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Colorado Potato Beetle Resistant Population Insight Using Single Insect Carboxylesterases (ALiE) Testing

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Abstract

Colorado potato beetle (CPB) is an extraordinary example of pest resistance to insecticides. It is proved that increased activity of CPB's ALiE / carboxylesterase is closely related to resistance to organophosphate insecticides. ALiE activity of different populations was tested, using spectrophotometry. The frequencies of ALiE activity of individual larvae were similar to the binomial distribution. For more resistant populations to organophosphates, the whole graph is shifted to the area with higher enzyme activity. Consequently, individuals with lower ALiE activity disappear from the population while individuals with higher activity appear more frequently. The analysis of single larvae ALiE activity showed a fairly high homogeneity of the examined populations, except for the population Kaona. Examination of single insect ALiE activity is viable and provides insight into the population, which is important for further genetic testing.

Resumen

El escarabajo de la papa de Colorado (CPB, por sus siglas en inglés) es un ejemplo extraordinario de resistencia de plagas a los insecticidas. Está comprobado que el aumento de la actividad de la ALiE de CPB / carboxilesterasa está estrechamente relacionado con la resistencia a los insecticidas organofosforados. Se probó la actividad de ALiE de diferentes poblaciones, utilizando espectrofotometría. Las frecuencias de actividad de ALiE de las larvas individuales fueron similares a la distribución binomial. En el caso de las poblaciones más resistentes a los organofosforados, toda la gráfica se desplaza a la zona con mayor actividad enzimática. En consecuencia, los individuos con menor actividad de ALiE desaparecen de la población, mientras que los individuos con mayor actividad aparecen con mayor frecuencia. El análisis de la actividad de ALiE de larvas individuales mostró una homogeneidad bastante alta de las poblaciones examinadas, a excepción de la población Kaona. El examen de la actividad de ALiE de un solo insecto es viable y proporciona información sobre la población, lo cual es importante para realizar más pruebas genéticas.

Keywords *Leptinotarsa decemlineata* · Carboxylesterases · ALiE · Potato · Fourth instar larvae

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Introduction

Colorado potato beetle *(Leptinotarsa decemlineata* Say) - CPB is a major and the most destructive pest in potato production worldwide (Hare 1990; Alyokhin et al. 2006, 2008, 2013; Jiang et al. 2010; Stanković et al. 2004a, 2012). Current CPB management and control practices include biological control, cultural practices, IPM (Zabel et al. 2002; Gökçe et al. 2006; Kostic et al. 2002, 2003, 2007, 2008, 2012; Trdan et al. 2009; Popovic et al. 2013; Stankovic et al. 2020; Lazarević et al. 2020), but still the most efficient are chemical (pesticide) treatments (Zabel et al. 2000; Igrc Barčić et al. 2006; Boiteau 2010). Simultaneously with the development of control measures, producers are facing rapid resistance development to all major groups of insecticides (Zhao et al. 2000; Stanković et al. 2003; Alyokhin et al. 2007; Węgorek et al. 2011; Stanković et al., 2012; Szendrei et al. 2012; Zhou et al. 2012), including organophosphates and carbamates (Stanković et al. 2004b; Kostic et al. 2015; Zabel et al. 2017). Insecticide resistance presence and level is measurable (Miyata 1983; Bishop and Grafius 1991; Stanković et al. 2004a) and that is important tool for insecticide resistance monitoring as part of insecticide resistance management (IRM) strategies and practices (IRAC 2022).

