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Effects of different insecticides on the antioxidative defense system of the European Corn Borer (*Ostrinia nubilalis* Hübner) (Lepidoptera: Crambidae) larvae

Filip Franeta[1], Dejan Mirčić[2,*], Dajana Todorović[4], Željko Milovac[1], Nihat Granica[2], Saša Obradović[3] and Vesna Perić-Mataruga[4]

[1] *Institute of Field and Vegetable Crops*, 21000 Novi Sad, Serbia

[2] *State University of Novi Pazar, Department of Biomedical Sciences*, Vuka Karadžića bb, 36300 Novi Pazar, Serbia

[3] *State University of Novi Pazar, Department of Chemical and Technological Sciences*, Vuka Karadžića bb, 36300 Novi Pazar, Serbia

[2] *Institute for Biological Research "Siniša Stanković", University of Belgrade*, 142 Bulevar despota Stefana, 11060 Belgrade, Serbia

***Corresponding author**: dmircic@np.ac.rs

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Abstract: The European corn borer (*Ostrinia nubilalis*) is one of the most important insect pests of maize, and has a significant impact on the production of this crop. In this work, we examined the effects of different insecticides on the antioxidative defense system of *O. nubilalis* larvae. The experimental setup consisted of a completely randomized block design with 4 replicates. Four experimental groups were formed as follows: control (C), indoxacarb (250 mL ha^{-1}), chlorantraniliprole (100 mL ha^{-1}) and the chlorantraniliprole+lambda cyhalothrin (200 mL ha^{-1}) group. Larvae from maize stems were collected 20 days after insecticide application and the whole larvae were homogenized. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST), and the total amount of free SH groups were assessed. Comparison of the experimental groups showed that indoxacarb significantly affected the activities of GST, GPx and the total amount of free SH groups, while chlorantraniliprole significantly affected the activities of SOD, CAT, GST and the total amount of free SH groups, while chlorantraniliprole+lambda cyhalothrin significantly affected the activities of CAT, GST and the total amount of free SH groups. The results show that exposure to insecticides considerably affects the antioxidative defense components of the European corn borer larvae, especially chlorantraniliprole (T2).

Key words: indoxacarb, chlorantraniliprole, lambda cyhalothrin, *Ostrinia nubilalis* larvae, oxidative stress

INTRODUCTION

The European corn borer (ECB) *Ostrinia nubilalis* (Hübner, 1796) is a widespread polyphagous moth that is primarily a pest of maize and as well as several other crops. It is indigenous to Europe; however, during the early 20th century it spread to North America where it has successfully adapted and become a genuine problem to corn growers [1]. The larvae feed by boring into the stalk and ear, damaging the stem and kernels, thus facilitating infections with fungal pathogens and mycotoxin contamination [2,3].

The reduction in corn yield that this species can cause is usually variable, but it is very often of economic significance, and insecticide use is generally required. Foliar broad-spectrum insecticides are conventionally applied on maize in many European countries (Spain, Hungary, Poland, Germany, Italy and France) to control mainly ECB larvae [4].

Maize is cultivated on approximately 1000000 ha in Serbia with a net production of 4000000 t [5] that accounts for 6.71% of the total European maize production, which makes this plant the most important crop in Serbia. It comes as no surprise that there is a need for detailed investigation of the different biological aspects of ECB in order to establish methods for a more efficient control of this pest.

In our trials we evaluated the effects of three insecticides on the antioxidative defense of the ECB. In-

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doxacarb (Avaunt®) belongs to the oxadiazine insecticides that show high selectivity and efficacy against lepidopteran pests [6]. Because class of indoxacarb is a prooxidant, it has to be metabolized to the decarboxylated form to become biologically active [7]. By blocking the neuronal sodium channels, the activated indoxacarb metabolites cause irreversible paralysis and disable food intake, thereby causing insect death [8,9]. Chlorantraniliprole (Coragen®) belongs to the anthranilic diamide class that acts on insect ryanodine receptors [10]. When ingested, it causes uncontrolled calcium release from sarcoplasmic reticulum deposits, leading to muscle paralysis, impaired food intake and development, and eventually to insect death [11]. Sublethal effects include latent toxicity, decrease in reproductive potential and life expectancy, behavioral changes and induction of enzyme production [12]. The third experimental group was treated with a combination of chlorantraniliprole and lambda cyhalothrin (Ampligo®). Lambda cyhalothrin is a synthetic pyrethroid insecticide used to control a wide range of pests in a variety of applications (crops, structural pest management and in public health applications) [13]. Its mode of action is based on the blockage of sodium channels of neurons and on prolonged sodium currents [14]. After testing the effectiveness of different chemical and biological compounds, Mazurek et al. [15] established that lambda cyhalothrin was the most effective in controlling ECB larvae.

