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Determination of organophosphate resistance status and mechanism in *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) from Turkey

Türkiye’deki *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae)’nin organofosfat direnç durumunun ve mekanizmasının belirlenmesi

**Abstract:** Objective: The objectives of this study were to determine resistance status to malathion and pirimiphos-methyl insecticides and to make biochemical analysis of resistance mechanism(s) developed to these insecticides in *Sitophilus zeamais* (*S. zeamais*) populations, collected from two different locations in Turkey. Two organophosphate insecticides, malathion and pirimiphos-methyl, were examined by bioassay using a discriminating dosage technique with impregnated filter papers. Mortality percentages were determined at the discriminating doses of these insecticides. In addition, esterase, glutathione S-transferase, and cytochrome P450 monooxygenase activities were also determined in this study in order to analyze detoxification mechanism(s) of tested insecticides in *S. zeamais*.

Methods: Bioassay experiments of malathion and pirimiphos-methyl insecticides in *S. zeamais* populations were performed according to the IRAC susceptibility test method No:006. Furthermore, enzyme activities of esterases, cytochrome P450 monooxygenases, and glutathione S-transferases were determined by using biochemical assays.

Results: The bioassay results of malathion and pirimiphos-methyl insecticides in *S. zeamais* populations showed that only Kırıkkale population of *S. zeamais* has resistance to both malathion and pirimiphos-methyl insecticides. However, no resistance was detected to malathion and pirimiphos-methyl insecticides in Samsun population of *S. zeamais*. Additionally, biochemical analysis displayed that while CYP450-PNOD activities showed an increase only in Kırıkkale population (3.0-fold), EST-PNPA activities showed an increase only in Samsun population (1.3-fold). Finally, GST-CDNB activities increased both in Kırıkkale (1.4-fold) and Samsun (2.2-fold) populations of *S. zeamais* compared to susceptible population.

Conclusion: Consequently, cytochrome P450 monooxygenases and glutathione S-transferases seem to play a role in organophosphate resistance in Kırıkkale population of *S. zeamais* from Turkey.

**Keywords:** *Sitophilus zeamais*, insecticide resistance, biochemical mechanisms, malathion, pirimiphos-methyl

**Özet:** Amaç: Bu çalışmanın amaçları Türkiye’de iki farklı yerden toplanan *Sitophilus zeamais* popülasyonlarının malatyon ve pirimifos-metil insektisitlerine karşı dayanıklılık durumlarının tespiti ve bu insektisitlere karşı gelişirdiğilen dayanıklılık mekanizmalarının yada mekanizmalarının biyokimyasal olarak analiz edilmesidir. İki organofosfatlı insektisitin (malatyon ve pirimifos-metil) biyoanalizini emdirilmiş filtre kağıtları kullanılarak ayırt edici doz tekniği ile test edildi. Bu insektisitlerin ayırt edici doz’daki ölmüş yüzdeleri belirlendi. Ayrıca, bu çalışmada *S. zeamais*’de test edilen bu insektisitlerin detoksifikasyon mekanizmalarının analizi için esteraz, glutatyon S-transferaz ve sitokrom P450 monokskizjenaz aktiviteleri de belirlendi.

Metod: *S. zeamais’in* malatyon ve pirimifos-metil insektisitleri ile biyoanaliz deneyleri IRAC hassasiyet testi
Sitophilus zeamais populations from Turkey could not be analyzed, as well. When the resistance occurs in insects, it requires high dose usage to control pests. Determination of resistance in insects by using bioassay or biochemical assays methods provide more efficient control of pests insects by using more efficient insecticides. It is also protect the human health and the environmental pollution by preventing the unnecessary insecticide usage.

In order to estimate resistance status of S. zeamais to organophosphate insecticides, method of impregnated filter paper bioassay was used. It is important to know resistance status of insecticides for resistance management and early detection of resistance development. Hence, determinations of resistance status in S. zeamais to malathion and pirimiphos- methyl insecticides (organophosphates) were carried out by using bioassay method.

Resistance development against continuously used and high dose applied insecticides generally occurs through three main mechanisms in insects. These mechanisms include decreased sensitivity of insecticide target site, decreased penetration of insecticides from insect cuticle and increased metabolism of applied insecticides through detoxification enzyme systems such as esterases (EST), glutathione S-transferases (GST), and cytochrome P-450 monoxygenases (CYP450) [3]. These detoxification enzyme systems in S. zeamais were analyzed by using biochemical activity assays, useful molecular techniques for understanding detoxification mechanism(s) in insects.

