Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

Nano-Insecticides Against the Black Cutworm *Agrotis ipsilon* (Lepidoptera: Noctuidae): Toxicity, Development, Enzyme activity, and DNA Mutagenicity

Mona Awad¹¶, El-Desoky S. Ibrahim¹, Engy I. Osman², Wael H. Elmenofy³, Abdel Wahab M. Mahmoud⁴, Mohamed A. M. Atia^{5*}, Moataz A. M. Moustafa^{1¶*}

¹ Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt.

² Department of Genetics, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt.

³ Agricultural Genetic Engineering Research Institute, ARC, Giza 12619, Egypt.

⁴ Plant Physiology Section, Botany Department, Faculty of Agriculture, Cairo University, 12613, Giza, Egypt.

⁵ Molecular Genetics and Genome Mapping Laboratory, Genome Mapping Department, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, 12619, Egypt.

***Corresponding authors**

¹ Moataz A. M. Moustafa. E-mail: moataz.moustafa79@gmail.com

² Mohamed A. M. Atia. E-mail: matia@ageri.sci.eg

¶ These authors contributed equally to this work.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

Abstract

High-frequency doses of chemical pesticides cause environmental pollution with high pesticide residues. 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 nanoinsecticides 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 at the molecular level of the polyphagous species of black cutworm *Agrotis ipsilon*. 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. The positive results obtained here have led us to apply these nano-forms in different insect models in additional studies.

KEYWORDS

Nanoinsecticides, toxicity, reproductive, enzymes, DNA mutagenicity, *Agrotis ipsilon*

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

Introduction

The black cutworm, *Agrotis ipsilon* (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 for insect pest control. 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]. Nanopesticides 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 nanopesticides raises questions about their fate, toxicity, and biodegradation [10], as well as how to assess the environmental risk of these materials.

The introduction of nanoinsecticides into the environment necessitates the careful identification of their potential. Generally, insecticide studies focus on evaluating the lethal and sublethal toxicity of insecticides 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

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 combat the adverse effects of ROS on cells. Several defense mechanisms have been developed by insects and include enzymatic and non-enzymatic components [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

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 nanoinsecticides 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 in a rearing room at 26 ± 1 °C, $65 \pm 5\%$ relative humidity under a reversed 16 L:8 D. The newly hatched larvae were kept in a clean glass jar (1 L) and provided castor oil leaves daily until the third instar larvae emerged. They were then transferred to larger, 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 till pupation occurred. The emerged moths were supplied with a 10% sugar solution as supplement dietary [11].

Insecticides and chemicals

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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.

Nano-insecticides preparation and size measurements

Nano-chlorine (chlorantraniliprole) and nano-sulfur (thiocyclam) were prepared by [28,29,30], with some modifications. Hydrochloric acid (HCl) and orthorhombic brava is 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. The obtained precipitation was filtered and washed thoroughly with deionized water, then the suspension solution was added to 16 ml of NaCl 0.2 M aqueous solution using forceful moving at a steady rate for 40 min. The reaction fusion was stirred for an additional 3 hours at ambient temperature. The precipitate was mixed with orthorhombic brava is w:w (2:1) in the presence of HCl 90%, then centrifuged at 1500 rpm for 30 min. Next, the solution was cooled in an ice bath, and then subsequently exposed to 1.5 psi pressure continuously for 6 hours.

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. 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. The precipitation was mixed with oxalic acid 1 M and trimethylammoniumbromide compound solution (molar ratio 1:3) under slow stirring at

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

33 °C for 6 hours. Afterwards, drop benzene sulphonate (molar ratio 2:3) was added, and the resulting 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 continuously for 3 days. The final nano-suspension was prepared in deionized water and left on a shaker for 2 days at 20 °C.

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, and water was used to control the larvae. Treated and untreated castor bean leaves were transferred after drying into a glass jar (0.25 L), and 10 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 *A. ipsilon* development

The sublethal and lethal concentrations (LC₁₅ and LC₅₀) for each form of chlorantraniliprole and thiocyclam were used 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 developmental 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%.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 sterilize 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 [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).

