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ORIGINAL ARTICLE

Resistance to common organophosphate and carbamate insecticides in *Aphis pomi* (Hemiptera: Aphididae)

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Abstract – Introduction. *Aphis pomi* (De Geer) has developed resistance to organophosphate and carbamate insecticides, as a result of long-term application of these insecticides in conventional apple orchards. For many years, the only mechanism of resistance identified in aphids was overproduction of insecticide-detoxifying esterases. **Materials and methods.** Insecticide resistance of *A. pomi*, collected from two conventional apple orchards (localities of Radmilovac – RA and Bela Crkva – BC) and one organic apple orchard (locality of Surčin – SU), was tested by bioassays and biochemical assays. **Results and discussion.** Compared with LC_{50} values for the susceptible population (organic orchard), both populations from the conventional orchards were highly resistant to pirimicarb (234.5 and 52.9 times) and moderately resistant to dimethoate (10.7 and 9.0 times). Increased esterase activity was determined in these two resistant aphid populations. Each of them also produced one esterase isoform more than the susceptible population, when 1-naphthyl acetate was used as a substrate for zymographic detection; when 2-naphthyl acetate was used as a substrate, only one resistant population produced two new esterase isoforms. In one of the resistant populations acetylcholinesterase (AChE) was significantly less inhibited by pirimicarb than in the other resistant population and the susceptible population, which indicates that this population developed another resistance mechanism – Modification of AChE (MACE). **Conclusion.** Detoxification of insecticides by the metabolic resistance mechanism of esterase enzymes and mechanism of modification of AChE was proven in one aphid population (RA). The other population (BC) has developed only metabolic resistance (enhanced metabolism by esterases), without modification of the insecticide target site (AChE). Development of insecticide resistance was caused by long-term application of acetylcholinesterase inhibitors (organophosphates and carbamates) in these conventional orchards.

Keywords: Serbia / apple / *Malus domestica* / *Aphis pomi* / pesticide resistance / pirimicarb / esterases

Résumé – Résistance aux insecticides organophosphorés et aux carbamates couramment utilisés sur *Aphis pomi* (Hémiptère : Aphididae). Introduction. *Aphis pomi* (De Geer) a développé une résistance aux organophosphorés et aux carbamates, à la suite de longues expositions à ces insecticides dans les vergers en culture conventionnelle. Pendant de nombreuses années, le seul mécanisme de résistance identifié chez le puceron était la surproduction des estérases de détoxification de l'insecticide. **Matériel et méthodes.** La résistance aux insecticides des pucerons collectés dans deux pommeraies en culture conventionnelle (localisés à Radmilovac - RA et Bela Crkva - BC) et dans une pommeraie en culture biologique (localisé à Surčin - SU), a été soumise à une batterie de bio-essais et de tests biochimiques. **Résultats et discussion.** Comparativement aux valeurs de la DL50 pour la population sensible (verger biologique), les deux populations des vergers conventionnels se sont montrées très résistantes au pirimicarbe (234,5 et 52,9 fois) et modérément résistantes au diméthoate (10,7 et 9,0 fois). Une augmentation de l'activité estérase a été mise en évidence dans ces deux populations de pucerons résistantes. Chacune d'elles a également produit un isoforme d'estérase de plus que la population sensible, lorsque l'acétate de 1-naphtyle a été utilisé comme substrat pour la détection zymographique. Lorsque l'acétate de 2-naphtyle a été utilisé comme substrat, une seule population résistante a produit deux nouveaux isoformes d'estérase. Dans l'une des populations résistantes, l'acétylcholinestérase (AChE) a été significativement moins inhibée

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par le pirimicarbe que dans l'autre population résistante ainsi que dans la population sensible, ce qui indique que cette population a développé un autre mécanisme de résistance – une modification de l'AChE (MACE). **Conclusion.** La détoxification d'insecticides par les mécanismes de résistance métabolique des enzymes estérases et de modification de l'acétylcholinestérase a été démontrée dans le cas d'une population de pucerons (RA). L'autre population (BC) a seulement développé une résistance métabolique (amélioration du métabolisme des estérases), sans modification du site cible de l'insecticide (AChE). Le développement de résistance aux insecticides est causé par l'utilisation à long terme des inhibiteurs d'acétylcholinestérase (organophosphorés et carbamates) dans ces vergers conventionnels.

