Preliminary Studies on the Susceptibility Level of *Ceutorchynhus assimilis* (Coleoptera: Curculionidae) to Acetamiprid and Chlorpyrifos in Poland and Resistance Mechanisms of the Pest to Acetamiprid

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ABSTRACT. The cabbage seed weevil, *Ceutorchynhus assimilis* (Paykull) (Coleoptera: Curculionidae) is a pest that more and more often causes large financial losses for rapeseed cultivators in Poland and other European countries. One of the reasons of these problems is the resistance of the pest to certain active substances of insecticides. The aim of the research was to assess the susceptibility level of the pest to chlorpyrifos, an organophosphate substance, and acetamiprid, a neonicotinoid, and to determine its enzymatic mechanisms of susceptibility to acetamiprid using synergists, i.e., blockers of particular enzyme groups. The presented research is the first to discuss the mechanisms of the resistance of the cabbage seed weevil to acetamiprid. The achieved results showed medium, high, or very high resistance of the cabbage seed weevil to acetamiprid and its lack of resistance to chlorpyrifos. The research on the mechanisms of the resistance of the pest to acetamiprid revealed the participation of hydrolytic enzymes blocked by S,S,S-tributylyphosphorotrithiate and glutathione transfers blocked by diethyl malonate in the metabolism of acetamiprid. The results did not show the participation of oxidative enzymes and esterases blocked by piperonyl butoxide in the detoxification of acetamiprid.

Key Words: cabbage seed weevil, acetamiprid, chlorpyrifos, resistance, resistance mechanism

Both Poland and other European countries strive to create integrated farm plant production programs. One of the more difficult problems is the control of agrophages that developed a certain level of resistance to the active substances present in pesticides (insecticides, herbicides, and fungicides) from different chemical groups. This phenomenon causes large financial losses and affects the natural environment.

Because of their resistance to some insecticides, it is currently very difficult to control two rapeseed pests that are of significant importance to the economy—the pollen beetle, *Meligethes aeneus* (F.) (Coleoptera: Nitidulidae), and cabbage seed weevil, *Ceutorchynhus assimilis* (Paykull) (Coleoptera: Curculionidae) (Heimbach et al. 2006a,b, Nauen 2007, Węgorek et al. 2009c, Zamojska et al. 2010). In the case of these pests, agronomic conditions need to be accounted for, including the increased area of rapeseed cultivation and the insecticide use history. Intensive chemical oilseed rape protection in Poland started in the 50s of the 20th century. To date, over 40 active substances have been used. Organochlorines and carbamates were withdrawn in 1998 and neeristine analogs in 2006. Nowadays, there are five insecticide chemical groups used in oilseed rape protection in Poland: neonicotinoids (with acetamiprid as the only active substance, used since 2004 and tiaclodiprid used in the mixture with deltamethrin), pyrethroids (10 active substances with deltamethrin as the first one, used since 1981), organophosphates (with chlorpyrifos as the only active substance used since 2000), oxadiazines (with indoxacarb as the only active substance, used since 1993), and azomethine pyridines (with pyrrozine as the only active substance, used since 2013) (Węgorek 2009, recommendations of the Polish Ministry of Agriculture). Regarding the last 10 yr, pyrethroids (deltamethrin, cypermethrin, alpha-cypermethrin, lambda-cyhalothrin, esfenvalerate, beta-cyfluthrin, zeta-cypermethrin, gamma-cyhalothrin, and tau-fluvalinate) and neonicotinoids (acetamiprid and tiachloprid) have mainly been used for *C. assimilis* control. Out of all insecticide active substances mentioned, tau-fluvalinate and acetamiprid are the most often used ones because they are not toxic to bees in the recommended doses (in Poland the period of controlling *C. assimilis* coincides with an intensive flight of bees). Depending on the density of oilseed rape pest populations, pyrethroids are used two or three times a season against cabbage seed weevil and pollen beetle but only once a season against *C. assimilis*. Acetamiprid is usually used only once a season. Chlorpyrifos does not exert selective pressure on *C. assimilis* because, due to its toxicity to bees, it is used only in the first treatment against the pollen beetle. All these recommendations result from the strategy preventing resistance and relying mainly on using a given active substance only once per season (Węgorek et al. 2013). In Poland, rapeseed is currently cultivated on 800 ha and is treated chemically numerous times each year.