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

197

198 **Molecular analysis**

199 **DNA extraction**

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

205 **ISSR polymorphism analysis**

206 For ISSR PCR amplification [38,39], a set of 15 ISSR primers were applied against the 9
207 treatments. The PCR was carried out in a total volume of 25 μl containing the following
208 components: 25 ng genomic DNA; 1X PCR buffer; 1.5 mM MgCl₂; 0.25 mM of each dNTPs;
209 1 μM of each primer; 1 U Go-Taq Flexi polymerase (Promega).

210 Thermocycling amplification was performed with a GeneAmp PCR system 9700 (Applied
211 Biosystem, Inc.). The amplification was programmed at 94 °C for 5 min for the initial
212 denaturation cycle, followed by 35 cycles with each cycle comprised of (94 °C for 1 min,
213 50 °C for 1 min, then 72 °C for 90 s) and a final extension at 72 °C for 7 min. The produced
214 PCR amplicons were electrophoresed using 1.5% agarose gel. A 100 bp plus DNA ladder and
215 1 kb were used as molecular size standards. PCR products were photographed using a Gel
216 Doc™ XR+ System (Bio-Rad®).

217

218 **Data analysis**

219 **Biological and biochemical data analyses**

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 multidimensional 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].

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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

Fig 1. Nano-chlorine

Fig 2. Nano-sulphur

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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	

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 LC₅₀ values were 0.058 and 9.20 mg/l for chlorantraniliprole 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).

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
Chlorantraniliprole	0.001 (0.000-0.003)	0.058 (0.010-0.159)	8.21 (2.17-141.89)	0.59 ± 0.12
Nano-Chlorantraniliprole	0.001 (0.000-0.003)	0.015 (0.006-0.038)	2.65 (0.57-48.27)	0.56 ± 0.09
Thiocyclam	0.052 (0.000-0.426)	9.209 (2.03-34.10)	1622.48 (233.63-38982.06)	0.57 ± 0.15
Nano-Thiocyclam	0.44 (0.11-0.89)	4.46 (2.87-6.78)	45.14 (23.29-154.65)	1.27 ± 0.21

^aLC₁₅: concentration causing 15% mortality

^aLC₅₀: concentration causing 50% mortality

^bLC₉₀: concentration causing 90% mortality

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 till the emergence) due to LC₁₅ and LC₅₀ exposure. The results showed elongation of the larvae developmental period under all LC₅₀treatments, but only a slight significance in the pupal stage duration. 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). Low significant differences were found in the female pupal weight under all treatments. No differences were found in the male pupal weight, sex ratio, or emergence% under all treatments (Table 3).

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

Treatments		*Larval duration	Pupation%	**Pupal duration	Pupal weight (mg)		Sex ratio		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

310 Values marked with the same letters are not significantly different ($P > 0.05$: Dunnett's multiple comparisons test).

311 * number of days from 2nd instar larvae till pupation

312 ** number of days from the pupation till the emergence

313

314

315

316

317

318

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 84.95, and 81.29% at LC₁₅, and 73.83, and 77.74% 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 (fecundity) showed no significant differences between treated and untreated larvae (Table 4).

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

Treatments		*Fecundity	**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

Values marked with the same letters are not significantly different (P > 0.05: Dunnett's multiple comparisons test)

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

**Fertility is calculated by counting of the emerged larvae from collected eggs batch.

Activity of oxidative stress enzymes

Table 5 shows that 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)

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

and nano-thiocyclam (43.0 U/g of protein). The SOD activity after treatment with the LC₅₀ of chlorantraniliprole, and nano-chlorantraniliprole were 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, respectively). The highest significance was observed for the LC₅₀ of chlorantraniliprole and nano-chlorantraniliprole, and the LC₅₀ of thiocyclam (ca. 5.56, 5.54, and 5.0-fold, respectively).