The breakdown of insecticides within the target organism through metabolic processes is a common defense mechanism (Hassall 1990; Matsumura 1983). Resistant populations often exhibit higher levels or more efficient forms of enzymes, regulated by specific genes (Terriere 1983; Liu and Scott 1998; Harshman and James 1998). In terms of metabolic resistance to insecticides, phosphoric triesters and carboxylesterases (ALiE esterases, non-specific or B-esterases) play a significant role (Stankovic and Kostic 2017). Structural mutations in mutant carboxylesterases have been extensively documented, leading to metabolic resistance against organophosphate and pyrethroid insecticides (Oakeshott et al. 1999, 2005; Russell et al. 2004; Wheelock et al. 2005; Hollingwort and Dong 2008). Instances of high-level esterase-mediated metabolic resistance to carbamates are relatively rare. The role of ALiE in the development of resistance to organophosphorus and other insecticides in insects has been investigated over the past few decades. In most tested insect species, there is a correlation between increased enzyme activity and insecticide resistance. Numerous authors (Zabel 1991; Argentine et al. 1994; Anspaugh et al. 1995; Feng et al. 2014) have confirmed the role of carboxylesterases in Colorado potato beetle resistance to insecticides. The primary function of this enzyme is the degradation of organophosphorus insecticides into non-toxic components (Dauterman and Hodgson 1978; Wheelock et al. 2006; da Silva et al. 2013). In our recent papers, we observed that increasing concentrations of average CPB enzymes (a mixture of enzymes extracted from 50 insects) led to higher production of 1-naphthol (1-N) when the substrate amount (1-NA) remained constant. Similarly, when the substrate concentration (1-NA) varied while the amount of average enzyme remained constant, the production of 1-N also increased. 1-N is the product of 1-NA decomposition and is directly related to esterase activity. Since these reactions are specific to carboxylesterases, our results indicate significant activity of carboxylesterases (ALiE) in the tested Colorado potato beetles (Stankovic and Kostic 2017).

Noticing the results of toxicological tests and measuring the activity of acetylcholinesterase (AChE) in populations of Colorado potato beetles (CPB) that are resistant to organophosphates (OPs) and carbamates is crucial (Kostic et al. 2015; Zabel et al. 2017). Interestingly, the resistance levels for OPs and carbamates were found to be completely opposite. The experiments clearly demonstrated that both ALiE and AChE activities in CPB populations were highly pronounced and easily measurable (Kostic et al. 2015; Stankovic and Kostic 2017). Given the importance of effective resistance monitoring programs for early detection and successful management of pest and resistance issues (Kadoić Balaško et al. 2020), our objective was to establish the validity of single insect ALiE activity testing as an accurate method for resistance testing and monitoring. The investigated CPB populations had previously been determined and confirmed to be resistant to organophosphate insecticides (OPs) through bioassays. Based on the obtained results, it can be concluded that single insect ALiE testing has the potential to be further developed and justified as a rapid method for determining the insecticide resistance status to OPs, which is attributed to the increased carboxyesterase activity.

Materials and Methods

Colorado potato beetle fourth instar larvae (L4) were collected from four locations with intensive field potato production and pest control: Futog, Dobanovci, Ratari and Kaona, except for a case where there was a need for a susceptible population from Sjenica (Mt. Giljeva). In the absence of normal susceptible strain of CPB (laboratory-bred susceptible CPB population) as a possible reference point, we have used the most susceptible available CPB strain, collected in high mountains, not exposed to pesticides as in a commercial potato fields). Before the experiment, the insects were kept on room temperature for minimum three hours to resume normal activity and to eat. Fourth-instar larvae (L4) of maximum vitality and uniform size were selected for testing.

Bioassay

For each locality (population) experiments were conducted in 4 replications with 10 larvae per replication, using at least 5 concentrations of insecticides plus control (min 240 insects per locality). Determination of CPB resistance to OP insecticides was conducted by a bioassay - insect dipping (immersion) method (FAO 1974) using quinalfos (trade name Ekalux 25, emulsion concentrate with 250 g a.i. in 1000 ml, "Syngenta"). For determination of LC_{50}, the mortality was recorded after 24 h within 4 days (96 h) interval.

Statistical analysis: Correction of results for mortality in control was done by using Abbott's formula (Abbott 1925). LC_{50} values were determined using probit analysis (Finney 1971) and computer program based on Raymond 1985.

Determination of Carboxylesterases (ALiE) Activity

Activity of Colorado potato beetle ALiE/ Carboxylesterase was determined, using spectrophotometry at wavelength of 585 µm (Gomori 1953; Kadoić Balaško et al. 2020) using UV-VIS Perkin-Elmer 130 spectrophotometer (Stankovic and Kostic 2017). Under experimental conditions, 1-naphthylacetate (1-NA) is commonly used as a suitable substrate for the ALiE enzyme (Zhang et al. 2004). Enzyme decomposes substrate 1-NA into 1 naphtol (1-N). After determination of the calibration line and adjustment of the experimental conditions, tests were carried out in the visible region of wavelengths of light, for different concentrations of 1-N.

