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Chapter 2

Role of AChE in Colorado Potato Beetle (Leptinotarsa decemlineata Say) Resistance to Carbamates and Organophosphates

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Additional information is available at the end of the chapter

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Abstract

Colorado potato beetle is one of the most important pests of potatoes and one of the most difficult insects to control. Over the years, none of the control techniques developed against this pest has provided long-term protection for potato crops. Worldwide, CPB is resistant to all major groups of insecticides, including organophosphates and carbamates. The target site of organophosphate (OP) and carbamate insecticides is the same; they inhibit the activity of AChE. The function of acetylcholinesterase (AChE) is degradation of acetylcholine (ACh - neurotransmitter) in the insect cholinergic synapses. Mutations in the AChE-encoding locus have been shown to confer target site insensitivity to organophosphate and carbamate insecticides, leading to modification of AChE (MACE). A range of other amino acid substitutions in AChE confer insecticide resistance, and these mutations typically reside near to or within the active site of the enzyme. Such AChE mutations, associated with insecticide resistance, mostly known as Ace in Drosophila, have also been observed in other species, including L. decemlineata. Based on bioassays and literature, modified/insensitive AChE confers two major patterns of resistance to OPs/carbamates. Pattern I resistance is characterized by significantly higher resistance ratios (RR) (much greater reduction in the sensitivity of AChE at the biochemical level) to carbamates than to organophosphate insecticides. Pattern II resistance is characterized by resistance ratios (and/or reductions in the sensitivity of AChE) that are approximately equivalent for both carbamates and OPs. There are also a few species for which an insensitive AChE has been reported and for which molecular data have been collected, but for which the resistance profiles for both OPs and carbamates have not been reported. For CPB, both patterns were registered.

Keywords: *Leptinotarsa decemlineata*, Colorado potato beetle, AChE, enzyme activity, resistance, insecticide, metabolism

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1. Introduction

Colorado potato beetle – CPB (Leptinotarsa decemlineata Say, Coleoptera: Chrysomelidae) is an oligophagous species that attacks numerous plants of cultivated and spontaneous flora in the Solanaceae family in North America, Europe, and parts of Asia [1, 2, 3]. Within the genus *Leptinotarsa*, the Colorado potato beetle has the widest host range, feeding on at least 10 species of wild and cultivated solanum [4]. Defoliation of potatoes, which is most intense during the bloom, makes huge losses in tuber yield, depending on the growth stage [5].

From the botanical aspect, the potato, *Solanum tuberosum* L., is a perennial plant, yet in practice it is often grown as an annual plant due to its vegetative method of propagation. The potato is a crop of the Western Hemisphere. It first appeared in Europe in 1573 in Spain, and in 1586 it reached England and Ireland. In Western Balkan, the potato did not come until 1759, when it arrived in Banat region. It is the third-largest crop in our country when it comes to growing areas, right after the maize and wheat. However, the total number of areas under potatoes is declining, which is a general tendency in Europe [6].

The potato is in all stages of its vegetation period susceptible to pests, the most important of which is the Colorado potato beetle (CPB). In Balkan region, this pest can also be found on tomatoes, aubergines, and peppers, or buttercups – when it comes to plants from spontaneous flora. Colorado potato beetle is a limiting factor to potato production in our region and in the rest of the world, while in some regions it is also a harmful pest of tomatoes and aubergines. In our conditions, CPB produces two generations per year (as well as incomplete 3rd generation), thus ensuring large populations for which 3-4 control treatments per year are necessary. Although there are some alternative methods [7], conventional insecticides are still most important in CPB management.

Adults can overwinter in the soil at the depth of 7.5–12.5 cm. After emerging from the soil in spring, they spread by walking and flying on adjacent fields, where they immediately start to feed on host plants. After 5–10 days, females lay eggs (in clusters of 20–60 eggs) on the underside of leaves. In laboratory conditions, females live for 120 days, and their maximal fecundity is over 4,000 eggs [1]. The eggs are hatched simultaneously, and the hatched larvae immediately start to feed. Larval development (four stages) lasts for 10–20 days, depending on temperatures. Feed consumption depends on plant hosts, and for one plant host it is relatively constant in all larval stages. Defoliation of potatoes, most intensive during the flowering period, makes great losses in tuber yields [8, 9, 5]. Considerably lower yields were a result of strong defoliation (20%) few weeks before harvest [10]. After feeding has stopped, the fourth instar larvae drop from the plant, burrow into the soil, and pupate. Imagoes eclode in the ground, from where they emerge, find the closest plant host, and start to feed. Depending on a number of factors (temperature, photoperiod, and the state of the plant host), imagoes mate and produce a new generation of beetles, fly to other fields or stop feeding and enter diapause [1].