An increase in lipid peroxidase activity was observed under exposure to the LC₅₀ of nano-chlorantraniliprole with 3.75 nmol/g of protein (ca. 16.30-fold). Meanwhile, the lipid peroxidase activity after treatment with the LC₅₀ of chlorantraniliprole, thiocyclam, and nano-thiocyclam were slightly significantly 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).

Table 5 shows that exposure to all the investigated insecticides and their nano-forms resulted in the significant stimulation of GR activity in the LC₁₅ and LC₅₀ of chlorantraniliprole (11.4 and 11.76 U/g of protein). GR activity was significantly high under the LC₁₅ and LC₅₀ nano-chlorantraniliprole treatments at 8.84 and 10.09-fold, respectively and under the LC₁₅ and LC₅₀ nano-thiocyclam treatments at 8.92, and 10.15-fold, respectively. No significant changes in enzyme activity was observed for the LC₁₅ and LC₅₀ thiocyclam treatments (3.73 and 6.41 U/g of protein) compared to the control.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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.

		Mean $\pm$ SE			
Treatments		SOD	CAT	Glutathione reductase	Lipid peroxidase
		U/g of protein	U/g of protein	U/g of protein	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

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 may have too few clusters. Meanwhile, two-dimensional heatmap plotting

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 inter relationships 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.

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.

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.

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

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

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 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).

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

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								

Table 7. Genetic similarities between the nine treatments based on Jaccard's similarity coefficient based on ISSR primers data. Symbols: C; Control, C15; chlorantraniliprole LC₁₅, C50; chlorantraniliprole LC₅₀, Cn15; nano-chlorantraniliprole LC₁₅, Cn50; Nano-chlorantraniliprole LC₁₅, T15; thiocyclam LC₁₅, T50; thiocyclam LC₅₀, Tn15; nano-thiocyclam LC₁₅ and Tn50; nano-thiocyclam LC₅₀.

	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%

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

Analysis of molecular phylogeny

A dendrogram based on the UPGMA cluster analyses of the ISSR data were 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).

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.

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

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-formulations may change the nature of chemical pesticides by increasing the efficiency of

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

insect pest control and reducing pesticide application frequency, per the Environmental Protection Agency's recommendation [51]. This technology is still insufficient and requires a better understanding of its mechanisms prior to field use. We investigated the effects of the lethal and sublethal concentrations of thiocyclam, chlorantraniliprole, and their nano-forms on *A. ipsilon* larval and pupal duration, larvae mortality, adult emergence, reproductive, sex ratio, and oxidative stress enzymes with particular emphasis on DNA mutagenicity.

Chlorantraniliprole (Coragen®) has a novel mode of action [52] that activates insect ryanodine receptors (RyRs), leading to paralysis and mortality in sensitive species [53]. Thiocyclam (Evisect-S®) 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 provides 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

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

reaction can damage foliage. Sulfur is non-toxic to humans and animals, unless ingested [57]. The nano-form of sulfur does not have the same caveats (at least currently); hence, there is no discrepancy or caveats regarding its application.

Similarly, thiocyclam is a highly effective insecticide against the field strain [58] of *Tuta absoluta* (9.98 mg/L). Likewise, the thiocyclam [59] had the highest efficiency in larvae mortality in field experiments in Iran. In contrast, *A. ipsilon* larvae were more susceptible to chlorantraniliprole and its nano-form than thiocyclam (Table 1). Other lepidopteran pests, including *Helicoverpa armigera*, *Spodoptera exigua*, and *Spodoptera littoralis* [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. Generally, pest susceptibility to chemical insecticides is affected by various factors, including nutrition type, size, and physiological status of the host [63].