Solutions:

- 0.02 M Phosphate buffer solution, pH 7.
- substrate preparation solution: 0.6 g of 1-naphthyl acetate in 100 ml of acetone;
- 1-naphthol in acetone, 300 mg of 1-naphthol in 100 ml of acetone.
- dodecyl sulfate, 5 g in 100 ml of distilled water.
- During work – prior to the application, a Fast Blue B Salt solution was prepared containing 1,500 mg of Fast Blue B Salt ($C_{14}H_{12}Cl_4N_4O_2Zn$ - o-Dianisidine bis (diazotized) zinc double salt - Sigma-Aldrich- D9805) dissolved in 150 ml of distilled water.
- From the basic solution of 1-naphthyl acetate in acetone, a substrate solution with a 0.02 M phosphate buffer was prepared, so that 100 ml contains 1 ml of the basic solution. 5 ml of the substrate solution was measured into separate glass vessels. 0.2 ml of a diluted enzyme was added to each vessel, after adding the enzyme, incubation was carried out for 30 min in a thermostat at a temperature of 27^0C.
- prior to use, the reagent solution was prepared by mixing 30 ml of Fast Blue B Salt solution and 70 ml of dodecyl sulfate in water. The reagent solution in the amount of 0.2 ml was added to each vessel separately.

After 10 min, absorption was measured. The blind trial was prepared in the same way, only the enzyme was omitted.

50 fourth instar larvae were tested. 0.5 ml of 0.02 M phosphate buffer was measured and poured into individual glass vessels. One larva was added to each glass container. The larvae were crushed with a glass rod.

The substrate was prepared by adding 1 ml of 1-naphthyl acetate to 100 ml of phosphate buffer. 5 ml of substrate was added to each glass vessel. Incubation was performed for each larva separately, at 27 0 C for 30 min measured from the time of adding the substrate.

Immediately prior to use, the reagent solution was prepared by mixing 30 ml of blue salt solution and 70 ml of dodecyl sulfate in water.

At the end of incubation, 0.2 ml of reagent was added. The total liquid was filtered using filter paper. 2 ml was taken from the filtrate, and 12 ml of distilled water was added to obtain a suitable dilution for measurement on the spectrophotometer.

The control variant was prepared in the same way, only the enzyme (larvae) was omitted.

Data were statistical processed with the analysis of variance (one-way ANOVA) and linear regression analysis using computer program based on Raymond 1985.

Results

Bioassay

Different resistance levels among five populations were determined. Bioassay showed that the most resistant population is the population from Futog, and then from Kaona, Ratari and Dobanovci, respectively (Table 1).

The results of bioassay showed that CPB population from Sjenica is susceptible to OPs and its susceptibility is likely to be similar to a normal susceptible strain. Results also demonstrated wide range of resistance among populations. However, comparing results among resistant population – 'relative' resistance ratios were within small range, up to 4,42.

Carboxylesterases (ALiE) Activity

Under constant conditions of the experiment and at the fully utilized size of the fourth instar larvae, the differences in the absorbances can be attributed to different ALiE activities in individual larvae.

Within each examined population the absorbance values of ALiE activity of individual larvae differ significantly.

The ALiE activity parameters of individual larvae in the examined populations exhibited significant differences, confirming previously identified variations. When comparing the ordered absorbance values of ALiE activity among individuals within each population (Table 2), notable distinctions in the lowest values were observed for each population. Depending on the population, individuals with lower ALiE activity values were replaced by those with higher values. The population Dobanovci had the lowest limit values for ALiE activity in individual larvae, while the population Kaona had the highest.

Similar trends were noted when comparing the highest absorbance values obtained for individual larvae. The highest value for larvae from the Kaona population significantly exceeded the corresponding value for larvae from the Dobanovci population. Additionally, the highest absorbance values for larvae of the Ratari and Futog populations differed significantly from those of the Kaona and Dobanovci populations.