The level of the susceptibility of the pollen beetle to many insecticides, both in Poland and in other countries, has been studied and monitored for a long time (Łąkocy 1969, Węgorek 2005 a,b, Hansen 2008, Richardson 2008, Węgorek and Zamojska 2008, Węgorek et al. 2009c, Zamojska et al. 2010). However, the data on the susceptibility of the cabbage seed weevil to active substances present in insecticides are limited and relate only to pyrethroids (Garthwaite et al. 1995, Buntin 1999, Heimbach et al. 2006a). Data on chlorpyrifos and acetamiprid are scarce (Zamojska et al. 2010).

As the resistance mechanisms are different in various species, with each of the mechanisms having its own characteristics, it is necessary to identify the mechanisms in agricultural and forest pests that are extremely important from the point of view of the economy, when designing plant protection strategies. The basic resistance mechanisms in pests include slower permeation of toxins through the coverings of insects, enzymatic detoxification of insecticides by enzyme systems, including especially mixed-function oxidases, hydrolases, and transferases (Malinowski 2003, Terra and Ferreira 2005), lowered susceptibility of target parts of the pests to insecticides, and behavioral mechanisms (Malinowski 2003, Węgorek 2009). There is no data in literature on the resistance mechanisms of the cabbage seed weevil to the active substances of insecticides.

The aim of the research was to specify the level of susceptibility of the cabbage seed weevil to chlorpyrifos, an organophosphate substance, and acetamiprid, a neonicotinoid, and to determine the participation of enzymatic systems in the resistance mechanisms of the pest to...
Materials and Methods

In the research, IRAC Susceptibility Test Method 7 was used. The method was described earlier for studies on pollen beetle (Węgorek et al. 2011a). The only differences included the tested species, plant material used (leaves and flower clusters of rape), and the number of the beetles placed in one container. In the case of the research presented in this publication, 50 cabbage seed weevils were placed in each container. The insects used in the research and the plant material were collected from fields that were not treated chemically at three locations in the Wielkopolska province: Września, Walcz, and Krotoszyn in 2008–2010. Tested populations have a very similar insecticide use history.

Insecticides (Commercially Available Products). Insecticide concentrations in ppm were calculated, assuming that 200 liters of water would be used per hectare.

Neonicotinoids (contact-gastric action): acetamiprid (Mospilan 20 SP with 20% of active substance): recommended dose of 0.12 kg/ha and recommended concentration: 120 ppm.

Organophosphate (contact-gastric and gas action): chlorpyrifos (Pyrinec 480 EC with 480 g/liter of active substance). Pyrinec 480 EC is not recommended for controlling cabbage seed weevils. The calculations were based on the recommended dose of 0.6 liter/ha (recommended concentration: 1,440 ppm) and the recommendations for the pollen beetle feeding on the winter rapeseed, often together with the cabbage seed weevil.

Laboratory conditions were consistent with the ones described for the Colorado potato beetle in the Journal of Plant Protection Research (Węgorek et al. 2011b, Zamojska et al. 2011). Also, statistical calculations were the same as the ones described in the mentioned publications—based on the percent mortality of the cabbage seed weevil at each dose, lethal concentrations LC50 and LC95 were calculated.

Resistance coefficient (RC) values were calculated as follows:

$$ RC = \frac{LC50/\text{recommended field dose}}{\text{the assumption that the recommended field dose had resulted in 100% mortality of insects at registration time}} $$

The following criteria for resistance assessment were assumed:

- RC ≤ 1—lack of resistance;
- RC = 1.1–2—low resistance;
- RC = 2.1–5—medium resistance;
- RC = 5.1–10—high resistance; and
- RC ≥ 10—very high resistance.