Mots clés : Serbie / pommier / *Malus domestica* / *Aphis pomi* / résistance aux pesticides / pirimicarbe / estérases

1 Introduction

Production of apple (*Malus domestica* Borkh.) in Serbia ranked in the 43rd position in the world, with 178,713 t from over 28,000 ha (FAOSTAT, 2012). In the apple variety assortment of Serbia, 'Idared' is the predominant cultivar [1]. One of the most limiting factors in the apple agro-system is the high number of biological aggressors - pests, diseases and weeds. The most common and damaging insect pests on apples in Europe are codling moth (*Cydia pomonella* L.) and green apple aphid (*Aphis pomi*), and the most economically damaging diseases on apple are scab and powdery mildew [2]. For many years pesticides have been widely used to control these pests and diseases in apples, most intensively in the USA [3] and France [4]. No wonder that pesticide resistance has become a big issue in apple production, making many pesticides useless for the control of many harmful organisms [5]. Insecticide resistance is very common to some long-used compounds for insect control [6]. In order to prevent insecticide resistance development, the IRAC (Insecticide Resistance Action Committee) recommended the management principles [6] to be followed in the long term.

Green apple aphid *Aphis pomi* (De Geer) is widespread in Europe, the Near East, North America and New Zealand [7]. It is an important pest in apple orchards in Serbia. Damage, consisting of curling leaves, honeydew contamination of foliage and fruit, and shoot and fruit malformations, are likely in 50% of shoots on a mature tree [8]. In most countries, recommended control products include various organophosphate and carbamate insecticides. Some populations of this pest have developed resistance to organophosphate and carbamate insecticides, as a result of long-term application of these insecticides in conventional orchards. Insecticide resistance has been investigated in many species of aphids, including *Myzus persicae* (Sulzer), *Schizaphis graminum* (Rondani) and *Aphis gossypii* (Glover), using biochemical and molecular techniques [9–14]. In the spectrum of resistance mechanisms in aphids, the most common are: the increased production of detoxifying esterase [9] and cytochrome p450 monooxygenase [15], target-site resistance involving changes in acetylcholinesterase [16, 17], the sodium channel [18, 19] and nicotinic acetylcholine receptor genes [20].

For many years, the only mechanism of resistance identified in aphids was overproduction of insecticide-detoxifying esterases. This mechanism of resistance was first implicated in the late 1960s by demonstration that all resistant strains showed an increased ability to hydrolyze the model esterase substrate, 1-naphthyl acetate [9, 21]. Production of different esterase isoforms, as well as different amounts of individual isoforms between susceptible and resistant strains of *A. gossypii*, were found to be a potential mechanism of insecticide resistance in aphids [22]. Many insects can overcome changes in environmental factors or food composition, including the presence of specific inhibitors, by the production of new enzyme isoforms [23–25].

Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine hydrolase, whose primary function is very efficient and specific termination of nerve impulses at cholinergic synapses, by breaking down the neurotransmitter acetylcholine [26]. AChE is the target for the two large groups of insecticides, organophosphate and carbamate compounds, which phosphorylate or carbamylate the active site of AChE to block the hydrolysis of acetylcholine, which leads to the death of an insect [27]. The alteration of AChE to an insensitive form was demonstrated as an important mechanism of insecticide resistance in many agricultural pests [9, 11, 28]. Intensive use of these insecticides over the last 50 years has led to the development of resistance in many pest species that are important in agriculture, or as vectors of human and animal diseases [29]. This frequently involves changes in AChE, rendering it less sensitive to the inhibitors.