Xenobiotic chemical substances such as the aforementioned insecticides generate a considerable amount of reactive oxygen species (ROS), which induce oxidative stress in insects. Namely, ROS, such as hydrogen peroxide (H_2O_2), superoxide anion ($O^{2\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), are generated as by-products in aerobic organisms during metabolic activities such as biosynthesis and biodegradation, cellular respiration and phagocytosis, and they can cause serious oxidative damage to DNA, proteins and lipids [16]. Exposure of organisms to the different pollutants and xenobiotics that enter the environment after emission by industry, agriculture or after accidental release, induces increased ROS production. As an example, the insecticide fipronil increases ROS formation, which leads to cell membrane lipid peroxidation and damage, and oxidative stress [17-19].

To decrease the toxic effects of oxidative stress, aerobic organisms, including insects, increase the production of protein and non-protein components of antioxidative protection. SOD catalyzes the dismutation of superoxide radicals to H_2O_2 with oxygen as the main response to dietary prooxidant exposure [20]; CAT reduces H_2O_2 to water and oxygen [21]; GST is involved in the detoxification of xenobiotics [22]; GR catalyzes the reduction of oxidized glutathione and helps to detoxify ROS; GPx metabolizes H_2O_2 and deleterious lipid peroxides [23]. The amount of SH groups in biological systems serves as a good indicator of the intensity of oxidative stress, because they express the level of protein oxidation after intoxication [24].

Considerable literature data examine the modes of action of insecticides on organisms at different levels of biological organization; however, very few studies have focused on the effects of indoxacarb, chlorantraniliprole and the combination of chlorantraniliprole and lambda cyhalothrin on the components of the antioxidative defense of ECB larvae. Being arguably the most economically significant pest of maize, a better understanding of the effects of insecticides on ECB larvae will increase our knowledge of the antioxidative defense strategies and other aspects of ECB biology, and will help to improve control strategies against this pest.

MATERIALS AND METHODS

Experimental design and larval sampling

The experiment was carried out in Rimski Šančevi near Novi Sad, Serbia (45°19'47.72"N, 19°51'1.95"E, altitude 78 m a.s.l.) during 2016 on calcareous chernozem soil. Three insecticide treatments were compared to an untreated control (C). The applied insecticides were as follows: experimental group T1 – Indoxacarb (Avaunt®, DuPont, Serbia, formulation: emulsifiable concentrate (EC)) at a concentration of 250 mL ha^{-1}; T2 – Chlorantraniliprole (Coragen®, DuPont, Serbia, formulation: suspension concentrate (SC)) at concentration of 100 mL ha^{-1}; T3 – Chlorantraniliprole+Lambda Cyhalothrin (Ampligo®, Syngenta, Serbia, formulation: SC and capsule suspension combination (ZC)) at a concentration of 200 mL ha^{-1}. The experiment setup consisted of a completely randomized block design with 4 replicates, according to EPPO guidelines (European and Mediterranean Plant Protection Organization) (nr. PP

1/13(3)). Each plot consisted of 4 rows of maize, separated from other plots by one untreated row on each side. The length of each plot was 10 m, with a spacing of 2 m between blocks.

Insecticide application was performed during peak flight of the ECB, using a backpack sprayer unit with a high clearance attachment with 6 nozzle booms (model 315-HCB-4) from Bellspray Inc dba R&D Sprayers. The working height of the sprayer is manually adjustable (0.6-4.2 m) and the spray volume is 400 L ha^{-1} at a pressure of 200 kPa with an operation speed of 4-6 km h^{-1}. ECB flight activity was monitored using a light trap (model RO Agrobečej, Serbia), which was active from 20:00 to 7:00 h every day. The sampled specimens were removed and counted on a daily basis.

The experiments were conducted on commercial dent hybrid NS 6030 (NS Seme), FAO group 600. Crop harvest and yield results were obtained using a Wintersteiger split combine. Maize was sown on 5 May 2016, and the insecticide treatment was applied on 3 August. The sampling of caterpillars was performed on 24 and 25 August.

