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ESTABLISHING THE RESISTANCE OF *MYZUS PERSICAE* (SULZER) BY MOLECULAR METHODS

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Abstract — In two years of investigating resistance of the peach-potato aphid *Myzus persicae* (Sulzer) by molecular methods, several types of resistance were established in the majority of individuals from peach and tobacco in Serbia and Montenegro. Most of the tested individuals had the *FE4* gene, which encodes production of FE4 esterase. The gene responsible for *kdr* (knock-down resistance) was found in the majority of individuals, but in the heterozygous state, while resistance based on formation of modified acetlycholinesterase (MACE) was least represented. Also, tests showed aphids from tobacco to be more sensitive to insecticide action than aphids from peach. Three tests were used in these investigations, e.g., the PCR - esterase, PCR - *kdr*, and RFLP - PCR tests, each for a single type of resistance.

Key words: Myzus persicae persicae, M. persicae nicotianae, resistance, esterase, *kdr*, MACE, Serbia, Montenegro

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INTRODUCTION

The peach-potato aphid *Myzus persicae* (Sulzer) is a very significant pest of peach, tobacco, vegetables, and flowers. Two subspecies have in recent years been established in Europe, viz., *Myzus persicae persicae* (Sulzer), whose primary host is peach, and *Myzus persicae nicotianae* (Blackman), which is adapted to tobacco, but the mechanisms of resistance are the same in both subspecies (Eastop and Blackman, 2005). *Myzus persicae* has been subjected to intensive treatment with insecticides, owing to its pronounced polyphagy, the large number of generations it produces annually, its capacity for parthenogenesis, and the existence of wingless and winged forms of it that are significant vectors of viruses. This constant pressure of insecticides has led to the development of resistance to many compounds.

Two mechanisms of resistance are present in *Myzus persicae*: increased detoxication of insecticides; and changes in sensitivity of the action sites. These two mechanisms provide resistance to a wide range of insecticides with different modes of action. Increased detoxication of insecticides occurs due to increased production of two related esterases designated E4 and FE4, as the genes encoding their production are also referred to (Field et al., 1989). Changes of action site sensitivity are involved in two other types of resistance. They are a consequence of point mutations of genes causing amino acid substitutions in protein synthesis. The first type of resistance results from formation of modified acetylcholinesterase or MACE, which occurs due to substitution of phenylalanine for serine near the catalytic part of the enzyme. The second type is knock-down resistance, which occurs due to substitution of phenylalanine for leucine, which results in mutations in Na-channels of the nerve synapse.

Esterase E4 and FE4 account for more than one percent of total proteins in the body of the aphid and perform hydrolysis of esters of insecticides and their capture before they reach the target in the nervous system. Increased activity of carboxylesterases is a consequence of amplification of their structural genes (Devonshire and Sawicki, 1979; cited

by Devonshire et al., 1998). This creates high resistance to organophosphates and pyrethroids and somewhat lower resistance to carbamates (Foster et al., 2000).

Another form of resistance is based on modified insensitive acetylcholinesterase (AChE) as the target of organophosphates and carbamates, which in *Myzus persicae* was first detected in 1990 (Moores et al., 1994). Acetylcholinesterase is a member of the serine hydrolase family of enzymes and is responsible for hydrolysis of the neurotransmitter acetylcholine (ACh) in nerve synapses. Organophosphates and carbamates act by inhibiting acetylcholinesterase, which causes repetitive discharges on postsynaptic nerves, leading to insensitivity of the nervous system and possible death of the organism (Javed et al., 2003). Modified AChE (MACE) creates high resistance to pyrimicarbs and triazamatimes, as well as to triazolimes, as was later established (Dewar et al., 1984).

The last discovered resistance mechanism is "knock-down" resistance (*kdr*), which creates resistance to pyrethroids and DDT (Martinez-Torres et al., 1999). It applies to changes in structure of the protein sheath of sodium channels in the nerve membrane. This form of resistance is referred to as *kdr* (knock-down resistance) because the instantaneous paralyzing effect of pyrethroids and DDT is called a knock-down. Resistance based on increased esterase production is of secondary significance in relation to *kdr* where pyrethroids are concerned.

Biochemical methods of establishing resistance revealed decreased sensitivity of *Myzus persicae* populations to the most often employed groups of insecticides and indicated that resistance is developed to a certain extent in populations from Serbia and Montenegro (Vučetić et al., 2007).

The purpose of the present investigations was to use molecular methods to detect the existence of genes responsible for the development of resistance and thereby establish the level of resistance of *M. persicae* to the most often employed groups of insecticides and obtain a more complete picture of the state of resistance of this aphid in Serbia and Montenegro.

MATERIAL AND METHODS

Samples were taken from a number of localities on the territory of Serbia and Montenegro during the years 2004 and 2005. Peach orchards, individual peach trees growing in yards and alongside roads, and tobacco fields were covered in the sampling. Leaves attacked by leaf aphids were brought into the Entomological Laboratory of the Faculty of Agriculture in Zemun, where the aphids were determined. One female from each sample was left in a phytotron for rearing under controlled conditions, while a greater number of females were put in a freezer or fixed in alcohol. Molecular investigations were carried out in the Laboratory of Entomology and Agricultural Zoology, University of Thessaly, Department of Crop Production in Volos, Greece. Molecular testing of the resistance level was performed on living individuals of *Myzus persicae*, ones fixed in alcohol (75 or 96%), or frozen specimens.

Extraction of DNA was carried out by the methods of Sannucks and Hales (1996) and Martinez-Torres et al. (1997). Testing of resistance by molecular methods involved establishing the site in the insect's genome responsible for one of the three existing types of resistance by means of PCR (the polymerase chain reaction).