Sublethal effects of insecticides could be considered common toxicological phenomena 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]. When second instar larvae of *A. ipsilon* are exposed to sublethal concentrations of chlorantraniliprole, thiocyclam, and their nano-forms, the developmental rates of the larvae and pupae were substantially extended (Table 2) in the original form of both insecticides compared to their nano-forms. These results agree with [62], who found that the development of *A. ipsilon* larvae was prolonged by low concentrations of chlorantraniliprole. Chlorantraniliprole has also been shown to prolong the development times of some lepidopteran insect pests, including; *Plutella xylostella*, *S. littoralis*, and *Spodoptera cosmioides* [65,66,67]. In this study, both insecticides and their nano-forms did not show any significant differences in other biological parameters, including pupal weight, pupation%, and emergence%, except for the LC₅₀ of nano-thiocyclam, which exhibits a significant

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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. Several studies have also shown that low concentrations of insecticides may affect the reproductive activity of insects (e.g., *Helicoverpa assulta*, *Mamestra brassica*, and *S. littoralis* [31,68,33]).

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 [69]. Pesticide intoxication causes a derangement of different antioxidant mechanisms in various tissues [69]; however, exposure to sublethal insecticide concentrations can induce oxidative stress enzymes to increase (e.g., SOD, CAT, and GR) [70]. 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 [71].

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 [72,73] 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 [74]. The accumulation of MDA can be reduced by antioxidant enzymes, such as

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

SOD and CAT. Other studies have demonstrated that toxic oxygen metabolism can mediate lipid peroxidation [75].

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 control (untreated). 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 [76]. 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 nano-the LC₁₅ of chlorantraniliprole compared to the control. In contrast, the most aggressive (highest) mutagenic effect was observed in the LC₅₀ of nano-chlorantraniliprole, followed by the LC₅₀ of nano-thiocyclam LC₅₀ compared to the control. It was elucidated that the PCA analysis of ISSR data revealed a consistent result to the 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

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

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 [77]. 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 nanoinsecticides. 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*.

Acknowledgments

Authors are thankful for the Egyptian Knowledge Bank (EKB) for improving the manuscript considerably, including English language and grammar.

References

1. Binning RR, Coats J, Kong X, Hellmich RL. Susceptibility to *Bt* proteins is not required for *Agrotis ipsilon* aversion to *Bt* maize. Pest Manag Sci. 2015; 71: 601–606.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

2. Abd El-Aziz SE, Omer EA, Sabra AS. Chemical composition of *Ocimum americanum* essential oil and its biological effects against, *Agrotis ipsilon*, (Lepidoptera: Noctuidae). Res J Agric Boil Sci. 2007; 3: 740–747.
3. Amin AH, Bayoumi AE, Dimetry AZ, Youssef DA Efficiency of Nano-formulations of neem and peppermint oils on the bionomics and enzymatic activities of *Agrotis ipsilon* larvae (Lepidoptera: Noctuidae). J Nat Resou. 2019; 4: 102.
4. Guedes C, Siqueira A. The tomato borer *Tuta absoluta*: insecticide resistance and control failure. Plant Sci Rev. 2012; 7: 1–7.
5. Kah M. Nanopesticides and nanofertilizers: emerging contaminants or opportunities for risk mitigation?. Front Chem. 2015; 3: 64.
6. Worrall EA, Hamid A, Mody KT, Mitter N, Pappu HR. Nanotechnology for plant disease management. J Agron. 2018; 8: 285.
7. Yan S, Cheng WY, Han ZH, Wang D, Yin MZ, Du XG, Shen J. Nanometerization of thiamethoxam by a cationic star polymer nanocarrier efficiently enhances the contact and plant-uptake dependent stomach toxicity against green peach aphids. Pest Manag Sci. 2021; 77: 1954–1962.
8. Kah M, Hofmann T. Nanopesticide research: current trends and future priorities. Environ Inter. 2014; 63: 224–235.
9. Pérez-de-Luque A, Rubiales D. Nanotechnology for parasitic plant control. Pest Manag Sci. 2009; 65: 540–545.
10. Chaturvedi M, Molino Y, Sreedhar B, Khrestchatisky M, Kaczmarek L. Tissue inhibitor of matrix metalloproteinases-1 loaded poly (lactic-co-glycolic acid) nanoparticles for delivery across the blood-brain barrier. Int J Nanomedicine. 2014; 9: 575–588.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