Preparation of larval homogenates

Frozen larvae were kept at -24°C until homogenization. Larvae were homogenized on ice in 0.25 M sucrose buffer (pH 7), 100 mg/2 mL, using an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) for 3×10 s at 2000 rpm, followed by 3×15 s sonication steps with a 50-W sonifier (Bandelin Sonoplus HD2070, Berlin, Germany). The homogenates were centrifuged using a Beckman L7-55 ultracentrifuge (Beckman, L7-55, Ultracentrifuge, Nyon, Switzerland) at 105000×g and 4°C. The supernatants were extracted and frozen at -24°C until use.

Determination of the activities of antioxidative enzymes and sulfhydryl group concentrations

The activity of SOD was determined according to the method described by Mistra and Fridovich [25]. This method is based on the ability of SOD to prevent adrenaline autoxidation in an alkaline medium. During the conversion of adrenaline to adrenochrome, superoxide anion radicals are released, which leads to the acceleration of the autoxidation reaction. Adrenaline autoxidation rate was determined spectrophotometrically by the absorption change at a wavelength of 480 nm at 25°C. SOD activity was expressed in units per mg protein.

CAT activity was determined according to the method of Beutler [26] by spectrophotometric determination of the dissolution of the standard concentration of H_2O_2 (10 mM) at 230 nm. The activity of the enzyme was expressed as the amount of dissolved H_2O_2 reduced per min per mg protein (in micromoles per min per mg protein).

GR activity was determined according to Glatzle et al. [27]. Changes in the amount of NADPH consumed by the reduction of a standard amount of oxidized glutathione (GSSG) were measured spectrophotometrically at 340 nm. Activity was expressed in nanomoles of NADPH per min per mg protein.

GST activity was determined by the Habig et al. method [28]. GST catalyzes the conjugation of 1-chloro-2.4-dinitrobenzene (CDNB) with the SH groups of glutathione (GSH). The amount of derived CDNB-glutathione complex was measured spectrophotometrically at 340 nm and expressed in nanomoles of GSH per min per mg of sample protein.

GPx was determined by following the oxidation of NADPH as a substrate with t-butyl hydroxyperoxide [29] and expressed in nanomoles of NADPH per min per mg of sample protein.

Determination of the amount of free SH groups was conducted according to Ellman [30], using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as a substrate. The product of the reaction was monitored at 412 nm. The amount of free SH groups in the sample was expressed in millimoles per mL and calculated from the standard curve using GSH as a standard.

Protein concentrations were determined according to Bradford [31] using bovine serum albumin as standard.

Statistical analysis

The results were processed using the statistical package SAS 9.1.3 (Service Pack 4, The SAS Institute. 2003;

Cary, NC, USA). The prerequisite for the analysis of variance was the normality of distribution within a group, which was achieved through logarithmic transformations of the traits [32]. Differences in the average values between the control and treatment experimental groups were assessed by one-way ANOVA and Fisher's LSD test. Differences with p-values less than 0.05 were considered as statistically significant for all treatments. Canonical discriminant analysis (CDA) was used to evaluate the differences between the experimental groups by analysis of the measured activities of SOD, CAT, GR, GST, GPx, and the concentration of the nonenzymatic components (SH groups).

RESULTS

A one-way ANOVA showed that SOD activity (Fig. 1A) changed significantly after all insecticidal treatments (F=14.42; p<0.005***) when compared to the control (C). The LSD *post hoc* test showed that chlorantraniliprole (T2) significantly affected the decrease in activity of this enzyme when compared to the control group, whereas in group T1, indoxacarb induced a considerable increase in SOD activity when compared to the other treatments (T2 and T3).

CAT activity in T2 (Fig. 1B) also changed significantly when compared to the other treatments. The treatment with chlorantraniliprole+lambda cyhalothrin (T3) significantly decreased CAT activity when compared to the control and T2 treatment.

Even though the activity of insecticides was significantly higher in treatment T2 when compared to T1, none of the insecticides' GR activity (Fig. 2C) was significantly affected (F=1.46; NS).

GST activity (Fig. 2B) was considerably affected by the insecticidal treatments (F=15.32; p<0.005***), and a significant increase in activity in all treatment groups as compared to the C was observed. The T1 treatment showed a significant increase in GST activity when compared to T3.

The activity of GSH-Px (Fig. 2A) was significantly altered in all insecticidal treatments (F=4.84**; p<0.01**), while the activity of the enzyme significantly increased in the T1 treatment when compared to the control (C) and the other two treatments (T2 and T3).