The enzymatic mechanisms of the resistance of the cabbage seed weevil to acetamiprid were determined using the method described for the Colorado beetle in the Journal of Plant Protection Research (Węgorek et al. 2011b, Zamojska et al. 2011), with the aforementioned changes. The research used synergists blocking three main groups of enzymes taking part in the metabolisms of toxins: piperonyl butoxide (PBO)—mainly oxidases blocker; S,S,S-tributylphosphorotrithioate (DEF)—esterases blocker; and diethyl malonate (DEM)—glutathione transferases blocker.

The dose of each if the synergists used was 100 ppm. Earlier research showed that the dose is nontoxic for the studied beetles. Inhibitors were applied at the same time as insecticides.

The synergism coefficient (SC) values were calculated as follows:

$$ SC = \frac{LC}{LC50} $$

The following criteria were accepted to assess synergism between deltamethrin and a given insecticide: SC < 1—antagonism; SC = 1—lack of synergism and the lack of antagonism; and SC > 1—synergism.

Results

The cabbage seed weevil susceptibility level to chlorpyrifos and acetamiprid is presented in Table 1.

The LC50 values for acetamiprid were high, ranging from 86.89 ppm (Walcz population in 2009) to 267.02 ppm (Września population in 2010). The lowest value was thus three times lower than the highest one. The biggest difference in the values was found in the Walcz population (the lowest value was 1.59 times lower than the highest one) in 2008 and 2009. Except for the Walcz population in 2009, the LC50 values slightly exceeded the concentration of acetamiprid recommended in Poland, with the values being from 1.01 to 2.23 times higher than the recommended dose. The LC95 values of acetamiprid in all the populations and in all the years of research exceeded the recommended dose, with the maximum value being 43 times higher than recommended dose (Walcz population in 2009). The LC95 values ranged from 422.46 to 5,753.4 ppm. The biggest difference between the values (with the lowest value being 4.44 times lower than the highest one) within one population was found in the research on the Września population in 2009 and 2010. The calculated values of the RC are presented in Table 1. In only one case, the resistance to acetamiprid was medium (Walcz population in 2009). In the remaining cases, the resistance was high (2008: Walcz and Krotoszyn and 2010: Walcz) or very high (2008: Września; 2009: Wręźnia and Krotoszyn; and 2010: Wręźnia and Krotoszyn).

The LC50 levels of chlorpyrifos ranged from 1.8 Wręźnia population (in 2008) to 7.86 ppm (Walcz population in 2010). The biggest difference within one population was found in the case of the Wręźnia population in 2008 and 2010, with the lowest value being twice lower than the highest one. The highest value of LC50 was 183 times lower than the highest one. The highest value of LC50 was 183 times lower than the highest one.

### Table 1. Susceptibility level of cabbage seed weevil adults to chlorpyrifos and acetamiprid

| Year | Active substance | LC50 (ppm) (confidence intervals, $P = 0.95$) | LC95 (ppm) | RC and resistance classification |
|------|------------------|------------------------------------------|------------|----------------------------------|
|      |                  | Września | Walcz | Krotoszyn | Września | Walcz | Krotoszyn | Wręźnia | Walcz | Krotoszyn | Wręźnia | Walcz | Krotoszyn |
| 2008 | Chlorpyrifos     | 1.80     | 4.38  | 2.48     | 8.35     | 28.14 | 10.31    | 0.006   | none  | none  | 19.97   | 8.14  | 8.61    |
|      | Acetamiprid      | 207.25   | 136.30| 165.59   | 2,396.4  | 976.27| 1,034.22 | 10.25   | very high | none  | high   | none  | 0.024  | 0.034  |
|      |                  | (142.75–348.73) | (100.27–193.84) | (120.91–242.82) | (129.61–219.07) | (3.94–5.06) | (3.21–7.69) | (1.85–3.93) | (1.93–3.07) | (1.59–7.69) | (1.85–3.93) | (1.93–3.07) |
| 2009 | Chlorpyrifos     | 4.49     | 5.34  | 2.92     | 16.59    | 34.91 | 20.04    | 0.011   | none  | none  | 0.024  | 0.034  |
|      | Acetamiprid      | 188.49   | 86.89 | 159.72   | 1,295.9  | 422.46| 1,230.12 | 10.79   | very high | none  | high   | none  | 0.024  | 0.034  |
|      |                  | (140.41–272.47) | (68.98–109.67) | (121.86–219.07) | (129.61–219.07) | (3.94–5.06) | (3.21–7.69) | (1.85–3.93) | (1.93–3.07) | (3.94–5.06) | (3.21–7.69) | (1.85–3.93) |
| 2010 | Chlorpyrifos     | 7.37     | 7.86  | 3.01     | 7.78     | 36.23 | 19.92    | 0.005   | none  | none  | 0.025  | 0.034  |
|      | Acetamiprid      | 267.02   | 191.80| 137.78–302.93 | 5,753.4  | 890.71| 1,223.5  | 47.94   | very high | none  | high   | none  | 10.19  | 0.034  |
|      |                  | (179.39–478.27) | (84.23–184.57) | (137.78–302.93) | (137.78–302.93) | (3.94–5.06) | (3.21–7.69) | (1.85–3.93) | (1.93–3.07) | (1.59–7.69) | (1.85–3.93) | (1.93–3.07) |