Remarkable ability of CPB has to adjust to adverse environmental conditions, manifested in its resistance to insecticide, which results in increased costs of potato production, environmental pollution, and a disturbance in biocenotic balance. A whole series of resistance

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mechanisms, such as lower permeability, enhanced metabolism of insecticides, change in target-site sensitivity, and change in behavior, make CPB very difficult to manage [11]. It has developed resistance to all classes of insecticides, rapidly shortening the period of resistance to new insecticides. The resistance of CPB to toxicological, biochemical, and genetic methods have been studied by numerous authors worldwide. A well-designed program of CPB management may protect crops successfully and extend the period of insecticide use. One of the basic measures of integrated pest management is resistance monitoring [6, 12].

2. Management of Colorado potato beetle

Rapid adjustment to new biotic and abiotic conditions is highly present within CPB populations. As a result, it is considered to be the most serious defoliator pest of potatoes in the world [11]. Nontreated pest populations can defoliate and completely destroy yields in the period of tuber formation [1]. Before CPB, we did not have a pest able to seriously harm potato crops. After coming to a new habitat, this pest finds conditions favorable for life and reproduction, which enables them to occur permanently and at a massive scale. The destructive activity of this pest is such that potato crops would be destroyed without efficient protection [13].

In most regions with dense CPB populations, chemical treatments are still the number one method in potato protection, considering that alternative measures are not efficient enough [11]. Most potato protection programs require decreasing CPB populations at the beginning and middle of the vegetation period, and tolerating higher populations at the end of the vegetation period. In general, the goal of such programs is to limit total defoliation to 10–25% during the most critical periods of potato development. Without chemical treatments, yield losses would amount to 74% [6].

In Balkan region, potatoes are treated with insecticides 3–4 times per year to ensure normal yields [14]. CPB management costs potato growers hundreds of million dollars a year [2]. Large numbers of pesticides from different chemical classes have been used so far to manage CPB, such as insecticides from the class of chlorinated hydrocarbons, organophosphates, carbamates, pyrethroids, nereistoxins, insect growth regulators, neonicotinoids, as well as bacterial products, GMOs, products of plant origin (azadirachtin), etc. Over the last few decades, only a few insecticides in our country have been satisfactorily efficient [15, 16, 17, 18].

3. Brief overview of CPB resistance

CPB has an amazing ability to adjust to toxicants from different chemical classes [19], by developing a range of different resistance levels to all classes of insecticides applied to manage this pest [1, 20, 21, 22, 23, 24]

The use of DDT and other organochlorine insecticides was efficient after World War II. The first information on DDT resistance dated back to early 1950s, specifically to 1952, reported by Quinton [19] and Hoffmaster and Waterfield [25]. Since then, CPB has developed resistance

to a wide range of insecticides, including arsenic compounds, organochlorine compounds, carbamates, organophosphates, and pyrethroids [1, 19] and more recently to neonicotinoids [13, 23, 26, 27, 28, 29]. Experimental proofs on development of resistance to *Bacillus thuringiensis* products and to transgenic plants, were also obtained [19, 30, 31]

In Balkan region, a significant level of CPB resistance was detected in 1967 to insecticides from the class of chlorinated hydrocarbons [32], which was proved for most localities of ex-Yugoslavia [33], where the resistance to organophosphorus insecticides and carbamates was detected in some CPB populations. Studying CPB resistance was continued in the years to follow [34, 35]. Remarkably high levels of resistance of the fourth instar larvae to quinalphos and carbaryl were recorded [36, 37]. Research on insecticide resistance level of CPB to most commonly used insecticides is ongoing [6, 17, 18, 38].

