agents to counter the damaging effects of ROS [70].

Similar to the elevated antioxidant levels, lipid peroxide activity was significantly increased in MDA, which is the main oxidation result from the peroxidation of unsaturated fatty acids. This represents the oxidative effect on different organisms [71,72] in the LC₅₀ of both insecticides and their nano-forms. We observed no significant differences between the insecticides and their nano-forms at the lowest tested concentration (LC₁₅), which reflects the protective effects of antioxidants in *A. ipsilon* larvae. The antioxidant system can reduce MDA levels, which contributes to the accumulation of active oxygen and inhibits antioxidant activity [73]. The accumulation of MDA can be reduced by antioxidant enzymes, such as SOD and CAT. Other studies have demonstrated that toxic oxygen metabolism can mediate lipid peroxidation [74].

The ISSR-PCR technique was used as an effective marker to investigate the genetic mutagenicity levels between the second instar larvae subjected to different insecticidal treatments (chlorantraniliprole, thiocyclam and their nano-forms) compared to the untreated control. One of the benefits of ISSR is that it performs a qualitative evaluation of DNA variability through the differences in amplification profiles [44]. Many studies reported that the exposure

of insects to particular insecticides at different concentration levels might lead to damages or changes in genomic DNA sequences (e.g., insertions, deletions, substitutions, or rearrangements), resulting in changes in the ISSR profile (e.g., the presence or absence of certain bands or variations of the band intensity) [25]. In addition, the possible occurrence of point mutations at the oligonucleotide annealing site may cause the absence of bands due to the loss of a priming site [75]. In this study, 15 ISSR primers were used to detect genetic mutagenicity levels and screen the degree of polymorphism between treated and untreated larvae. Furthermore, to establish the relationship between the insecticides, the ISSR study results were used to create a dendrogram. The results showed that the lowest mutagenic insecticidal effects on the insect DNA was observed in the LC₁₅ of nano-chlorantraniliprole treatment compared to the control. In contrast, the highest mutagenic effect was observed in the LC₅₀ of nano-chlorantraniliprole, followed by the LC₅₀ of nano-thiocyclam compared to the control. The PCA analysis of ISSR data revealed a consistent result to that obtained by the dendrogram topology. Moreover, it was found that the LC₁₅ of nano-chlorantraniliprole and the LC₅₀ of thiocyclam exhibited almost the same mutagenic effects as the control.

It is expected that the polymorphic differences observed in the ISSR patterns may be attributed to changes in primer binding sites or DNA structures, or to DNA damage caused by insecticide exposure. In addition, it could also be due to the blocking of DNA replication, the presence of a large number of chromosomal lesions, such as large rearrangements (e.g., deletion, inversion, or translocation), or un-repaired DNA damage due to direct exposure to different insecticidal treatments [76]. The ISSR studies demonstrated their ability and effectiveness to detect DNA damage and changes caused by the studied insecticides.

Conclusion

In summary, we developed insecticide nanometerization to improve the biological activity of conventional insecticides. Our obtained results represent a promising step toward developing safe and efficient nano-insecticides. Further investigations are still needed to determine their effects on the environment. To the best of our knowledge, this research is a pioneer case-study that examined and analyzed the genome-wide DNA mutability, biochemical effects, and toxicity levels of chlorantraniliprole, thiocyclam, and their nano-forms for the control of *A. ipsilon*.

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