Biochemical and toxicity tests have been conducted for some aphids in many parts of the world [14, 30–33]. Insecticide resistance in green apple aphids has been very little studied. There are several articles with the results of the acute toxicity parameter LC_{50} of imidacloprid, pirimicarb, dimethoate, lambda-cyhalothrin and pymetrozine, for different populations of *A. pomi* originating from conventional and organic apple orchards in North America [33, 34]. One study indicated a significant correlation between amounts and activity of esterase enzymes and pirimicarb and dimethoate resistance of *A. pomi* and *Aphis spireacola* (Patch), based on biochemical tests [35].

This article reports on the first examination in Serbia of insecticide resistance of *A. pomi*, collected from three different localities. The dimethoate and pirimicarb resistance level of *A. pomi* was investigated by bioassays and biochemical tests, using three strains. The aim of this investigation was to study the differences between resistant and susceptible strains, in terms of determining toxicity parameters (LC_{50} values), as well as the underlying biochemical parameters: total esterase activity, differences in produced esterase isoforms and AChE resistance to insecticide.

Table I. Concentration series of insecticides (mg a.i. L^{-1}) used in the bioassays for *Aphis pomi*, collected from the localities of Radmilovac (RA), Bela Crkva (BC) and Surčin (SU).

RA		BC		SU	
Pirimicarb	Dimethoate	Pirimicarb	Dimethoate	Pirimicarb	Dimethoate
315.00	400.00	56.25	400.00	1.76	50.00
220.50	280.00	39.38	280.00	1.23	35.00
154.35	196.00	27.56	196.00	0.86	24.50
108.05	137.20	19.29	137.20	0.60	17.15
75.63	96.04	13.51	96.04	0.42	12.01
52.94			67.23	0.30	8.40

2 Materials and methods

2.1 Chemicals

All standard chemicals were of analytical grade (or higher) and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Floradur B Fine was used as potting soil (Floragard GmbH, Oldenburg, Germany). FitoFert Ultra 20:20:20 + ME, produced by Fertico, Serbia, was used as fertilizer. The insecticides pirimicarb (Pirimor 25WG, Syngenta, Germany) and dimethoate (Sistemin 40-EC, Bayer, Germany) were used for bioassays. Pirimicarb of analytical standard was purchased from Fluka and was used for biochemical characterization of enzymes.

2.2 Plant material

Aphids were collected from two conventional apple orchards (*Malus domestica* Borkh. cv. Idared), with extensive application of insecticides, in the localities of Radmilovac (RA) and Bela Crkva (BC), and from one organic apple orchard (cv. Idared) in the locality of Surčin (SU) in Serbia.

In conventional apple cropping systems, depending on pest population densities, insecticides are applied 8 to 10 times per year. The average annual frequency of insecticide spraying, starting from planting (1993) and according to chemical groups, was as follows:

- In the locality of RA: organophosphates – 2 to 3 times, pyrethroids – up to 2 times, carbamates (only pirimicarb) – 1 to 2 times, neonicotinoids – 1 to 2 times, other insecticides – up to 2 times;
- In the locality of BC: organophosphates – up to 2 times, pyrethroids – up to 2 times, carbamates (triazamate or pirimicarb) – once, neonicotinoids – up to 2 times, other insecticides – 3 to 4 times.

Apple seedlings (cv. Idared) used for raising aphids were grown in a growth chamber in 5-L plastic pots, containing a soil mixture of three parts commercial potting soil (Floradur B Fine) and one-half part perlite. A dilute solution of fertilizer (FitoFert Ultra 20:20:20 + ME) was applied weekly, as was previously described by Lowery *et al.* [33].