The rate of resistance development increases progressively with the introduction of new, synthetic insecticides. When it comes to pyrethroids, this resistance occurred 2–4 years after pyrethroids were put into practice and widely used. Physiological and genetic mechanisms of CPB resistance have been little studied. It is expected that in the future CPB will develop resistance to all newly introduced insecticides [1].

4. Insect resistance to insecticides

According to IRAC (Insecticide Research Action Committee), resistance may be defined as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species."

Cross-resistance may be described as an ability to simultaneously develop tolerance to substances they have never been exposed to (resistance to one insecticide confers resistance to another, newly introduced insecticide). Due to large populations and numerous descendants (they breed quickly), there is always a risk that insecticide selection pressure will ultimately result in insecticide resistance. Insecticide resistance may evolve rapidly, especially when field application of insecticides is misused or overused.

The resistance occurs with behavior change, when the insects avoid contacts with the toxicant [39], or physiological change [40], where they survive toxicant exposure. Most important mechanisms of resistance are reduced cuticle penetration, increased excretion, increased metabolic detoxification and altered target-site sensitivity. Knowing molecular basis for emergence and development of resistance is very important for developing appropriate measures and strategies to slow down the resistance [41].

There is never only one cause of resistance. Hence, regardless of the type, the cause of resistance to different pesticides can vary from substance to substance.

According to Hassall [42], factors that lead to resistance are:

1) morphological, 2) physiological and biochemical, and 3) behavioral.

4.1. Morphological changes

Such changes lead to a reduction of the amount of a pesticide that comes into an insect's body in a unit of time, compared to the amount that comes into the body of a susceptible insect. The change in lipid content of the insect's cuticle can result in reduced absorption, as a factor for overall resistance development. This so-called penetration resistance is quite significant since it often goes along with other resistance mechanisms, enhancing their effect. In its simplest form, this can be explained by the fact that different speed of intake is a factor that, combined with excretion, provides a high level of resistance, even during usual enzyme activity [43].

4.2. Metabolic changes

Populations of resistant insects may detoxify or destroy the toxin faster than susceptible insects, or quickly rid their bodies of the toxic molecules. This type of resistance is the common mechanism and often presents the greatest challenge. Resistant populations may possess higher levels or more efficient forms of these enzymes. In addition to being more efficient, these enzyme systems also may have a broad spectrum of activity (i.e., they can degrade many different insecticides).

Changes in insect metabolism could be manifested as:

- Change in metabolic rate of a certain insecticide. Resistant strains increase the amounts of enzymes or their activity (a wide range of impact). These changes come as a result of the organism's reaction to stimulants. When it comes to transcription control, the metabolism is reformulated in order to increase the level of transcription, which leads to better supply of enzymes (gene amplification, P450 for example).
- Loss of sensitivity of the target-site, receptors, or enzymes as a responsive reaction to a stimulant, or a change in transcription control, i.e., change in encoding gene expression (in structural genes, direct change occurs in the structure of the enzymes, i.e., there are different forms of P450, ALiE, GST, AChE) [44].

4.2.1. Gene amplification

Gene amplification is the multiple copying of structural genes that manage the synthesis of enzymes, thus ensuring hundreds of copies of structural genes. Increased detoxification can be a result of better supply of enzymes, and this quantitative increase is caused by gene amplification [45]. Gene amplification has been determined as a key factor for increased esterase production or change in sensitivity of AChE and Na+ channels (the target-site of 90% of insecticides), but not GST [46].

In the first stage, the metabolism of insecticides is manifested through many reactions, most important of which are oxidation, reduction, and hydrolysis. In the second stage, conjugates are formed, which are practically nontoxic. Selective toxicity of insecticides mostly comes from the balance of the reactions included in activation and detoxification [6].

4.2.1.1. Oxidative processes

Oxidative processes have a dominant role in the metabolism of insecticides present in living organisms. These reactions are catalyzed by enzymes of multifunctional oxidases (MFO) or mono-oxygenises, located in the endoplasmic reticulum, i.e., in microsoms. Cyt P450 is the active center of MFO. MFO mechanism has evolved due to the need of living organisms to protect themselves from many natural toxicants they are constantly exposed to [47, 48]. It is clear that several forms of Cyt P450 exist both in resistant and susceptible strains, while there is a quality difference in different strains [49].