In summary, the objectives of this study were: (1) to determine resistance status of malathion and pirimiphos-methyl insecticides in S. zeamais; and (2) to specify possible detoxification mechanism(s) of S. zeamais populations, obtained from grain stores in Kırıkkale and Samsun cities in Turkey.

In this study, the combination of organophosphate bioassays and specific enzyme activity assays enabled to pinpoint detoxification events (esterases, glutathione S-transferases and CYP450s) as the major players of metabolic resistance in S. zeamais populations.

Materials and Methods

Insects

Insect populations of S. zeamais were obtained from grain stores in Kırıkkale and Samsun cities in Turkey in 2014. They were reared on whole wheat grain free of insecticide in the laboratory in glass boxes at 60% ± 5 room humidity and 25±2°C in the dark. Similarly, laboratory population of
S. zeamais was also reared under the same conditions at different glass boxes. The population used as susceptible population was the population which was collected from the field (Mersin), reared in the laboratory for a long time, tested 3–4 times in different times with discriminating dose and obtained as death at the rate of 100%. Insects were allowed to breed for several generations until sufficient insect numbers were available for resistance testing. Lastly, whole adult S. zeamais larvae were used for bioassay experiments and cytosol preparation for biochemical activity assays.

Chemicals

The insecticides used in this study were malathion and pirimiphos-methyl. The technical grade (96%) of malathion was obtained from the company of Safa Agricultural Chemicals in Turkey. Pirimiphos-methyl (99.5%) was obtained from Labor Dr. Ehrenstorfer, Germany. Pure acetone was obtained from Sigma Chemicals (Novagentek-Turkey). Fluon was obtained from DuPont Agrochemical Company, Turkey. All other chemicals were purchased from Sigma-Aldrich (Novagentek, Turkey). All solvents were of analytical grade.

Insecticide bioassays

All populations were tested by using discriminating doses of malathion and pirimiphos-methyl. Discriminating dose is the dose killing 95–99% of susceptible population. Adult beetles (2–4 weeks) were tested in terms of toxicity of various insecticides by using filter paper technique with discriminating doses, 1.5 mg/disc for malathion and 2.0 mg/disc for pirimiphos methyl [4]. All populations of S. zeamais were tested with this discriminating dose. In other words, single dose (discriminating dose) was used for each insecticide in trials. Being collected by sieving the infested commodity prior to testing, beetles were placed in vials in groups of 40. The procedure was repeated until the required number was reached to minimize batch variation. These insects were kept without food for 1 h at 25±2°C and approximately 70% room humidity before assigning batches randomly to treatments.

Insects were treated by exposing them to filter papers impregnated with insecticide with acetone. Technical grade (96%) malathion and pirimiphos-methyl (99.5%) were dissolved in acetone. Whatman No: 1, 5.5 cm filter paper discs were treated with 0.5 ml of the appropriate solutions to obtain 1.5 mg malathion and 2.0 mg pirimiphos-methyl on each disc [4]. Treated papers were air-dried for 30 minutes to evaporate the solvents. For control insects, only acetone impregnated filter paper was used.

The filter paper discs treated with discriminating dose solutions (1.5 mg malathion/disc or 2.0 mg pirimiphos-methyl/disc) were laid on a clean glass plate and glass rings with 5-cm diameter and 2.5-cm height were placed centrally on the top of papers. The inside surface of rings was painted with a thin film of fluon to prevent escape for S. zeamais. Then, ten insects were placed on test papers inside rings. Approximately 80–100 insects were used for each experiment. Rings were covered with fine-mesh screen to prevent immigration or escape by flight. Then, insects were maintained under the conditions at 25±2°C and approximately 70% room humidity. Mortality was assessed after the recommended exposure period (according to species). This period was 6 hours for Sitophilus spp. [4]. Insects were counted as dead if they were ataxic and unable to right themselves. A gentle push with forceps is usually sufficient to categorize individuals.

Data analysis of bioassay results

Mortality percentages were calculated in population treated with discriminating doses of insecticides. The dead or alive S. zeamais larvae was scored after 6-hour incubation on insecticide absorbed papers. S. zeamais, which was unable to make coordinated movement from gentle stimulus with a seeking pin, were considered as “dead”. Mortality in the controls was ≤2% in all cases. Mortality percentages were calculated from the experiment results without using Abbott’s formula because the control mortality did not exceed 5%. The correction was virtually negligible when the control mortalities were below 5% [5].