2.3 Insect material

Aphid larvae were sampled once, immediately after their appearance in early April, from the conventional orchards (RA and BC), previously untreated with insecticides, and from the organic orchard (SU). The aphid larvae were reared on excised leaf disks in Petri dishes until maturity. Adult aphids were then transferred to caged apple seedlings held in a growth chamber at 24 °C and a photoperiod of 16:8 h (light:dark). Aphids from a single clone were used to establish the laboratory culture for bioassays and biochemical assays.

The aphids used in biochemical assays had been previously stored in triplicate ($n = 50$), in 50% glycerol solution (w/w) at −20 °C.

2.4 Insecticide resistance – Bioassays

Toxicity of pirimicarb and dimethoate, expressed by the median lethal concentration or LC_{50}, was tested according to the IRAC protocol, as described below. The concentration series of these insecticides were chosen according to preliminary laboratory experiments and are shown in *table I*.

Non-infested apple leaves were dipped in test liquids for 5 sec. Control leaves were dipped in water. The trial was conducted in five replications. The leaves were placed individually in Petri dishes, each infested with 20 adult aphids after drying the surface water, and then kept in a thermostat at 20 °C and relative humidity (RH) of 40–50%. Mortality was assessed after 24 h and LC_{50} values were estimated by probit analysis [36]. The resistance ratio (RR) was calculated according to the formula:

$$RR = \frac{LC_{50} \textit{value of the RA (BC) population}}{LC_{50} \textit{ value of the SU population}}.$$

2.5 Insecticide resistance – Biochemical assays

2.5.1 Aphid homogenization and enzyme extraction

Homogenization was performed with a handheld homogenizer. Samples of 50 individual aphids were homogenized in 0.1 mL buffer (20 mM phosphate buffer pH 7, 0.1% Triton X-100) and incubated for 30 min at 4 °C for enzyme extraction. Enzyme extracts were obtained after centrifugation for 1 min at 14,000 rpm. Each sample was prepared in triplicate.

2.5.2 Total esterase activity assay

Esterase activity was tested by spectrophotometric assay, using 1-naphthyl acetate (0.3 mM in the homogenization buffer) as a substrate, according to Devonshire *et al.* [37], with slight modifications. The reaction was conducted for 5 min at room temperature and stopped by DBLS reagent (Fast Blue Salt 10 mg mL^{-1} and 5% sodium dodecyl sulfate in the ratio of 1.0:2.3). Color was developed in 30 min and absorbance was measured at 620 nm.

2.5.3 Zymography detection of esterase activity

Esterase isoforms were detected by in-gel activity staining following isoelectric focusing (IEF), using 1-naphthyl acetate and 2-naphthyl acetate as substrates, according to Owusu *et al.* [7,13], with slight modifications. The IEF was performed using the Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology), according to the manufacturer's instructions, on 7.5% polyacrylamide gel with ampholytes (3–10 pH range). The Broad pI kit (GE Healthcare) was used to provide pI markers. Zymograms were analyzed using GelAnalyzer. Proteins in gel were also stained with Coomassie Brilliant Blue (CBB).

2.5.4 Acetylcholinesterase activity assay

Acetylcholinesterase activity was detected at 25 °C, in a continual assay using acetylthiocholine iodide (AChI) as a substrate, according to Moores *et al.* [4, 10], with slight modifications. 150 μL of 50 μM AChI solution in the homogenization buffer and 150 μL of 75 μM DTNB solution in the homogenization buffer were added to the enzyme extract (80 μL). The increase in absorbance was monitored every 10 sec, at 405 nm for 10 min.

Another acetylcholinesterase activity assay was also performed as described above, by adding 0.4 mM pirimicarb to AChI solution. The concentration of the inhibitor was chosen according to the results obtained by preliminary experiments (enzyme assays in the presence of pirimicarb in the concentration range 8×10^{-3}–8 mM).

2.6 Statistical analysis

The bioassay data were analyzed by the probit method [36], using Probit Analysis-MSChart 2011 software to estimate LC_{50} values and their 95% confidence intervals (CI), slopes with standard errors (SE) and chi-squares (X^2). Ratios of LC_{50} values with their lower and upper 95% confidence limits were used to determine significant differences in sensitivity to insecticides.