Similar absorbance value ratios were obtained when comparing the average absorbance values as well as the corresponding medians. When the average absorbance values were compared, the following sequence and ratios among average absorbance (μm) values for populations were obtained: Dobanovci: Futog: Ratari: Kaona = 1.0: 1.508: 1.583: 1.892.

The statistical analysis of the collected data revealed significant differences in all sets, except for the values pertaining to the sets of individuals from the populations of Ratari and Futog. Notably, all other sets exhibited significant distinctions from one another. Upon analyzing the sorted data on the absorbance values of ALiE activity in individual larvae from the examined populations, it was observed that in larvae from Dobanovci, absorbances showed fluctuations only in groups with the lowest and highest ALiE activity. In the remaining value range, the absorbance values displayed a very smooth and steady increase.

The frequencies of absorbances for individual insects in the ALiE analyzed populations are visually represented in Fig. 1, forming plateaus of varying sizes. The Ratari population sample created a plateau that was smaller in individuals with low ALiE activity and larger in individuals with higher ALiE activity, with gradual increases connecting the plateaus. In the Futog population sample, a higher number of plateaus were observed, occurring throughout the entire range and interconnected by absorbance values that gradually changed. In the Kaona population sample, a larger number of smaller plateaus were evident in the range of medium and high ALiE activities, with noticeable jumps in value between each plateau.

To conduct a more in-depth analysis of the ALiE activity structure within the populations, the absorbance values for individual larvae from all examined populations were assessed. The lower limit of the absorbance values was set at 0.01 μm, and the upper limit at 0.6 μm. The range was then divided into twenty equal classes, and the number of individuals with corresponding ALiE activity (frequencies) was determined for each population within each class.

Population Dobanovci

Based on the absorbance values for the ALiE activity of individual larvae in the Dobanovci population, the larvae were distributed across 8 different classes. The frequency distribution (Fig. 2A) reveals that the majority of values were concentrated within three adjacent classes, accounting for 88.89% of the absorbance frequencies. This suggests a high degree of homogeneity in ALiE activity among the individuals, with most falling within a similar activity range. Notably, 35.19% of the total tested individuals belonged to a single class. Only 3.7% of individuals exhibited ALiE activity significantly higher or lower than the majority. The mean absorbance value and median were nearly identical (0.18113 and 0.182), indicating a pronounced homogeneity in ALiE activity within the Dobanovci population. The standard deviation was very low (0.036615), further supporting the population's uniformity in ALiE activity.

For all examined populations, the same upper and lower limits of the interval were applied to classify absorbance values. In the Dobanovci population, all frequencies were concentrated in the first half of the selected range. Two classes had low frequencies and less active ALiE than most individuals, while three classes included individuals with higher activity but also with low frequencies.

Population Ratari

The absorbance frequencies for the ALiE activity of the Ratari population were also divided into 8 classes (Fig. 2B), with a distribution differing from that of the Dobanovci population. The class closest to the average absorbance value comprised 34.55% of the total larvae. On both sides of this central class, the number of absorbance frequencies gradually decreased, creating a distribution pattern resembling a normal or binomial distribution. The average value and median were very similar (0.28669 and 0.283), and they were higher than the absorbances for the Dobanovci population, indicating an overall higher enzyme activity. The standard deviation was also higher (0.049482), signifying a less homogeneous ALiE activity within the Ratari population compared to the Dobanovci population. However, unlike the frequencies in the Dobanovci population, here the largest number of frequencies was concentrated in one class.

Population Futog

The absorbance frequencies reflecting ALiE activity in potato beetle larvae from the Futog population were categorized into 11 different classes (Fig. 2C). The class housing both the average value and the median, which were very close (0.27317 and 0.2715), constituted only 20.37% of the tested individuals. Progressing towards the upper limit of the range, six more classes emerged, while four classes appeared towards the lower limit. Similar to the Ratari population, the average value and median were positioned roughly in the middle of the ranking range. However, the participation percentage was notably lower, indicative of increased frequencies and the number of classes towards the upper ranking limit. The standard deviation was significantly higher (0.064342) compared to values obtained for the Dobanovci and Ratari populations, signaling a greater heterogeneity in the populations concerning ALiE activity. Additionally, apart from having the highest percentage of absorbances in the middle of the ranking range, a considerable percentage of the absorbance values (42.58%) was positioned above, towards the upper limit.