The amount of free SH groups (Fig. 3) was also significantly affected by all insecticide treatments (F=4.32; p<0.01**), with the concentration being significantly lower in all treatment groups when compared to the control.

CDA showed separation between the experimental groups (Fig. 4). The first canonical function (Root 1) in the analysis accounted for 68% of total variance. The second canonical function (Root 2) accounted for 32% of total variance.

DISCUSSION

The ECB is one of the most destructive pests of maize in Europe and North America. The annual costs of control of this pest and grain losses in the USA are estimated to exceed 1.85 billion USD [33]. A consider-

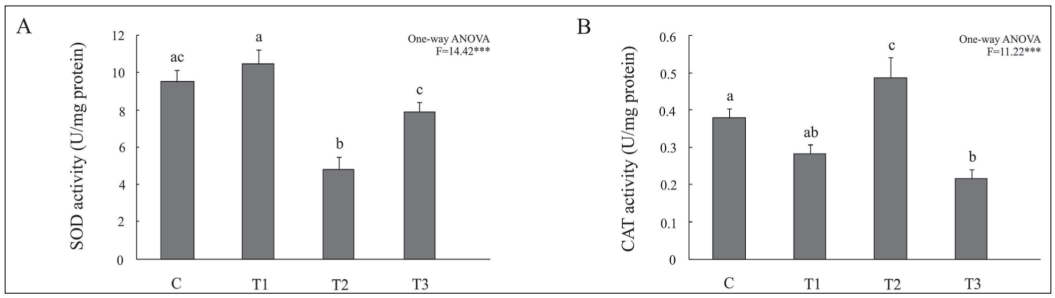

Fig. 1. Effects of insecticides (T1 – indoxacarb, T2 – chlorantraniliprole and T3 – chlorantraniliprole+lambda cyhalothrin) on SOD (**A**) and CAT (**B**) in *O. nubilalis* larvae. Bars represent the mean±standard error of mean. Significance of the effects on larvae was tested by one-way ANOVA (F values are presented) and *post hoc* compared by Fisher's least significant difference test (LSD test). Values indicated by different letters (a, b, c) are significantly different. ***p<0.001 (significant differences from the control – C).

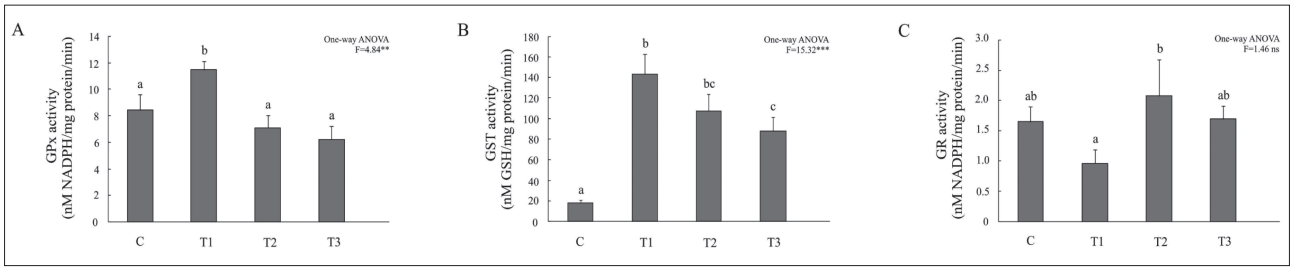

Fig. 2. Effects of insecticides (T1 – indoxacarb, T2 – chlorantraniliprole and T3 – chlorantraniliprole+lambda cyhalothrin) on GSH-Px (**A**), GST (**B**) and GR (**C**) in *O. nubilalis* larvae. Bars represent the mean±standard error of mean. Significance of the effects on larvae was tested by one-way ANOVA (F values are presented) and *post hoc* compared by Fisher's least significant difference test (LSD test). Values indicated by different letters (a, b, c) are significantly different. ***p<0.001, **p<0.01, ns – not significant (significant differences from the control – C).