The results of biochemical assays were statistically interpreted by GraphPad Prism 5, using one-way analysis of variance (ANOVA), repeated measurements, and the Bonferroni test at a significance level of $P < 0.05$.

Table II. LC_{50} (mg L^{-1}) values of pirimicarb, based on probit analysis of mortality rates, 24 h after insecticide application on *A. pomi* originating from the localities of Radmilovac (RA), Bela Crkva (BC) and Surčin (SU).

A. pomi group	Slope (S.E.)	LC_{50} (95% CI)	X^2 (*P*)	RR*
RA	2.99 (0.25)	134.16 (122.65–146.75)	0.40 (0.98)	235.4
BC	3.04 (0.31)	30.17 (27.49–33.25)	1.24 (0.74)	52.9
SU	3.12 (0.25)	0.57 (0.52–0.62)	0.30 (0.99)	–

* RR: resistance ratio.

3 Results and discussion

3.1 Insecticide resistance – Bioassays

The results of the bioassays indicated that the BC and RA aphid populations were resistant, while the SU population was susceptible to both insecticides. Aphids from the RA group showed higher resistance to pirimicarb insecticide than aphids from the BC group, but the resistance to dimethoate insecticide was not significantly different between these two groups.

Susceptibility to pirimicarb of the three *A. pomi* populations was determined in bioassays (*table II*). The pirimicarb resistance ratios of the RA and BC populations were 235.4 and 52.9, respectively, and the LC_{50} values ranged from 134.16 mg L^{-1} to 30.17 mg L^{-1}, while the LC_{50} value of the susceptible SU population was 0.57 mg L^{-1}, confirming the resistance of the RA and BC aphid groups. Aphids from the RA group were more tolerant to pirimicarb than those from the BC group. All clones differed significantly based on non-overlapping 95% confidence intervals for LC_{50} values.

Different resistance mechanisms have been reported in certain populations of several aphid species, causing high levels of resistance to many carbamate and organophosphate insecticides [10, 38]. Resistance of *A. pomi* to pirimicarb and dimethoate, as well as to other insecticides, was also reported [33–35]. One study showed similar results to ours on dimethoate susceptibility of various green apple aphid populations from North America, with the highest RR of 11.53 times [34]. Reported resistance to pirimicarb is much lower in American than in Serbian green apple aphid populations, with a maximum RR of 4.58 times.

The resistance ratios of the RA and BC aphid populations for dimethoate were 10.7 and 9.0, respectively, at the LC_{50} values of 189.96 mg L^{-1} for RA, 158.92 mg L^{-1} for BC and 17.71 mg L^{-1} for SU aphids (*table III*). The BC and RA populations did not differ significantly based on overlapping 95% confidence intervals for LC_{50} values, but they were significantly different from the susceptible SU population.

3.2 Insecticide resistance – Biochemical tests

3.2.1 Total esterase activity

According to the results of the total esterase activity test and bioassay, aphids derived from the SU population were