Population Kaona

The frequency distribution of absorbances in potato beetle larvae ALiE from the Kaona population revealed a highly heterogeneous structure in relation to ALiE activity, with absorbances distributed across 10 different classes (Fig. 2D). The class closest to the average value and median exhibited a significantly lower number of frequencies compared to the class with the average absorbance from other localities, constituting only 21.74%, except for the Ratari population.

The average and median, with similar values (0.34274 and 0.324), were further shifted towards the upper limit into the second half of the classification range. The standard deviation was significantly higher (0.071317) than for individuals from the Ratari population and almost twice as high as the standard deviation values for the Dobanovci population, indicating a pronounced heterogeneity regarding ALiE activity.

A class incorporating the average value was one rank higher than the class with the highest frequency but contained a very small number of individuals or frequencies. The highest frequency for these populations was in the third fifth of the band, slightly less in the fourth, but with frequencies in the second and last fifth of the range. The data suggested that this population was much more heterogeneous than the others.

Discussion

Over the last few decades, it is obvious that Insecticide Resistance Monitoring (IRM) is critical to resistance management, but very little discussed regarding design of monitoring programs. Some general considerations show that the LD_{50} (as well as LC or LT), a standard measure for IRM, is very inefficient compared with diagnostic tests that accurately distinguish between resistant and susceptible individuals (Roush and Miller 1986). According to IRAC (2022) monitoring results can provide an early warning of resistance evolution, advance the understanding of factors that drive resistance evolution, document the effectiveness of IRM strategies, and provide relevant information to guide implementation of effective pest management practices. However, even with diagnostic doses, sample sizes at any given location must often be very large (on the order of hundreds of individuals per population) to reliably detect resistance, especially when it is present at frequencies below < 10%. Resistance detection may not be a practical component of IRM for those species where it is difficult to collect large numbers of individuals (Roush and Miller 1986).

Toxicological, biochemical, molecular and genetic diagnostics for monitoring insecticide resistance are present for major pests worldwide over last decades (Bishop and Grafius 1991; Roe et al. 1994; Kadoić Balaško et al. 2020; Mavridis et al. 2023).

For population resistance level to OPs, we have used bioassay. In the absence of laboratory susceptible population, we have used the most susceptible field population of CPB – Sjenica (Mt. Giljeve) for following reasons: high altitude (1617 m), absence of pesticide use in potato production, lower temperature for CPB development resulting in only one generation per year. After comparing other populations with susceptible one (calculated RR - resistance ration), we have introduced Relative Resistance Ratio (Table 1), as a more useful reference point for further analysis and compares among populations.

Individual larval ALiE activity was measured to obtain absorbance data. Data analysis provided information on both the overall level of ALiE activity for the population and the structure/distribution of activities within it. Comparison of ALiE activity data for individuals across all populations revealed significant differences in the lowest values for each specific population. The populations examined showed varying levels of ALiE activity, with the lowest values observed in Dobanovci, followed by Futog, Ratari, and individuals from Kaona. Depending on the locality, individuals with lower ALiE activity disappeared from populations, while those with higher activity appeared. Differences in ALiE activity were found among groups of individuals, depending on their place of origin. The highest ALiE activity was observed in individual larvae from population Kaona, which was significantly higher than the corresponding value for larvae from population Dobanovci. The absorbance values of larvae from the Ratari and Futog populations were similar to each other and to the corresponding values for the Kaona and Dobanovci populations. The ratios of the average absorbance values and corresponding medians were also similar. The ratios of the average values of the samples compared to the lowest were as follows: It is important to note that no new content has been added to the text. Resistance ratios of populations - Dobanovci: Futog: Ratari: Kaona was found to be 1.0: 1.508: 1.583: 1.892, which is similar to the previously determined ratios of ALIE enzyme activity at different time intervals and the ratios in organophosphate resistance determined by the bioassay (Kostic et al. 2015; Zabel et al. 2017; Stankovic and Kostic 2017).