MFO activity of CPB (epoxidation, N- and O-demethylation) is 2–3 times higher in resistant species than in susceptible species. Resistant species have two different types of mfo. Type 1 mfo provides resistance to permethrin, and weak cross-resistance to azinphosmethyl and carbofuran. Type 2 provides resistance azinphosmethyl and carbofuran, but not to permethrin [50]. This mechanism is comprised in the resistance of CPB larvae and adults to imidacloprid [29].

4.2.1.2. Hydrolytic processes

Insecticide detoxification primarily unfolds through molecule hydrolysis on different sites, thereby breaking ester, carboxyl-ester, amide, and other chemical bonds. Pyrethrins, pyrethroids, organophosphates, carbamates, and other insecticides are degraded by hydrolysis. This is the basis for the selective effect of insecticides and for insects' resistance mechanisms. The most important hydrolytic enzymes are phosphoric triesters and carboxylesterases (ALiE esterases, nonspecific or B-esterases) [50].

Esterase-related insect resistance is based on the following:

- Increase in the total amount of esterase – by altering regulatory genes or regulatory loci combined with structural genes, which results in change in enzyme synthesis in the organism [51] or amplification of genes responsible for DNA methylation.
- Change in their activity – by altering structural genes that directly determine the nature of enzymes.

The impact of nonspecific esterase on the level of resistance to carbamates has not been confirmed [52], which was also [36] indicated in the case of CPB. The role of esterase in CPB resistance was confirmed [29, 36, 53].

4.2.1.3. Conjugation processes

Forming of conjugates almost always implies detoxification, but sometimes there are some cases of toxicant reactivation. The most important conjugation reactions are: glutathione conjugation, glucoside or glucuronide conjugation and amino acids conjugation. A change in GST activities depends on modifying a series of enzymes, rarely on only one enzyme, as in esterase. In this class of enzymes, there is no proof that enzyme amplification and resistance are related [54]. When determining the amount of GST-metabolite of azinphosmethyl in

resistant CPB strains, no direct impacts of GST to resistance were found. The authors think that the share of GST in total resistance is manifested through the transformation of toxic oxidative metabolites of azinphosmethyl, whose levels are higher in resistant strains [46].

4.2.2. Change in target-site sensitivity

There are four basic groups of macromolecules, depending on the neurotoxic insecticide target-site:

Target-site	**Active ingredient**
Acetylcholinesterase (AChE)	Organophosphates, carbamates
Na^+ channels	Pyrethroids, DDT
GABA receptors	Lindane, cyclodienes, fipronil
ACh receptors	Neonikotinoides, nereistoxins

4.2.2.1. Acetylcholinesterase (AChE)

AChE is a target-site for organophosphate and carbamate insecticides. The structure of AChE has undergone some changes that resulted in different levels of transformation of differently structured AChEs. Modified forms of AChE differ among species. As a result, many different forms of cross-resistance are possible [42]). It is important to determine kinetic constants, especially Michaelis constant (Km). It is a constant that measures the enzyme's affinity toward the substrate (ACh, butyrylcholine, and ATCh). During the 1980s and 1990s, some authors [42, 55, 56, 57] indicated that altered AChE causes the resistance to carbamates and organophosphate compounds.

Studies on resistance to organophosphates and carbamates have shown that AChE activity of CPB is quite pronounced and easily measured. The AChE activity of the fourth instar larvae was determined by measuring the absorption, at 585μm wavelength. Total AChE activity was correlated with the determined resistance to carbamate insecticides [36].