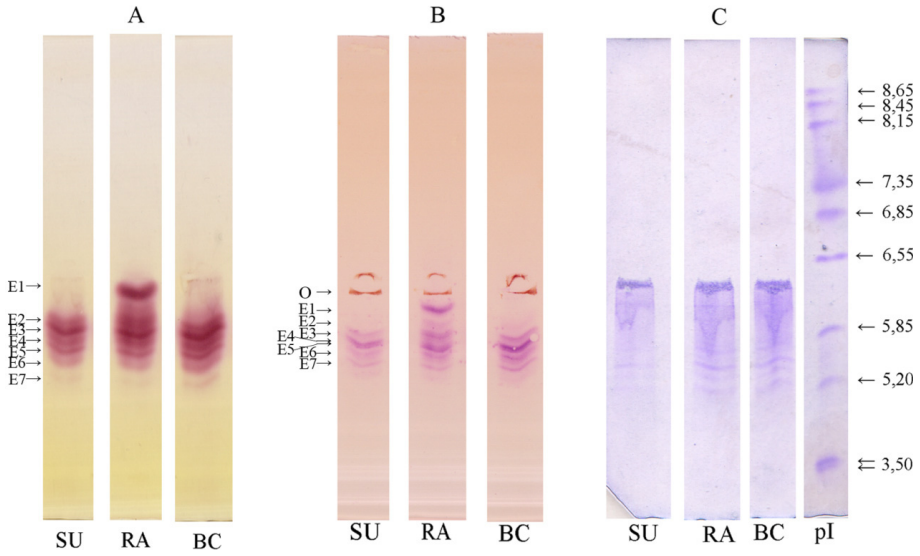

Figure 1. Zymographic detection of total esterase activity in extracts of three aphid groups: A) with 1-naphthyl acetate; B) with 2-naphthyl acetate; C) protein staining with CBB. Arrows on the left indicate the position of isoforms, marked with E1 to E7. O-Original place of sample application. Positions of pI markers are indicated on the right (Broad pI kit, GE Healthcare). *Aphis pomi* groups: RA-Radmilovac, BC-Bela Crkva, SU-Surčin.

Table III. LC_{50} (mg L^{-1}) values of dimethoate, based on probit analysis of mortality rates, 24 h after insecticide application on *A. pomi* originating from the localities of Radmilovac (RA), Bela Crkva (BC) and Surčin (SU).

A. pomi group	*Slope* (S.E.)	LC_{50} (95% CI)	X^2 (*P*)	RR*
RA	2.62 (0.29)	189.96 (170.23–211.26)	0.15 (0.98)	10.7
BC	2.78 (0.24)	158.92 (144.31–174.63)	0.20 (1.00)	9.0
SU	3.06 (0.25)	17.71 (16.15–19.32)	0.31 (0.99)	–

* RR: resistance ratio.

Table IV. Resistance levels of *A. pomi*, according to total esterase activity (A_{620}). Absorbance values are the means of three measurements per independent aphid sample from the same group. Each assay was conducted in duplicate. Data followed by the same letters were not significantly different according to ANOVA. The first letter refers to the linkage between the SU and RA groups. The second letter refers to the linkage between the SU and BC groups.

A. pomi group[x]	Esterase activity (A_{620}) ± SEM[y]	Category of resistance	Relative activity
SU	$0.400 \pm 0.017^{a, a}$	S	1.0
RA	$0.685 \pm 0.024^{b, a}$	R_2	1.7
BC	$0.649 \pm 0.019^{a, a}$	R_1	1.6

[x] RA-Radmilovac, BC-Bela Crkva, SU-Surčin.
[y] SEM-Standard Error of Measurement.

susceptible (S), while those from the BC and RA populations proved resistant (R_1 and R_2), as shown in *table IV*. There are significant statistical differences among the tested groups according to ANOVA (**P* = 0.0224, $F_{2,5}$ = 5.688).

According to the results obtained by the bioassay and total esterase activity test, the RA and BC populations were resistant, while the SU population was susceptible to the two applied insecticides. Similar classification was described by Devonshire [37]. Increased esterase activity is the main mechanism of insecticide resistance in many aphid species [12,13,21, 39–41]. This mechanism of insecticide resistance was first discovered in *M. persicae* clones [21]. *Aphis spiraecola* (Patch) and *A. pomi* are morphologically similar and often present together on apple trees, but their susceptibility to insecticides is different. *A. spiraecola* showed higher tolerance to pirimicarb and dimethoate than *A. pomi* [35]. There were differences in the amount and activity of detoxifying esterase enzymes between these two species and between different populations within these species, with a certain correlation between esterase activity and tolerance to insecticides [35].