- 608 11. Kandil MA, Abdel-kerim RN, Moustafa MAM. Lethal and sublethal effects of bio-and
609 chemical insecticides on the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera:
610 Gelechiidae). Egypt J Biol Pest Control. 2020; 30: 1–7.
- 611 12. Kodrík D, Bednářová A, Zemanová M, Krishnan N. Hormonal regulation of response to
612 oxidative stress in insects. Inter J of Mol Sci. 2015; 16: 25788–25816.
- 613 13. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide
614 and survival. J Cell Physiol. 2002; 192: 1–15.
- 615 14. Felton GW, Summers CB. Antioxidant systems in insects. Arch Insect Biochem Physiol.
616 1995; 29: 187–197.
- 617 15. Rudnev II. Antioxidant system of black sea animals in early development. Comp
618 Biochem Physiol. 1999; 122: 265–271.
- 619 16. Weirich GF, Collins AM, Williams VP. Antioxidant enzymes in the honey bee, *Apis*
620 *mellifera*. Apidologie. 2002; 33: 3–14.
- 621 17. Afiyanti M, Che H-J. Gatalase activity is modulated by calcium and calmodulin in
622 detached mature leaves of sweet potato. J Plant Physiol. 2014; 171: 35–47.
- 623 18. Yoshida K, Kaothien P, Matsui T, Kawaoka A, Shinmyo A. Molecular biology and
624 application of plant peroxidase genes. Appl Microbiol Biotechnol. 2003; 60: 665–670.
- 625 19. Couto N, wood J, Barder J. The role of glutathione reductase and related enzymes on
626 cellular redox homoeostasis network. Free Radic Biol Med. 2016; 95: 27– 42.
- 627 20. Board PG, Menon D. Glutathione transferases, regulators of cellular metabolism and
628 physiology. Biochim Biophys Acta Gen Subj. 2013; 1830: 3267–3288.
- 629 21. Parsons BJ, Newbury HJ, Jackson MT, Ford-Lloyd BV. Contrasting genetic diversity
630 relationships are revealed in rice (*Oryza sativa* L.) using different marker types. Mol Breed
631 1997; 3: 115–125.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

22. Van Droogenbroeck B, Kyndt T, Maertens I, Romeijn-Peeters E, Scheldeman X, Romero-Motochi JP, et al. Phylogenetic analysis of the highland papayas (*Vasconcellea*) and allied genera (*Caricaceae*) using PCR-RFLP. *Theor Appl Genet.* 2004; 108: 1473–1486.
23. Joshi P, Dhawan V. Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. *Biol. Plant.* 2007, 5, 22–26.
24. Perez de Castro A, Blanca JM, Diez MJ, Vinals FN. Identification of a CAPS marker tightly linked to the tomato leaf curl disease resistance gene Ty-1 in tomato. *Eur J Plant Pathol.* 2007; 117: 347–356.
25. Lindroth EJ. Population genetics of the western bean cutworm (*Striacosta albicosta* Smith) across the United States. *Ann Entomol Soc Am.* 2011; 105: 685–692.
26. Sharma K, Agrawal V, Gupta S, Kumar R, Prasad M. ISSR marker-assisted selection of male and female plants in a promising dioecious crop: jojoba (*Simmondsia chinensis*). *Plant Biotechnol Rep.* 2008; 2: 239–243.
27. Hundsdoerfer AK, Wink M. New source of genetic polymorphisms in Lepidoptera. *Z Naturforsch.* 2005; 60 c: 618–624.
28. De Oliveira JL, Campos EnVR, Gonçalves da Silva CM, Pasquoto T, Lima R, Fraceto LF. Solid lipid nanoparticles co-loaded with simazine and atrazine: Preparation, characterization, and evaluation of herbicidal activity. *J Agric Food Chem.* 2015; 63: 422–432.
29. Cota-Arriola O, Onofre Cortez-Rocha M, Burgos-Hernández A, Marina Ezquerro-Brauer J, Plascencia-Jatomea M. Controlled release matrices and micro/nanoparticles of chitosan with antimicrobial potential: Development of new strategies for microbial control in agriculture. *J Sci Food Agric.* 2013; 93: 1525–1536.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