4.2.2.2. Na+ channels – Sodium channels

In some cases where resistance cannot be explained by other causes, one can assume there has been a modification in the target-site structure. The exception is the resistance of flying insects that can lead to diminishing of the knockdown effect. In houseflies, this property is carried by the kdr gene and it can be associated with the alternation of receptors in the nerve cell membrane. Pyrethroids can predominantly affect synaptic sites, which are less sensitive in resistant housefly strains. There is some evidence that the term "change in target-site sensitivity" was coined to explain the kdr resistance factor [58, 59, 60]. The target-site inactivity of motor nerves' ends to permethrin and deltamethrin is also proved. The insensitivity of binding-site of resistant strains can be a result of multiple insecticide receptors. It is also possible that weakened binding can be a result of structural changes in proteins or changes in the structure of lipids adjacent to ion pumps in kdr-resistant strains, which can cause minor problems in the

mechanism of the ion pump or diminish the process of repetitive polarization, typical for unchanged receptors. It is not clear whether the changes detected in lipid structures of neural membranes of kdr and super-kdr strains of houseflies are a factor that reduces the sensitivity of Na+ channels or these are just compensation changes, necessary for normal functioning of modified Na+ channels [56, 61], as a cause of pyrethroid resistance, which point out changes in the target-site (modification of Na+ channels), detoxification increased by oxidation, hydrolysis, and specific proteins.

4.2.2.3. GABA receptors

By using subcellular products of the neural tissue of insects, several studies have shown that cyclodienes and lindane have a neurotoxic impact by blocking the GABA receptor complex. Studies on brain tissues of cockroaches showed that resistant strains had 90% lower sensitivity of GABA receptors to cyclodienes. It was found that mutations of Rdl-genes that encode the GABAa receptor subunit caused the resistance of Hypothenemus hampei Ferrari to endosulphan [62].

4.2.2.4. ACh receptors

Acetylcholine (ACh) is a neurotransmitter that regulates a large number of vital functions. Its activity is enabled by two types of postsynaptic ACh receptors (muscarinic and nicotinic – nAChR). Nicotinic receptors mainly act as ACh activity modulators [63, 64]. The activity of insecticides is manifested through nAChR activation or blocking. The basic structure of nAChR consists of five protein subunits, mostly two identical alpha-subunits and three beta-subunits that give it a pentagonal shape. So far, scientists have detected ten different nAChR genes in insects. The number of nAChR genes implies there are much more nAChR protein subunits, whose main role is recognition when binding the receptor on one side and ACh or insecticide on the other side [65].

Every modification in the protein structure, even the smallest one, can reduce the affinity of nAChR. This mechanism can be a reason for reduced CPB sensitivity to imidacloprid when oxidative and hydrolytic enzymes are blocked [29].

4.3. Behavioral changes

This type of resistance implies the evolution of behavior, manifested in reduced exposure to toxic compounds or in the insect's ability to survive in toxic or some other kind of fatal environment. Flying insects can acquire the instinct not to dwell long on contaminated surfaces [42]. This resistance mechanism has been recorded for many insecticide classes [66]. Insects simply stop feeding or leave the treated surface.

In CPB, where management is increasingly based on growing transgenic potatoes that contain Bacillus thurigiensis δ- endotoxin, the correlation between these two resistance mechanisms is very significant. More pronounced physiological resistance was recorded [67] in the larvae that avoided transgenic potato crops [31] grown in the same field with nontransgenic potato crops.

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4.4. Cross-resistance and multiresistance

Insect populations exposed to certain insecticides have an ability to simultaneously develop tolerance to other substances they have never been exposed to. Insecticide resistance is model of rapid evolution within populations and typical example of directional selection. Eradication of susceptible genotypes from field populations increases both the frequency of resistant genes, which became dominant especially in the absence of susceptible insect refugees, and the application dose of insecticide, needed to keep pest below economically damaging levels. Same physiological or biochemical mechanisms of resistance to one group of insecticides, in some cases, leads to resistance to insecticides from other group/class; such phenomenon is commonly known as cross-resistance [4].

Cross-resistance enables resistant insects to survive the exposure to insecticides with similar chemical composition to the one they are resistant to. In general, cross-resistance results in detoxifying or changing sensitivity to common biochemical and physiological damage. It also happens when one enzymatic system detoxifies more than one class of insecticides [68]. Hence, cross-resistance does not necessarily spread to all members of the same group of insects or it is limited to only closely related pesticides. Similarities in their mode of action or, sometimes the similarity of their enzymatic systems, are more important for their degradation.