The frequencies of ALiE activity indicate that the samples from the Dobanovci, Ratari, and Futog populations were quite homogeneous but differed in activity. The ALiE larval activity distribution in the Kaona population was highly heterogeneous, with the highest enzyme activities. Additionally, there was a significant correlation between the frequency of individuals with a certain ALiE activity and their resistance to organophosphates, as determined by bioassay.

Conclusions

Efficient insecticide resistance management requires accurate, effective, and timely monitoring of pest populations, particularly in commercial potato production areas. The use of organophosphorus (OPs) insecticides in CPB control remains significant worldwide. Therefore, it is essential to develop precise and efficient monitoring methods for OPs insecticide resistance. Measuring ALiE activity of individual insects can be a component of systematic monitoring of resistance in field populations. This involves collecting populations from specific locations at fixed time intervals to accumulate historical data. Such data enables the assessment of susceptibility shifts from the baseline over time and the evaluation of field resistance evolution (IRAC 2022).

Examination of ALiE activity in individual potato beetle larvae provides insight into population structure. The activity of this enzyme is important for further testing. As resistance to organophosphates increased, individuals with lower ALiE activity disappeared from the population and were replaced by individuals with higher activity. The analysis of ALiE activity in individual larvae revealed a high degree of homogeneity in the examined populations, except for the Kaona population. The population of Futog exhibited the highest resistance to organophosphate insecticides, followed by Kaona, Ratari, and Dobanovci in descending order. The biochemical test results were proportional to the results obtained from the bioassay. It can be concluded that the insecticide was more effectively decomposed with an increase in ALiE activity, which is a key mode of resistance to OPs.

These findings align with the requirements of systematic monitoring of resistance of field populations (IRAC 2022), making a single insect ALiE activity test a suitable method for monitoring insecticide resistance.

Appendix

Table 1 Bioassay: Organophosphate toxicity to L_4 larvae of *L. decemlineata*

Population	Correlation coefficient	SE	LC_{50} (mg l^{-1})	Regression line	Resistance ratio (RR)	Relative RR
Dobanovci	0.94	0.136	877.00	Y = 0.92 + 1.386 * X	109.76	1,00
Ratari	0.97	0.197	1218.40	Y = 0.43 + 1.482 * X	152.49	1,39
Futog	0.98	0.155	3876.20	Y = 0.27 + 1.317 * X	485.13	4,42
Kaona	0.95	0.727	2758.10	Y = 4.25 + 2.688 * X	345,19	3,14
Sjenica	0.85	8.390	7.99	Y = 3.88 + 1.239 * X	1.00	-

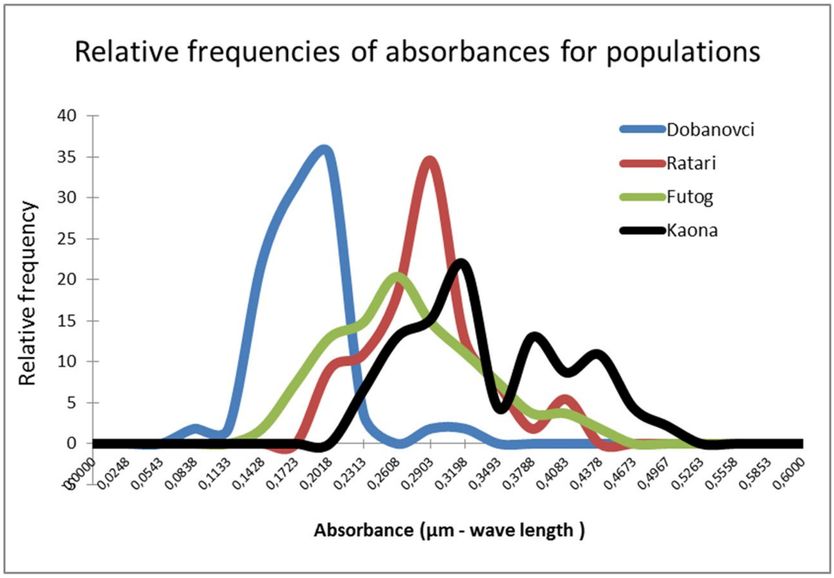

Fig. 1 Distribution of absorbances for individual insects for investigated populations of Colorado potato beetle