- 655 30. Xu ZP, Stevenson GS, Lu C-Q, Lu G-Q, Bartlett PF, Gray PP. Stable suspension of
656 layered double hydroxide nanoparticles in aqueous solution. J Am Chem. Soc. 2006; 128:
657 36–37.
- 658 31. Moustafa MAM, Fouad EA, Abdel-Mobdy Y, Hamow KÁ, Mikó Z, Molnár BP, et al.
659 Toxicity and sublethal effects of chlorantraniliprole and indoxacarb on
660 *Spodopteralittoralis*(Lepidoptera: Noctuidae). Appl Entomol Zool. 2021; 56: 115–124.
- 661 32. Hamada HM, Awad M, EL-Hefny M, Moustafa MMA. Insecticidal activity of garlic
662 (*Allium sativum*) and ginger (*Zingiber officinale*) oils on the cotton leafworm, *Spodoptera*
663 *littoralis* (Boisd) (Lepidoptera: Noctuidae). Afr Entomol 2018; 26: 84–94.
- 664 33. Moustafa MMA, Kákai Á, Awad M, Fónagy A. Sublethal effects of spinosad and
665 emamectin benzoate on larval development and reproductive activities of the cabbage moth,
666 *Mamestra brassicae* L. (Lepidoptera: Noctuidae). Crop Prot. 2016; 90: 197–204.
- 667 34. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine
668 and a simple assay for superoxide dismutase. J Biol Chem. 1972; 247: 3170–3175.
- 669 35. Aebi H. Catalase in vitro. Meth Enzymol 1984; 105: 121–126.
- 670 36. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by
671 thiobarbituric acid reaction. Anal Biochem.1979; 95: 351–358.
- 672 37. Goldberg DM, Spooner RJ. Glutathione reductase. Meth Enzymol. 1983; 3: 258–265.
- 673 38. Atia MA, Sakr MM, Mokhtar MM, Adawy SS. Development of sex-specific PCR-based
674 markers in date palm. Methods Mol Biol. 2017a; 1638: 227–244.
- 675 39. Atia MA, Sakr MM, Adawy SS. Assessing date palm genetic diversity using different
676 molecular markers. Methods Mol Biol. 2017b; 1638: 125–142 .
- 677 40. Rousseeuw PJ. Silhouettes: a graphical aid to the interpretation and validation of cluster
678 analysis. J. Comput. Appl. Math. 1987; 20: 53–65.
- 679 41. Wickelmaier F. An introduction to MDS. SQRU. 2003; 46: 1–26.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