On the other hand, multiresistance is resistance to insecticides from different classes. It depends on different mechanisms, so insects can develop resistance to a large number of insecticides from different classes, regardless of their chemical structure. Each new insecticide can cause one or more resistance mechanism to develop, and each developed mechanism results in resistance to similar insecticides. Multiresistance can occur when the organism develops more than one mechanisms of resistance, such as change in AChE sensitivity combined with multienzymatic detoxification, as in the case of organophosphates [68]. Rapid development of multiresistance is the most important reason why organisms quickly become resistant to new compounds introduced to replace inefficient insecticides, which is a result of persistent R-genes and their interactions manifested in several mechanisms of resistance [69].

Recorded cases of negative cross-resistance are very important. Increased resistance to one compound can lead to increased susceptibility to another [70]. Negative cross-resistance has still not been commercially exploited in field conditions, but knowledge on this mechanism can potentially be very important in practice.

Cross-resistance limits the choice of available insecticide, whereas multiresistance represents a rapid overview of insecticide selection that prevents us from reusing insecticides on resistant species for a longer period [69].

5. Role of AChE in Colorado potato beetle resistance to carbamates and organophosphates

Organophosphates and carbamates are neural toxins, characterized by high toxicity and a quick action. They act inhibitory on acetylcholinesterase (AChE), during neural transmis-

sion. Organophosphates phosphorylate enzymes, whereas carbamates form an enzyme-inhibitor complex. The forming of this complex is not irreversible but the reactivation rate is 10^5–10^6 slower when compared to a similar reaction in case of ACh and AChE. The enzyme cannot disintegrate new ACh. Although organophosphates and carbamates have a similar mode of action, there are also some pronounced differences between them, mainly as a result of different binding site in the active center and due to different geometries of nucleophilic attack [71].

Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme in the nervous system (55), terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. Carboxylesterases belong to a multifunctional carboxylesterase/cholinesterase superfamily (CCE). They are ubiquitous in most living organisms, including animals, insects, plants, and microbes. CCEs, regarding their physiological and biochemical functions could be divided in three groups: dietary/detoxification, hormone/semiochemical processing, and neurodevelopmental [72]. AChE is the major target for organophosphate and carbamate insecticides, which inhibit enzyme activity. Such inhibition of AChE causes excessive excitement in nerves, a blockage of neurotransmission, and the death of insects. Insensitivity of AChE to organophosphates and carbamates is one of the important mechanisms for insecticide resistance. Changes in AChE lead to an efficient mechanism of resistance to OP and carbamate insecticides and that is site insensitivity at the target enzyme, acetylcholinesterase (AChE) [73, 74].

For testing the resistance to organophosphates and carbamates, caused by altered AChE, the determination of kinetic constants, especially the Michaelis-Menten constant (Km) is of great importance. It measures the affinity of the enzyme to its substrate [42].

The first case of AChE with decreased susceptibility to pesticides was described by Smissaert in 1961 [75]. Ioannidis et al. [76] first characterized a field population of the carbofuran-resistant CPB. The resistance was determined to be autosomal and monofactorial, leading to a decrease in AChE sensitivity to carbofuran inhibition. A study of a crude enzyme preparation [77] showed that AChE from the AZ-R strain had a 2.4-fold reduction of affinity to acetylthiocholine (ATCh) compared with AChE from a susceptible (SS) strain.

As previously recorded, altered acetylcholine esterase plays a critical role in resistance to organophosphates and carbamates [76, 78, 79]. The measurement of AChE activity is commonly used as a biomarker of exposure to different pesticides [80]. Kinetic analysis of AChE was used to explain the resistance of some insect strains and the selectivity of some organophosphate and carbamate insecticides. A lot of different studies have described CPB resistance to OPs and CBs [46, 77, 81, 82]. Azinphosmethyl resistance has been reported in CPB; high level of resistance (136-fold) in a nearly isogenic CPB strain (AZ-R) was due to multiple resistance mechanisms, including reduced penetration, enhanced xenobiotic metabolism, and target site insensitivity [83]. Russel et al. [84] suggest that interspecific comparisons of bioassay and biochemical data suggest two major patterns of resistance to OPs/CBs resulting from an insensitive AChE: Pattern I resistance, which is generally more effective for carbamates, and Pattern II resistance, at least as effective for organophosphates as it is for carbamates and may even be specific to organophosphates in some cases.