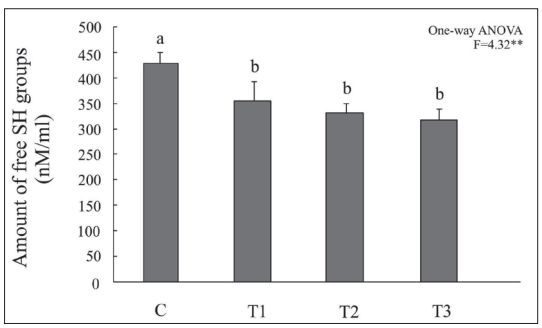

Fig. 3. Effects of insecticides (T1 – indoxacarb, T2 – chlorantraniliprole and T3 – chlorantraniliprole+lambda cyhalothrine) on free SH groups in *O. nubilalis* larvae. Bars represent the mean±standard error of mean. Significance of the effects on larvae was tested by one-way ANOVA (F values are presented) and *post hoc* compared by Fisher's least significant difference test (LSD test). Values indicated by different letters (a,b) are significantly different. **p<0.01 (significant differences from the control – C).

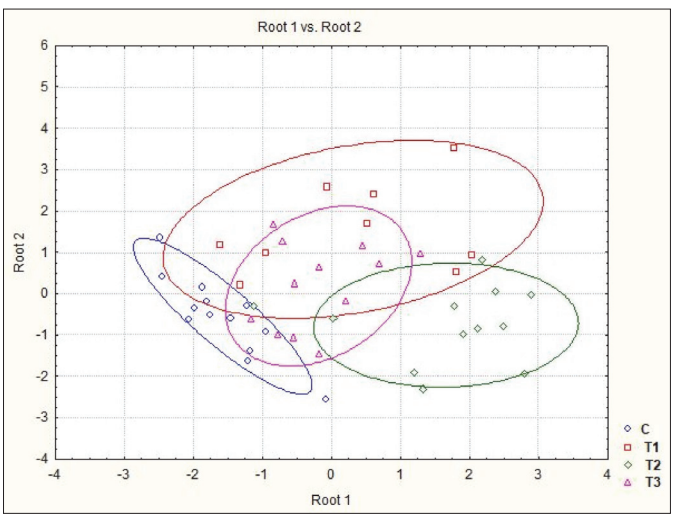

Fig. 4. Canonical discriminant analyses for experimental groups (C, T1, T2 and T3) and biochemical markers in *Ostrinia nubilalis* larvae.

able number of different insecticides are used in the management of ECB and other pests, many of which induce oxidative stress as part of their insecticidal activity. Some of them, like indoxacarb, chlorantraniliprole and chlorantraniliprole+lambda cyhalothrin, even though relatively new on the market, are widely used to control lepidopteran pests. The effects of insecticides lead to changes in larval development and decrease the reproductive potential and life expectancy of insects. These changes also occur as a consequence of the tradeoff between life history traits and antioxidative defense induction. Because of this, the induction of stress defense mechanisms has a primary role in the survival strategy of insects. This means that the physiological changes that lead to the synthesis of detoxifying components represent the first insect response to stress related to insecticide exposure. The insecticide-induced oxidative stress increases the production of ROS, which leads to changes in activities of SOD, CAT, GR, GST and GSH-Px, as the first line of defense against oxidoreduction processes [34].

Indoxacarb is an oxadiazine insecticide and the first commercial pyrazoline insecticide type, developed in the late 1990s and registered for use in 2000 in the USA and Australia, and in 2006 in the EU [35-37]. Indoxacarb is characterized by high efficacy and low toxicity to nontarget organisms. It is effective against insects that have resistance to other major classes of neurotoxic insecticides, such as pyrethroids, carbamates and organophosphates [38]. In our trial, the ECB larvae treated with indoxacarb showed increased

GST activity compared to the control group and T3 treatment. GPx levels were also increased when compared with T2, T3 and control treatments. Mirhaghparast et al. [39] obtained similar results in their experiments on the effects of indoxacarb on 3rd instar larvae of the cotton bollworm *Helicoverpa armigera*, where the activity of GST increased considerably 24 h after exposure to indoxacarb as compared to the control group; after a longer exposure period, the activity of GST started to decrease [40]. GST belongs to a group of multifunctional enzymes involved in the antioxidative and detoxification processes of poisonous compounds and is a phase II biotransformation enzyme according to the classification of biomarkers [41]. Thus, elevated activity shows its early involvement in the detoxification metabolism to eliminate this insecticide.

In their experiments, Demirci et al. [7] observed that indoxacarb did not considerably affect the activity of SOD, CAT and GR in *Ostrinia nubilalis* larvae when compared to the control group, while the same enzymes, including GST, were considerably increased in the aquatic crustacean *Gammarus kischineffensis*. In contrast, in our experiments, ECB larvae treated with indoxacarb (T1) showed significantly lower activities of CAT and GR when compared to the group treated with chlorantraniliprole, while indoxacarb induced SOD to a considerably higher activity than the two other insecticides (T2 and T3).