- 680 42. Abouseadaa HH, Atia MA, Younis IY, Issa MY, Ashour HA, Saleh I, et al. Gene-targeted
681 molecular phylogeny, phytochemical profiling, and antioxidant activity of nine species
682 belonging to the family Cactaceae. Saudi Boil Sci. 2020; 27: 1649–58.
- 683 43. Jaccard, P. Nouvelles recherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. 1908
684 , 44, 223–70.
- 685 44. Atia MA, Osman GH, Elmenofy WH. Genome-wide in silico analysis, characterization
686 and identification of microsatellites in *Spodoptera littoralis* multiple nucleopolyhedrovirus
687 (SpliMNPV). Sci Rep. 2016; 6: 1–9.
- 688 45. Liu BH. Statistical genomics: linkage, mapping, and QTL analysis. CRC press. 2017.
- 689 46. Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in
690 man using restriction fragment length polymorphisms. Am J Hum Genet. 1980; 32: 314.
- 691 47. Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, et al. The comparison
692 of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol
693 Breed. 1996; 2: 225–38.
- 694 48. Tessier C, David J, This P, Boursiquot JM, Charrier A. Optimization of the choice of
695 molecular markers for varietal identification in *Vitis vinifera* L. Theor Appl Genet. 1999; 98:
696 171–177
- 697 49. Prevost A, Wilkinson MJ. A new system of comparing PCR primers applied to ISSR
698 fingerprinting of potato cultivars. Theor Appl Genet 1999; 98: 107–12
- 699 50. Rodriguez-Saona L, Giusti MM, Shotts M. Advances in infrared spectroscopy for food
700 authenticity testing. Advances in Food Authenticity Testing, 2016; 71-116.
- 701 51. Gopal M, Kumar R, Goswami A. Nano-pesticides - A recent approach for pest control. J
702 Plant Prot Sci. 2012; 4: 1–7.
- 703 52. Insecticide Resistance Action Committee. IRAC Mode of Action Classification Scheme.
704 IRAC. 2020; v 9.4 .

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

53. Cordova D, Benner EA, Sacher MD, Rauh JJ, Sopa JS, Lahm GP, et al. Anthranilicdiamides: A new class of insecticides with a novel mode of action, ryanodine receptor activation. *Pestic Biochem Physiol.* 2006; 84: 196–214A.
54. Lihl C, Heckel B, Grzybkowska A, Dybala-Defratyka A, Ponsin V, Torrentó C, et al. Compound-specific chlorine isotope fractionation in biodegradation of atrazine. *Environmental Science: Processes & Impacts.* 2020; 3: 792–801.
55. Camargo JA. (1991). Toxic effects of residual chlorine on larvae of *Hydropsyche pellucidula* (Trichoptera, Hydropsychidae): A proposal of biological indicator. *Bull Environ Contam Toxicol.* 1991; 47: 261–265.
56. Institute of Medicine of the National Academies. Chapter 7: Dietary Reference Intakes for Water, Potassium, Sodium, Chloride, and Sulfate; National Academies Press: Washington, DC. 2005
57. UConn Home and Garden Education Center. (<http://www.ladybug.uconn.edu/FactSheets/insecticides--low-toxicity-options.php>). 2017.
58. Radwan EM, Taha HS. Efficacy of certain pesticides against larvae of Tomato Leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Egypt Acad J Boil Sci.* 2017; 9: 81–95.
59. Hosseinzadeh A, Aramideh S, Ghassemi-Kahrizesh A. Efficacy of bio-insecticides on *Tuta absoluta* (Meyrick) (Lep.: Gelechiidae) in laboratory and field conditions. *Agric Eng Int.* 2019; 21: 164–170.
60. Lai T, Su JY. Effects of chlorantraniliprole on development and reproduction of beet armyworm, *Spodoptera exigua* (Hübner). *J Pest Sci* 2011; 84: 381–386.
61. Cao GC, Lu Q, Zhang L, Guo F, Liang G, Wu K, et al. Toxicity of chlorantraniliprole to Cry1Ac-susceptible and resistant strains of *Helicoverpa armigera*. *Pestic Biochem Physiol.* 2010; 98: 99–103.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