3.2.2 Zymography detection of total esterase activity

Differences between resistant and susceptible aphids were detected by using 1-naphthyl acetate and 2-naphthyl acetate in zymograms, and by CBB staining of proteins (*figure 1*). The results of qualitative and quantitative analyses of the zymogram bands obtained are shown in *table V*. Each resistant aphid population (RA and BC) produced one esterase isoform more than the susceptible population (SU) if 1-naphthyl acetate was used as a substrate (*figure 1A*). A new isoform with pI 6.13 was induced in the RA population, while another acidic isoform, with pI 5.06, was induced in the BC group. Relative distributions of all other common esterase isoforms in the RA and BC

Table V. Analysis of position (pI) and distribution of esterase isoforms in *A. pomi* tested groups in zymography detection: a) using 1-naphthyl acetate as a substrate; b) using 2-naphthyl acetate as a substrate, by the GelAnalyzer program. Relative distribution refers to representation of individual isoforms (percentage in a pool of all isoforms present in a specific sample). *Aphis pomi* groups: RA-Radmilovac, BC-Bela Crkva, SU-Surčin.

	SU		RA		BC	
Isoform	pI	Relative distribution (%)	pI	Relative distribution (%)	pI	Relative distribution (%)
a) 1-naphthyl acetate as substrate						
E1	–	–	6.13	32.0	–	–
E2	5.78	22.6	5.81	23.7	5.72	18.5
E3	5.66	26.6	5.63	19.6	5.61	20.5
E4	5.55	21.4	5.55	3.5	5.49	17.4
E5	5.44	15.0	5.41	10.8	5.39	20.0
E6	5.30	14.4	5.25	10.3	5.24	11.4
E7	–	–	–	–	5.06	12.1
b) 2-naphthyl acetate as substrate						
E1	–	–	5.92	30.1	–	–
E2	–	–	5.76	12.5	–	–
E3	5.64	27.0	5.62	21.8	5.60	29.8
E4	5.53	18.3	5.50	8.3	5.49	14.6
E5	5.49	12.6	5.47	5.3	5.47	13.6
E6	5.41	20.4	5.39	11.6	5.36	18.4
E7	5.28	21.7	5.24	10.3	5.23	23.6

Table VI. Acetylcholinesterase activity in aphid extracts, with and without pirimicarb. Enzyme activity is presented as the mean of the values for three independent aphid samples from the same group. Each assay was conducted in duplicate. Data followed by the same letters were not significantly different according to ANOVA. The first letter refers to the linkage between the SU and RA groups. The second letter refers to the linkage between the SU and BC groups ($P \leqslant 0.0001$***, $F_{5,2,10} = 432.3$).

A. pomi group[x]	Enzyme activity ± SEM[y] (U mL^{-1})	
	without pirimicarb	with pirimicar
SU	$14.5 \pm 0.57^{a, a}$	$8.6 \pm 0.27^{a, a}$
RA	$16.4 \pm 0.71^{b, a}$	$11.0 \pm 0.39^{b, a}$
BC	$5.2 \pm 0.10^{b, b}$	$3.0 \pm 0.07^{b, b}$

[x] RA: Radmilovac, BC: Bela Crkva, SU: Surčin.
[y] SEM: standard error of measurement.

populations were also different from such distribution in the SU population (*table Va*).

Use of 2-naphthyl acetate as a substrate for zymograms distinguished the RA group (seven detected esterase isoforms) from the SU and BC groups (five detected isoforms each) (*figure 1B*). Two new isoforms were on the pI 5.76 and 5.92 positions. Esterase isoforms in the BC group differed from those in the SU group only in relative distributions of the esterase isoforms E3 and E7, which were wider in the BC than in the SU group (*table Vb*).