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The role of acetylcholinesterase (AChE) as the primary mechanism for removing the excitatory neurotransmitter, acetylcholine (ACh), from cholinergic synapses and its role as the target site for organophosphate and carbamate inhibitors and accumulation of ACh that results from the inhibition of AChE leads to the prolonged stimulation and, in many cases, the desensitization of the ACh receptors, eventually to severe neurological disruption, and ultimately to death [85]. Since AChE causes death, irreversible inhibitors have been developed as insecticides: organophosphates and carbamates. They have similar properties to acetylcholine but are hemisubstrates ultimately leading to irreversible inhibition of the enzyme. This inhibition leads to an accumulation of acetylcholine in the synapses (active site of the enzyme is therefore occupied and incapable of hydrolyzing its normal substrate) which in turn leaves the acetylcholine receptors permanently open, resulting in the death of the insect [75].

CPB populations resistant to azinphosmethyl contained two mutations in the AChE (S291G and R30K), which made the enzyme less sensitive to azinphosmethyl and carbofuran [73, 86]. In the strain resistant to carbofuran, the presence of two mutations (I392T and S291G) did not result in resistance, but the presence of just one (S291G) conferred high resistance to carbofuran and medium resistance to azinphosmethyl [73]. Compared to the susceptible strain, due to altered acetylcholine esterase, strain resistance to azinphosmethyl had a reduced substrate affinity for ATCh and azinphosmethyl oxon [87]. Modifications in acetylcholine esterase, resulting in resistance, may be selective. It was found that while one resistant strain was highly insensitive to arylcarbamates, another strain with the same affected enzyme was highly insensitive to organophosphates, but not arylcarbamates. Such changes in AChE made yet another resistant strain more sensitive to α-chaconine, a glycoalkaloid present in potatoes and an inhibitor of AChE. Additionally, modified AChE also had increased sensitivity to tomatine, which is also glycoalkaloid present in tomatoes [78].

Zhu and Clark [77] demonstrated that the less bulky substrates, such as ATCh, interact poorly with AChE from the AZ-R strain than AChE from the SS strain of CPB. Such structure–activity relationships may be an indication that a similar alteration in amino acid residues has taken place in the acyl pocket size in AChE from the AZ-R strain and has resulted in the altered substrate and inhibitor profile.

The target site of organophosphate (OP) and carbamate insecticides is the same; they inhibit the activity of AChE. The function of acetylcholinesterase (AChE) is degradation of acetylcholine (ACh – neurotransmitter) in the insect cholinergic synapses. Mutations in the AChE-encoding locus have been shown to confer target site insensitivity to organophosphate and carbamate insecticides, leading to modification of AChE (MACE). A range of other amino acid substitutions in AChE confer insecticide resistance, and these mutations typically reside near to or within the active site of the enzyme. Such AChE mutations, associated with insecticide resistance, mostly known as Ace in Drosophila, have also been observed in other species, including *L. decemlineata*. Based on bioassays and the literature, modified/insensitive AChE confers two major patterns of resistance to OPs/carbamates [84]. Pattern I resistance is characterized by significantly higher resistance ratios (RR) (much greater reduction in the sensitivity of AChE at the biochemical level) to carbamates than to organophosphate insecti-

cides. Pattern II resistance is characterized by resistance ratios (and/or reductions in the sensitivity of AChE) that are approximately equivalent for both carbamates and OPs. There are also a few species for which an insensitive AChE has been reported and for which molecular data have been collected, but for which the resistance profiles for both OPs and carbamates have not been reported. For CPB, both patterns were registered.

In a few cases of each pattern, gene sequencing has identified the molecular nature of the alteration leading to the lowered sensitivity to inhibitors. Although it is not possible yet to relate with full confidence the mechanism by which these structural changes alter sensitivity to inhibitors. Pattern I mutations may involve changes in the active site, such as a common Gly^Ser mutation in the oxyan–ion hole, whereas Pattern II changes may result in a constriction of the cleft leading to an active site that limits the access of inhibitors and, presumably, of ACh itself. Insensitivity to inhibitors may be accompanied by a reduced ability to hydrolyze ACh. Whether this is always deleterious to the organism is unclear since it is generally considered that, as in vertebrates, AChE is present in insects at a level considerably in excess of that needed for basic neurological functions under normal physiological conditions.