Chlorantraniliprole is a relatively new insecticide belonging to the anthranilic diamides group, which blocks the activity of the ryanodine receptors located in the sarcoplasmic reticulum and disrupts the release of intracellular calcium stores, which are critical for muscle contraction, thus preventing food intake and causing muscle paralysis and death in insects [11]. Chlorantraniliprole was demonstrated to have species-specific properties as the insecticide showed lethal effects only on ECB larvae, while it had no effect on its predators, pirate bugs *Orius* spp. [42].

Sublethal doses are known to decreases larval weight, reproductive potential and viability, and to induce behavioral changes and changes in enzyme production in the larval stages of *H. armigera*, *Spodoptera exigua* and *Plutella xylostella* [12,43-45]. The treatment with chlorantraniliprole (T2) produced a considerable effect on the ECB larval antioxidative defense components. The activity of SOD was considerably lower, while CAT activity was significantly higher when compared to the other treatments. The effect of chlorantraniliprole on the production of the superoxide anion radical (O^{2-}) is variable, and depending on the experimental conditions, an increase or decrease of ion concentration, which acts as a substrate for SOD, was observed.

The decrease in activity of the antioxidative defense system enzymes is usually connected with damage to different cellular molecules, such as DNA, lipids and proteins [46], which leads to a lower enzyme production. The results of our experiment showed that the activity of GST in ECB larvae treated with chlorantraniliprole was significantly higher when compared to the control group, while the activity of GR was considerably higher when compared to the group treated with indoxacarb. An increased activity of GR in three amphibian species treated with glyphosate- and methidathion-based pesticides was shown by Güngördü et al. [47]. However, another study established that the same enzyme was inhibited in the fish species *Piaractus mesopotamicus* treated with a mix of endosulfan and lambda cyhalothrin [48].

When used in combination with the pyrethroid insecticide, lambda cyhalothrin, chlorantraniliprole led to a significant decrease in CAT activity when compared to the control and T2 treatment (where only chlorantraniliprole was applied). The activity of GST was higher in the T3 treatment when compared to the control, but the activity of this enzyme as well as the activities of SOD and GPx were significantly lower when compared to the indoxacarb treatment (T1). Mahmoud et al. [49] established that at higher concentrations pyrethroid insecticides induced a decrease in CAT activity in the sea snail *Hexaplex trunculus*. It was suggested that pyrethroid insecticides can exhibit considerable differences dependent on sex, time, type of tissue and concentration in both vertebrates and invertebrates [50]. Bacchetta et al. [48] showed that the activity of GST was increased only in the liver of *Piaractus mesopotamicus* that were exposed to a combination of pesticides with lambda cyhalothrin. The Colorado potato beetle *Leptinotarsa decemlineata* was shown to react to exposure to lambda cyhalothrin, fipronil and endosulfan by producing higher amounts

of GST [51]. Also, research on the diamondback moth *P. xylostella* confirmed the higher activity of GST in resistant individuals, which indicates that GST has a very important role in detoxification strategies in Lepidoptera and other insect groups [52,53].

The activities of the antioxidative defense enzymes depend on the type of insecticide applied. CDA showed that SOD, CAT and GR activities significantly contributed to differences between the experimental groups vs. the treatments.

CONCLUSION

Insecticides such as indoxacarb, chlorantraniliprole as well as chlorantraniliprole combined with lambda cyhalothrin are used to control a number of crop pests, including *Ostrinia nubilalis*. These insecticides affect various aspects of insect biology, including the induction of oxidative stress, which leads to shifts in the life strategies of the insect. Induction of antioxidative defense decreases their development and reproduction fitness. All three insecticides used in our trial altered the biochemical physiology of *O. nubilalis* larvae in response to oxidative stress. They also all displayed different potentials for oxidative stress induction. The larval biochemical response to the insecticides depended on the insecticide type. The complex reaction of the larvae to the three insecticides included mechanisms of antioxidative defense and detoxification, and increased levels of GST activity. On the other hand, SOD, CAT and GR activities varied, depending on the used insecticide type. Further research into the connection between oxidative stress and insecticide effects is expected to lead to a better understanding of pest biology and will help improve control strategies against insects.

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Author contributions: FF, VPM and ŽM conceived the research. DM, DT and NG conducted the experiments. FF and ŽM contributed the material. SO analyzed the data and conducted the statistical analyses. FF and DM wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

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