Results expressed in LC50, LC95, and RC.
The experiments were carried out on two populations: Wrzesień and Krotoszyn. Chlorpyrifos on the cabbage seed weevil ranged from 7.78 (Wrzesień population in 2009) to 5.19 (Wrzesień population in 2010). The biggest difference within one population was found within the Wrzesień population (with the highest value being 2.1 times higher than the lowest one) in 2009 and 2010. None of the LC95 values exceeded the dose of 1,440 ppm, with the highest LC95 value being 87 times lower than the recommended dose. The LC95 values in the research on the influence of acetamiprid showed a synergism with this substance. In all the experiments, the LC95 values ranged from 1.4 (Wrzesień population in 2009) to 5.15 (Wrzesień population in 2010). None of the LC95 values exceeded the dose of 1,440 ppm, with the highest LC95 value being 87 times lower than the recommended dose. The RC values for chlorpyrifos in all the cases indicated that the pest is not resistant to this substance.

The results of experiments devoted to the resistance mechanisms of the cabbage seed weevil to acetamiprid are presented in Table 2 and in Fig. 1. The experiments were carried out on two populations: Wrzesień and Krotoszyn in 2009 and 2010.

The results of experiments devoted to the resistance mechanisms of the cabbage seed weevil to acetamiprid are presented in Table 2 and in Fig. 1. The experiments were carried out on two populations: Wrzesień and Krotoszyn in 2009 and 2010.

The experiments designed to determine the interaction of acetamiprid and PBO did not show any significant synergistic action of these substances. The SC for LC50 revealed both a slight antagonism in each of the studied populations (Wrzesień population in 2009 and Krotoszyn population in 2010), with the values being, respectively, 0.95 and 0.94, and a slight synergism (Krotoszyn population in 2009 and Wrzesień population in 2010)—the values were, respectively, 1.21 and 1.31. The values of the SC for LC95 were similarly ambiguous, showing antagonism (Wrzesień population in 2009 and Krotoszyn population in 2010) or a slight synergism (Krotoszyn population in 2009 and Wrzesień population in 2010). The SC for LC95 was >1, with its values ranging from 2.35 (Wrzesień population in 2009) to 5.15 (Wrzesień population in 2010). The SC for LC95 ranged from 2.21 (Wrzesień population in 2009) to 3.13 (Wrzesień population in 2010). In all the experiments, it resulted in a change of the resistance classification of the studied populations from very high to high. The results indicate the participation of glutathione transferases in the processes of acetamiprid detoxification in the studied populations of the cabbage seed weevil.

To summarize, the experiments concerning the resistance mechanisms of the cabbage seed weevil to acetamiprid prove that the esterases blocked by PBO in the acetamiprid detoxification processes in the cabbage weevil in the studied populations.