Biochemical studies using an affinity-purified AChE from the SS strain established that the AChE associated with CPB possessed typical characteristics of other AChEs and consists of two different molecular forms: the major form (92%) was a hydrophilic dimer, whereas the minor form (8%) was an amphiphilic dimer. Both molecular forms had virtually identical molecular weights and isoelectric points. Amino acid analysis indicates that the mole percentages of amino acids of the AChE from CPB were highly comparable to those previously reported for AChE from Drosophila [77].

According to Zhu and Clark [77], affinity (Km) and hydrolyzing efficiency (Vmax) of AChE purified from a near-isogenic azinphosmethyl-resistant (AZ-R) strain of CPB to selected substrates, including acethylthiocholine, acetyl-(5-methyl) thiocholine, and propionythiocholine, were lower than those of AChE purified from a susceptible (SS) strain. AChE from the SS strain was significantly inhibited by higher amounts of acethylthiocholine and acetyl-(fj-methyl) thiocholine, whereas AChE from the AZ-R strain was activated by higher amounts of all four substrates examined.

Finally, it is important to notice results on toxicological tests and measuring activity of AChE of CPB populations in Serbia, resistant to OPs and carbamates [88]. The order of resistance levels for OPs and carbamates was completely opposite. Experiments showed that acetylcholinesterase (AChE) activity of CPB was very pronounced and easily measured. At a constant AChE concentration, increasing the substrate concentration will cause a positive, linear, and dependent increase in the reaction. The same applies in the reaction with constant substrate concentration and increased enzyme concentrations. AChE activity is significantly affected not only by location, but also by substrate concentration (acetylthiocholine iodide ATChI). Considering that ATChI (substrate) in increased concentrations inhibits normal AChE activity, it can be concluded that altered AChE affected the change in the population order. The total AChE activity is in correlation with the determined resistance to carbamates.

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6. An integrated approach to CPB control

The IRAC recommendation [89] is that the most effective strategy to combat insecticide resistance is to do everything possible to prevent it from occurring in the first place. It is recommended to develop and apply IRM (Integrated Resistance Management) programs as one part of a larger IPM program. Field researchers and entomologists should be focused on three basic components: pest monitoring, economic injury levels, and integration of multiple control strategies. It is essential to widely implement Economic Thresholds (ET) (use of insecticides only if pest populations are able to cause economic losses that exceed the cost of the insecticide plus application, or where there is a threat to public health). Integrated Control Strategies: Incorporate as many different control strategies as possible including the use of synthetic insecticides, biological insecticides [90, 91, 92, 93, 94], beneficial insects (predators/parasites) [2, 95], cultural practices, transgenic plants (where allowed), crop rotation, pest-resistant crop varieties, and chemical attractants or deterrents [96, 97, 98, 99, 100].

Applications of insecticide must be timed correctly, targeting the most vulnerable life stage of the insect pest. The use of spray rates and application intervals recommended by the manufacturer and in compliance with local agricultural extension regulations is essential.

Integrated pest management (IPM), with the reduced application of synthetic insecticides, has an increasing need for alternative methods of plant protection. Together with systematic insecticide resistance monitoring [6, 12, 89], application of plant extracts with antifeedant and repellent effects could be one of the tools in efficient IPM program [101, 102, 103].

According to Boiteau [7], our understanding of the potato ecosystem and a number of preventive and curative control methods is sufficient to undertake a holistic approach to insect pest management. The harmonization of concepts should stimulate the integration of insect control methods beyond the level of the single pest. The greatest challenge is to bring together bad and good control methods, as well as conventional and sustainable (or organic) crop protection. This will require learning how to manage the unpredictability (uncertainty) of ecologically based IPM methods. Active adaptive management (AAM) is one approach that has been suggested to manage the different types of uncertainty [104]. Research and openness to new ideas will be essential for harmonization of the different insect control approaches and potato crop protection systems [7].

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