Two esterase substrates (1-naphthyl and 2-naphthyl acetate) were used for zymogram detection of esterase isoforms since using multiple test substrates provides more accurate information for making predictions on insecticide resistance mechanisms and recommendations for optimal management practice, which is shown in the study on *A. pomi* esterase enzyme assays [35]. The results obtained by zymogram detection of esterase isoforms of all tested aphid populations, using both substrates, indicate that both *A. pomi* populations collected from the two conventional orchards developed mechanisms of resistance. Expression of new esterase isoforms seems to be in direct correlation with the resistance mechanisms developed. The RA population even produced two new esterase isoforms if 2-naphthyl acetate was used as a substrate for zymogram detection, while both the RA and BC groups produced one, mutually different, new isoform each, if 1-naphthyl acetate was used. These results are in correlation with the results obtained by bioassays and the total esterase activity test. All the results presented show that zymogram detection of esterase isoforms could be used for distinguishing resistant from susceptible aphid populations, as well as different resistance levels between different resistant populations. Qualitative and quantitative differences in esterase isoforms were also determined in resistant and susceptible populations of *A. gossypii* [13, 22]. The expression of different esterase isoforms could be caused by post-transcriptional modification, e.g. glycosylation, as observed in a resistant strain of *Laodelphax striatellus* [42], or by production of new enzyme isoforms. Further analyses would provide more detailed information on esterase isoform expression in *A. pomi*.

3.2.3 Acetylcholinesterase (AChE) activity assay

Acetylcholinesterase activity was examined with and without the presence of the most common carbamate insecticide,

pirimicarb, and its values are shown in *table VI*. Without pirimicarb, AChE activity was the highest in the RA population; slightly lower activity was recorded in the SU population, while the BC population of *A. pomi* showed significantly lower AChE activity. In the presence of 0.4 mM pirimicarb, AChE activity was considerably lower in all tested aphid groups. The RA population showed a decrease in AChE activity of 34%, while in the BC and SU populations it was 42% and 41%, respectively.

Modification of AChE (MACE) was first reported in *M. persicae* by Moores *et al.* [10]. MACE has occurred later in the evolution and has always been followed by amplification of esterase genes [9], and represents the second most important mechanism of resistance in aphids. Point mutations in the AChE gene cause changes in the AChE structure by replacement of some amino acids near the catalytic site of the enzyme [9]. The MACE mechanism of resistance in aphids has been described in several articles during the last 20 years [10, 11, 16, 17].

Our results suggest that MACE did not develop in the SU aphid population. In the presence of pirimicarb, the inhibition process of AChE was on the same level in the SU and BC populations, and it should be noted that the SU population proved susceptible. AChE was significantly less inhibited by pirimicarb in the RA population than in the BC and SU populations. It indicates that AChE activity was modified in the resistant RA population of *A. pomi*. According to our results, the resistant BC population did not develop the MACE mechanism of resistance.

4 Conclusion

A. pomi has developed the two most common resistance mechanisms to the most frequently used insecticides in Serbia, as shown in this paper. Detoxification of insecticides by the metabolic resistance mechanism of esterase enzymes and mechanism of modification of AChE was proven in one aphid population (RA). The other population (BC) has developed only metabolic resistance (enhanced metabolism by esterases), without modification of the insecticide target site (AChE). Development of insecticide resistance was caused by long-term application of acetylcholinesterase inhibitors (organophosphates and carbamates) in these conventional orchards. This phenomenon of insecticide resistance is an inevitable process with constant use of insecticides with the same mode of action, and it is a matter of time when certain compounds will become useless for pest control. In order to postpone the development of resistance to effective insecticides, it is recommended to introduce integrated pest management and resistance management principles in pest control. This means, among all other measures, alternating compounds with different modes of action classified by the IRAC (Insecticide Resistance Action Committee), applying more selective insecticides and other pest management tools, which preserve beneficial arthropods (predators and parasitoids) in orchards, and including effective biological and non-chemical control practices. Implementation of these measures would decrease insecticide resistance risk, making fruit production much safer.

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