Preparation of cytosols from S. zeamais larvae for cytochrome P450 monooxygenase, glutathione S-Transferase, and esterase assays

Each 25 mg batch of S. zeamais was homogenized in 1 ml homogenization solution, including 0.1 M potassium phosphate buffer, pH 7, 4, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, and 1 mM phenyl methyl sulfonyl fluoride, with an ultraturrax homogenizer. Then, homogenates were centrifuged at 10,000 x g for 30 min at 4°C. After centrifugation, supernatants were centrifuged again at 10,000 x g for 5 min at 4°C. Supernatant
parts of second centrifuge were used as enzyme source for CYP450s, GSTs and ESTs enzyme activity assays. Finally, protein concentrations of these supernatants were determined by using Bradford method [6].

**Determination of cytochrome P450 monooxygenase activity (CYP450-PNOD)**

Cytochrome P450 monooxygenases O-demethylation activities towards p-nitroanisole substrate was determined according to the method of Rose et al., (1995) [7]. Enzymatic reactions were done in micro plates by monitoring p-nitrophenol formation as described in detail in Konus (2014) [3]. CYP450-PNOD activities were calculated with the help of standard curves of p-nitrophenol.

**Determination of glutathione S-transferase activity (GST-CDNB)**

Glutathione S-transferase (GST-CDNB) activities were measured according to the modified Habig et al., (1974) [8] method by using 1-chloro-2,4-dinitrobenzene (CDNB) substrate. Each enzymatic reaction mixture contains 0.1 M potassium phosphate buffer, pH: 7.4, 1 mM reduced glutathione, and 1 mM CDNB. Calculations of GST-CDNB activities were made with the help of extinction coefficient of CDNB at 340 nm (9.6 mM cm⁻¹) as described in detail in Konus (2014) [3].

**Determination of esterase activity (EST-PNPA)**

Esterase enzyme activity (EST-PNPA) was determined according to van Asperen (1962) [9] method in 96 well micro plates. EST-PNPA activity was measured at 405 nm by using p-nitrophenyl acetate (PNPA) substrate. Reaction mixture of EST-PNPA assay contained 0.1 M potassium phosphate buffer, pH: 7.0, 0.05 % Triton X-100, 4 mM PNPA. EST-PNPA activity was calculated by preparing standard curves with p-nitrophenol.

**Statistical analysis of enzyme activity assays**

Statistical analysis of measured CYP450-PNOD, GST-CDNB, and EST-PNPA activities were performed with student-t test by using MINITAB 16.0 statistics software.

**Results and Discussion**

Table 1 illustrates mortality percentages of *S. zeamais* populations treated with discriminating doses of malathion and pirimiphos-methyl insecticides.

**Malathion**

In bioassays, it was found that while discriminating dose of malathion (1.5 mg malathion/disc) killed all susceptible *S. zeamais* insects, this dose killed only 72.2% and 92.2% of *S. zeamais* insects from Kırıkkale and Samsun populations, respectively (Table 1). Hence, our results suggested that while Kırıkkale population of *S. zeamais* seemed to be resistant to malathion, Samsun population of *S. zeamais* was not resistant to malathion insecticide. Similar results have also been obtained in other studies. For example, in Brazilian populations of *S. zeamais*, resistance was not observed to organophosphate insecticides such as malathion and pirimiphos-methyl, widely and frequently used in 1970s and 1980s in that country [10]. However, resistance to organophosphate insecticides such as malathion, pirimiphos-methyl, and fenitrothion were also reported for *S. zeamais* together with other stored products insects such as *Rhyzopertha dominica* (F.), *Sitophilus oryzae* (F.),

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**Table 1:** Mortality percentages of *Sitophilus zeamais* populations that treated with discriminating doses of malathion and pirimiphos-methyl insecticides.

| Insecticide       | Population                  | Discriminating dose (mg/disc) | Mortality (%) |
|-------------------|-----------------------------|-------------------------------|---------------|
| Malathion         | *Sitophilus zeamais*-Kırıkkale | 1.5                           | 72.2          |
|                   | *Sitophilus zeamais*-Samsun  | 1.5                           | 92.2          |
|                   | *Sitophilus zeamais*-Susceptible | 1.5                          | 100           |
| Pirimiphos-methyl | *Sitophilus zeamais*-Kırıkkale | 2.0                           | 70            |
|                   | *Sitophilus zeamais*-Samsun  | 2.0                           | 95            |
|                   | *Sitophilus zeamais*-Susceptible | 2.0                          | 100           |
Pirimiphos-methyl