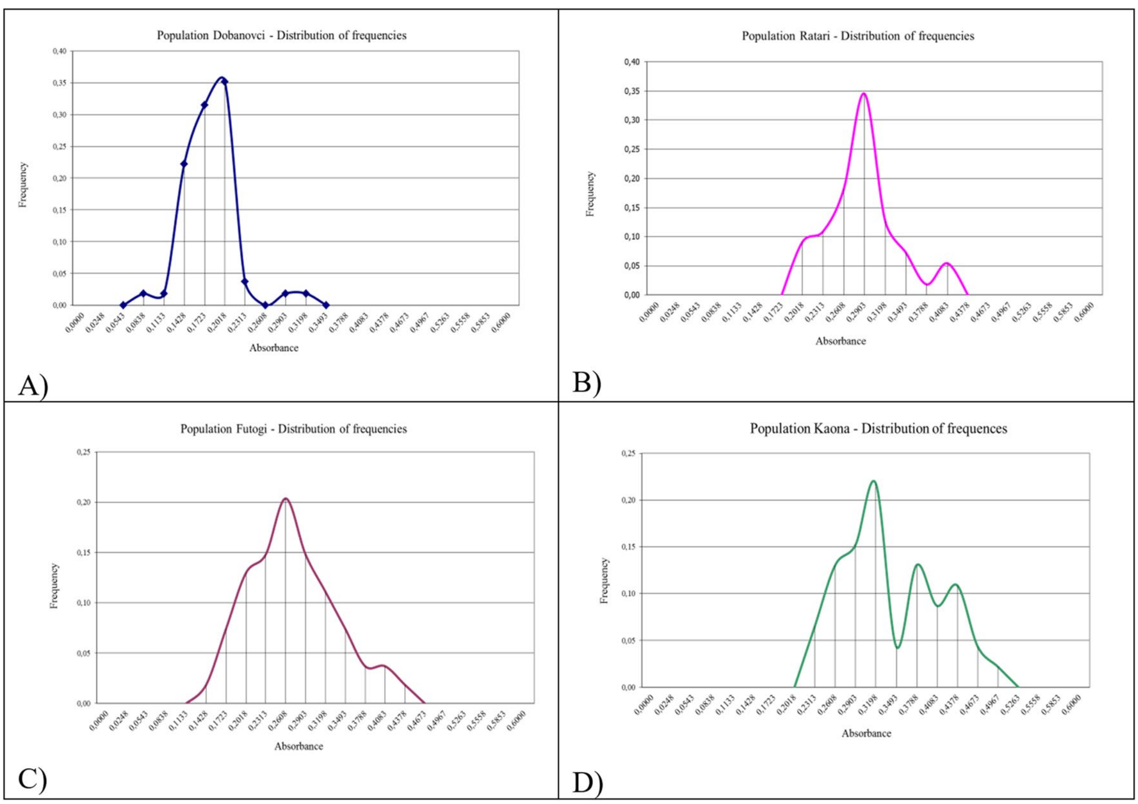

Fig. 2 Distribution of frequencies and absorbances (in µm) for population: Dobanovci (**A**) Ratari (**B**), Futog (**C**) and Kaona (**D**)

Table 2 Absorbances for individual insects for investigated populations of Colorado potato beetle

Population	The lowest absorbance (μm)	The highest absorbance(μm)	Average absorbance (μm)	Median (μm)	Standard deviation (SD)
Dobanovci	0.0870	0.1850	0.18113	0.1820	0.036615
Ratari	0.1950	0.2850	0.28669	0.2830	0.049482
Futog	0.1570	0.2750	0.27317	0.2715	0.064342
Kaona	0.2360	0.3700	0.34274	0.3240	0.071317

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Author Contributions For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, S.S., S.J., D.S. and S.K.; methodology, S.S., M.M. and S.K.; formal analysis, S.S. and D.S.; investigation, S.S., S.J., M.M. and S.K.; resources, S.S.; data curation, S.S.; writing—original draft preparation, S.S., S.K., N.Dj. and D.S.; writing—review and editing, S.S., S.J. D.S. and V.C.; visualization, S.S.; supervision, S.S.

All authors have read and agreed to the published version of the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

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