The results of experiments devoted to the interaction of DEF and acetamiprid showed a synergism with this substance. In all the experiments, the SC for LC50 was >1, with its values ranging from 2.35 (Wrzesień population in 2009) to 5.15 (Wrzesień population in 2010). The SC for LC95 ranged from 2.21 (Wrzesień population in 2009) to 3.13 (Wrzesień population in 2010). In all the experiments, it resulted in a change of the resistance classification of the studied populations from very high to high. The results indicate the participation of glutathione transferases in the processes of acetamiprid detoxification in the studied populations of the cabbage seed weevil.

Discussion

The research on the resistance of the Colorado beetle and pollen beetle in Poland and around the world has a long history, and many aspects of this phenomenon in both these species have been explained. There are not, however, many works discussing the resistance of the cabbage seed weevil to insecticides.
The results of the research on the level of the susceptibility of the cabbage seed weevil to acetamiprid showed that the susceptibility of the pest is surprisingly low. The dose of acetamiprid recommended in Poland (120 ppm) may even not be sufficient to achieve a mortality rate of 50%. In the presented research, only one experiment with a concentration lower than the recommended one (120 ppm)—87 ppm showed a mortality rate of 50%. To achieve the mortality rate of 95% in the case of the cabbage seed weevil, it was necessary to use acetamiprid doses that were on average 2–12 times higher than the ones for the pollen beetle (Węgorek 2009, J.Z., unpublished data), and in comparison to the Colorado beetle, the concentrations were 20-100 times higher (Węgorek et al. 2011b). It proves that the cabbage seed weevil metabolizes acetamiprid more efficiently than the two other mentioned species and that it is necessary to take it into account when planning rapeseed protection programs. Cabbage seed weevils appear and feed on the rapseseed from the flowering period, i.e., when the highest number of honey bees, tolerating very high doses of acetamiprid, appears at the plantations (J.Z. and P.W., unpublished data). The resistance of the cabbage seed weevil to this insecticide potentially eliminates this active substance from the cabbage seed weevil control program when the harmfulness level values are exceeded significantly. This phenomenon is a negative one and needs to be taken into account when designing rapeseed protection strategies using insecticides. There is no research publications related to the resistance of the discussed pest to acetamiprid, except for the author’s own publication, which included limited information on this subject (Zamojska et al. 2010). The resistance of other species to the active substances from the group of neonicotinoids has been presented in many publications in the last 10 yr, including Choi et al. (2001), Daborn et al. (2001), Nauen et al. (2002), Wang et al. (2002), Byrne et al. (2003), Foster et al. (2003), Zeven et al. (2003), Nauen and Denholm (2005), and Gorman et al. (2008).

Resistance level of *C. assimilis* to acetamiprid might be caused by two factors. The first one is an evolutionary-created capability of metabolizing acetamiprid and the other one is a strong selective pressure of acetamiprid in the last 10 yr in Poland. These conclusions derive from the research on other insect species and theories of the cumulation of resistance genes (Malinowski 2003). However, the conclusion requires further research.

The values of the RC showed that the cabbage seed weevil is not resistant to chlorpyrifos. The research performed by Węgorek (2009) proves that the toxic effect of this active substance on the pollen beetle, related to the oxidizing desulfuration of chlorpyrifos, which in turn leads to increased toxicity, persists on fields for about 8 d after the treatment. The residues of chlorpyrifos on rapseseed decrease to <20 ppm on the third day after the treatment. The reaction of the cabbage seed weevil to chlorpyrifos is similar to that of the pollen beetle (Węgorek 2009). The LC50 level in the cabbage seed weevil in this research was similar, ranging from 1.8 to 7.9 ppm, whereas the LC95 level was significantly higher, ranging from 7.8 to 36.2 ppm, compared with the pollen beetle.