Bioassay results of pirimiphos-methyl showed similar pattern for malathion bioassay. It was found that while 2.0 mg pirimiphos-methyl (discriminating dose) killed all susceptible *S. zeamais* insects, the discriminating dose killed only 70% and 95% of *S. zeamais* insects from Kırıkkale and Samsun populations, respectively (Table 1). Like malathion insecticide, Kırıkkale population of *S. zeamais* seemed to be resistant to pirimiphos-methyl and Samsun population of *S. zeamais* was not resistant to pirimiphos-methyl insecticide (Table 1). Petar and Ilija (2005) [14] also reported that high levels of resistance to pirimiphos-methyl were reported mostly for populations of *S. oryzae* in Serbia and Montenegro.

Resistance to DDT and pyrethrroids was reported in the early 1990s and, more recently, a few cases of organophosphate as well as pyrethroid resistance have been reported [10,15]. Perez-Mendoza (1999) [16] also described resistance in population of *S. zeamais* in Mexico. In this country, maize weevil resistance to deltamethrin and permethrin was in its initial stages because these insecticides were registered as grain protectants only after 1992. Comparison of data is not easy because of the different bioassay protocols employed and the huge variety of insecticides used [17]. Insects have successfully adapted to most insecticides by becoming physiologically or behaviorally resistant [18]. In postharvest ecosystems, the development of insecticide resistance is a major concern for many countries [1]. Cases of resistance of stored products insect to grain protectants [19,20] and fumigants [21] have been well documented.

Tables 2, 3 and 4 illustrate biochemical assay results of CYP450-PNOD, GST-CDNB, and EST-PNPA activities of *S. zeamais* strains, respectively. It was previously reported that insecticides from different classes might be metabolized by cytochrome P450 monooxygenases in insects [22]. Recently, it has also been reported that CYP450s may have role in resistance development in stored-grain pests against insecticides [1]. Additionally, GSTs can metabolize organophosphate insecticides [23,24] and decrease oxidative stress by detoxifying lipid peroxides and oxidized DNA bases induced by increased insecticide metabolism [25,26]. Biochemical assay results demonstrated that malathion and pirimiphos-methyl resistant Kırıkkale population showed significant (p<0.05) increases in CYP450-PNOD (3.0-fold) and GST-CDNB activities (1.4-fold) compared to the susceptible strain (Table 2 and Table 3).

However, EST-PNPA activity was not changed significantly (p<0.05) in Kırıkkale population compared to the susceptible population (Table 4). Consequently, it is proposed that CYP450s together with GSTs seem to be involved in resistance development to malathion and pirimiphos-methyl insecticides in Kırıkkale population of *S. zeamais*.

Although Samsun population of *S. zeamais* was not resistant to both pirimiphos-methyl and malathion insecticides, it showed 1.3-fold and 2.2-fold more EST-PNPA and GST-CDNB activity, respectively, compared to the susceptible population (Table 3 and Table 4). As esterases might be involved in organophosphate, carbamate, and pyrethroid resistance in insects [27], increased EST-PNPA activity along with increased GST-CDNB activity could be related with resistance to other insecticide(s) from organophosphates or insecticide(s) from different chemical classes in Samsun population of *S. zeamais*.

Monitoring of resistance is important in adopting proper control measures regarding pest management for stored product pests and protect the human health.
Conclusion

In conclusion, the results obtained in these bioassays suggested that Kırıkkale population has resistance to both malathion and pirimiphos-methyl insecticides. CYP450s and GSTs might have role in resistance development against organophosphate insecticides (malathion and pirimiphos-methyl) in S. zeamais from Turkey. In addition, it was suggested that there was not any resistance development against malathion and pirimiphos-methyl insecticides in Samsun population of S. zeamais. Monitoring of resistance is important in adopting proper control measures regarding pest management for stored product pests. It is also protect the human health providing less insecticide usage.

It is the first report that organophosphate resistance was resistance determined in Turkish field populations of S. zeamais. Besides, it was found that CYP450s and GSTs might have an important role in resistance development in field populations of S. zeamais from Turkey.

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Conflict of Interest: The authors have no conflict of interest.

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