- 729 62. He F, Shiang S, Haili T, Xiao S, Chao Q, Shoumin J, et al. Chlorantraniliprole against the
730 black cutworm *Agrotis ipsilon* (Lepidoptera: Noctuidae): from biochemical/ physiological to
731 demographic responses. Sci Rep. 2019; 9: 1–17.
- 732 63. Yin X-H, Wu Q-J, Li X-F, Zhang Y-J, Xu B-Y. Sublethal effects of spinosad on *Plutella*
733 *xylostella* (Lepidoptera: Yponomeutidae). Crop Prot. 2008; 27: 1385–1391.
- 734 64. Wang P, Zhou L-L, Yang F, Li M, Liu X-M, Wang Y, et al. Sublethal effects of
735 thiamethoxam on the demographic parameters of *Myzus persicae* (Hemiptera: Aphididae). J
736 Econ Entomol. 2017; 110: 1750–1754.
- 737 65. Lutz AL, Bertokaccini I, Scotta RR, Curis MC, Favaro MA, Fernandez LN, et al. Lethal
738 and sublethal effects of chlorantraniliprole on *Spodoptera cosmioides* (Lepidoptera:
739 Noctuidae). Pest Manag Sci. 2018; 74: 2817–2821.
- 740 66. El-Dewy MEH. Influence of some novel insecticides on physiological and biological
741 aspects of *Spodoptera littoralis* (Boisduval). Alex Sci Exchange J. 2017; 38: 250–258.
- 742 67. Han WS, Zhang S, Shen F, Liu M, Ren C, Gao X. Residual toxicity and sublethal effects
743 of chlorantraniliprole on *Plutella xylostella* (Lepidoptera: Plutellidae). Pest Manag Sci. 2012;
744 68: 1184–1190.
- 745 68. Dong JF, Wang K, Li Y, Wang SL. Lethal and sublethal effects of cyantraniliprole on
746 *Helicoverpa assulta* (Lepidoptera: Noctuidae). Pestic Biochem Physiol. 2017; 136: 58–63.
- 747 69. Banerjee BD, Seth V, Ahmed RS. Pesticide-induced oxidative stress: perspectives and
748 trends. Rev Environ Health. 2001; 16: 1–36.
- 749 70. Afolabi OK, Aderibigbe FA, Folarin DT, Arinola A, Wusu AD. Oxidative stress and
750 inflammation following sub-lethal oral exposure of cypermethrin in rats: Mitigating potential
751 of epicatechin. Heliyon. 2019; 5: 125–134.

Effects of chlorantraniliprole and thiocyclam and their nano-form on *Agrotis ipsilon*

- 752 71. Bednářová A, Kodrík D, Krishnan N. Adipokinetic hormone exerts its anti-oxidative
753 effects using a conserved signal-transduction mechanism involving both PKC and cAMP by
754 mobilizing extra- and intracellular Ca²⁺ stores. Comp Biochem Physiol. 2013; 158: 142–149.
- 755 72. Valavanidis T, Vlahogianni M, Dassenakis M. Scoullos Molecular biomarkers of
756 oxidative stress in aquatic organisms in relation to toxic environmental pollutants Ecotoxicol
757 Environ Saf. 2006; 64: 178–189.
- 758 73. Draper HH, Squires EJ, Mahmooch H, Wu S, Agarwal M, Handley A. Comparative
759 evaluation of thiobarbituric acid methods for the determination of malondialdehyde in
760 biological materials. Free Radic Biol Med. 1993; 15: 353–363.
- 761 74. Wu GC, Bornman JE, Bennett SJ, Clarke MW, Fang ZX, Johnson SK. Individual
762 polyphenolic profiles and antioxidant activity in sorghum grains are influenced by very low
763 and high solar UV radiation and genotype. J Cereal Sci. 2017; 77: 17–23.
- 764 75. Zhang QM, Zhu LS, Wang J, Xie H, Wang JH, Han YN, et al. Oxidative stress and lipid
765 peroxidation in the earthworm *Eisenia fetida* induced by low doses of fomesafen. Environ Sci
766 Pollut Res. 2013; 20: 201–208.
- 767 76. Loewe L, Hill WG. The population genetics of mutations: good, bad and in different.
768 Philos T R Soc B. 2010; 365: 1153–1167.
- 769 77. Michod RE. Eros and Evolution. A Natural Philosophy of Sex, Addison-Wesley
770 Publishing, USA.1995.

Fig 1

Fig. 2

Fig. 3

Fig. 4

File #

53-6

Fig. 7

Fig 8

Figure