Both in Poland and in Europe, research has been conducted on the mechanisms of the resistance of different insect species, most frequently the pollen beetle and Colorado beetle, to pyrethroids and other insecticides (Węgorek 2002, 2004, 2009; Nauen 2007; Skillman 2007; Philippou et al. 2010; Węgorek et al. 2011a,b; Zamojska et al. 2013). Although many publications stress the significant role of the oxidative enzymes blocked by PBO in the detoxification of insecticides (Węgorek 2009, Philippou et al. 2010, Węgorek et al. 2011a, Zamojska et al. 2013), the participation of esterases cannot be excluded, as PBO also blocks this group of enzymes (although to a smaller extent; Gunning et al. 1998, Philippou et al. 2010). The issue of the role of esterases and glutathione transferases in the detoxification of insecticides in many insect species has also been discussed in world literature (Clark et al. 1986, Devonshire and Field 1991, Whyard et al. 1994, Parker et al. 1996, Gunning et al. 1998, Ranson and Hemingway 2005).

The research on the mechanisms of the detoxification of acetamiprid in the cabbage seed weevil did not prove that oxidative enzymes in this species play a significant role in the metabolism of the studied active substance. In turn, it was observed that esterases and glutathione transferases play a certain role in this process. However, the considerable difference in the toxicity of acetamiprid in the cabbage seed weevil compared with the Colorado beetle and pollen beetle (Węgorek 2009, Węgorek et al. 2011b) may stem from the differences in the molecular structure and affinity of the acetylcholine receptor to this toxin. Acetamiprid and other neonicotinoids have been used for the protection of rapeseed and potato since 1999, and the selective pressure to the studied species has not been long or strong. Thus, the cabbage seed weevil was probably better evolutionarily adapted and less susceptible to acetamiprid than the two other species of the beetles. In Poland, the Colorado beetle is not resistant to this insecticide, and the resistance of the pollen beetle is usually low. Both the species display natural differences in the tolerance to this insecticide. In turn, in the United States, the Colorado beetle has developed resistance to other neonicotinoids—relatively high resistance to imidacloprid (Mota-Sanchez et al. 2000, Dively 2006, Alyokhin et al. 2007) and low resistance to thiamethoxam (Grafius 2006). The resistance resulted from the oxidative mechanism blocked by PBO (Mota-Sanchez et al. 2006). The metabolic resistance to acetamiprid was observed in the diamondback moth (*Plutella xylostella* (L.)). Both PBO and DEF increased the toxicity of this active substance (Ninsin and Tanaka 2005). The fact that the cabbage seed weevil survives the recommended doses of acetamiprid is a negative phenomenon, potentially making it difficult to use this insecticide in the integrated rapeseed protection programs in Poland.

The fact that oxidative enzymes are not an important factor responsible for acetamiprid detoxification in the tested insect species can be the result of the lack of isoenzymes capable of metabolizing the acetamiprid molecule. Oxidative enzymes are very common in detoxification processes in different insect species, but their effectiveness is dependent on different enzymes versions. For example, the western honey bee (*Apis mellifera* (L.)), similar to the cabbage seed weevil, is resistant to high doses of acetamiprid and is susceptible to most pyrethroids and chlorpyrifos (Zamojska et al. 2010, J.Z. and P.W., unpublished data). It has been reported that PBO will sensitize honeybees to the effects of some pyrethroid insecticides (Johnson et al. 2006), but not to tau-fluvalinate (Moore et al. 2012). The level of synergism with PBO is dependent on molecular structure of the active substance in pyrethroids (Gunning et al. 2007). Our experiments suggest similar metabolism processes in the cabbage seed weevil. Also, different esterases’ isoenzymes can be of various effectiveness to different active substances of insecticides (Capineri et al. 2008, Montella et al. 2012). This may be the reason why esterases responsible for acetamiprid detoxification are not involved in chloropyrifos resistance development. This result proves that insect resistance is a greatly complex matter.

The problem of the resistance of any pest species is a complicated one and should be analyzed as a separate issue and research subject. The conclusions of the research on one species cannot be used in the case of other species. One characteristic of many of the studied species is the participation of at least a couple detoxification metabolic pathways with different levels of expression (Pospischil et al. 1999, McAbee et al. 2004). The results of the research are of particular importance for the protection of rapeseed, in the case of which the cabbage seed weevil and pollen beetle have to be controlled at the same time using active substances, to which the two pests react differently. The developed integrated production and plant protection programs are and will be based on monitoring and preventing the resistance of the pests.

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