The PCR-esterase test was used to establish whether genes (E4 and FE4) responsible for resistance exist in the aphid's genome. Results were confirmed by horizontal electrophoresis in 1% agarose gel, by staining with ethidium bromide, and in the presence of an appropriate marker. Aphids with an amplified E4 gene always give a PCR product with a value of 572 bp, whereas aphids with an amplified FE4 gene give one with a value of 865 bp, which show up as clear lines and are easy to distinguish in agarose gel. Sensitive aphids either give no result of any kind after electrophoresis in agarose gel or else give two pale lines indicating small amounts of amplified genes, one with a value of 572 bp, the other with a value of 865 bp (Field and Devonshire, 1998).

The PCR - *kdr* (knock-down resistance) test was used to establish existence in the aphid's genome of a mutation responsible for creation of the knock-down type of resistance, i.e., to establish whether mutation leading to the amino acid substitution responsible for changes in the structure of Na-channels in the nerve membrane occurred in the genome. The test was carried out according to the procedure of Martinez-Torres et al. (1999). Results were confirmed by horizontal electrophoresis in 1% agarose gel, by staining with ethidium bromide, and in the presence of an appropriate marker. Fragments with a value of 300 bp indicate resistance, whereas fragments with a value of 600 bp indicate sensitivity. If both lines appear in a sample, this means that the gene in it is found in the heterozygous state (RS).

The RFLP (restriction fragment length polymorphism) - PCR test was used to establish the site in the genome responsible for changes in the structure of acetylcholinesterase. Based on identification of the site of the point mutation that leads to amino acid substitution and creation of modified AChE, it also indicates whether the mutation is in the homozygous or the heterozygous state. By means of PCR with the aid of appropriate primers, an amplified DNA segment measuring 1269 bp is obtained which constitutes 2/3 of full length of the DNA sequence that encodes AChE. In the wild type, further digestive restriction enzyme action yields three fragments with values of 435, 780, and 54 bp, whereas the 780 and 54 fragments are fused in the type in which mutation occurred (S431F). Restriction enzymes belong to the nuclease group, enzymes that cut the phosphoester bonds of polynucleotide chains. They are endonucleases whose activity is strictly confined to a specific sequence of nucleotide pairs in DNA (Marinković et al., 1991). Isolation of restriction enzymes made controlled fragmentation of chromosomes possible.

The protocol for this method was devised by Cassanelli et al. (2004), and the results are read in vertical electrophoresis in polyacrylamide gel and staining with silver nitrate. DNA fragments of different size will be arranged differently, and on the basis of their position in the presence of an appropriate marker, it can be clearly seen whether the enzyme cut DNA and if so whether the sites cut by it are in the homozygous or heterozygous state.

RESULTS AND DISCUSSION

Aphids from tobacco growing at 33 localities were tested. In aphids from three samples (Mladenovac 1, Čoka 8, and Bački Petrovac, Serbia), amplification of the FE4 and E4 genes occurred, which indicates sensitivity. The FE4 gene was found in all other samples. The gene responsible for *kdr* was not found in three samples (Bački Petrovac, Mladenovac 2, and Čoka 1), whereas it was found in the heterozygous state in the remaining 30 samples. As for resistance based on modified acetylcholinesterase, the test showed that only one sample (Senta 1) possessed this gene, but in the heterozygous state, whereas its presence was not established in the other tested aphids (Table 1).

Table 2 presents the results of tests performed on peach. Aphids from 20 localities were tested.

Amplification of the FE4 gene occurred in all of the tested aphids. The gene responsible for *kdr* was found in the homozygous state in aphids from one sample (Podgorica 2), whereas it was found in the heterozygous state in the other samples. The presence of MACE resistance was not established in any of the tested samples.

Analyzing the results of all tests, we are able to conclude that resistance based on increased production of carboxylesterase (here caused by only the single gene FE4) is dominant. It is followed by *kdr*, which was not registered in only three samples. Modified acetylcholinesterase turned out to be the rarest form of resistance. However, in view of the fact that a certain number of individuals possessed the gene encoding its production, the possibility exists that it will undergo expansion as a result of selection of such individuals.

It was established by means of the PCR - esterase test that amplification of the FE4 and E4 genes occurred (horizontal electrophoresis yielded pale lines measuring 865 and 572 bp) in three samples from tobacco, and it can be asserted that the aphids in question are sensitive, since it was demonstrated

Table 1. Results of molecular tests of resistance performed on aphids from tobacco (*Myzus persicae nicotianae*).

Locality	Esterase	kdr	RFLP-PCR
Mladenovac 1	**FE4+E4**	RS	-
Čoka 8	**FE4+E4**	RS	-
Bački Petrovac	**FE4+E4**	**SS**	-
Senta2	FE4	RS	-
Mladenovac 2	FE4	**SS**	-
Beška	FE4	RS	-
Ostojićevo	FE4	RS	-
Čoka 1	FE4	**SS**	-
Čoka 4	FE4	RS	-
Čoka 5	FE4	RS	-
Senta0	FE4	RS	SS
Senta1	FE4	RS	**RS**
Senta2	FE4	RS	SS
Senta3	FE4	RS	SS
Senta4	FE4	RS	SS
Senta5	FE4	RS	SS
Kanjiža	FE4	RS	SS
Kanjiža1	FE4	RS	SS
Kanjiža2	FE4	RS	SS
Kanjiža - Mali Pesak	FE4	RS	SS
Kanjiža - M.Pesak2	FE4	RS	SS
Kanjiža - M. Pijace	FE4	RS	SS
Kanjiža - M.Pijace5	FE4	RS	SS
Kanjiža - M.Pijace6	FE4	RS	SS
B. Vinogradi	FE4	RS	SS
Šid - Kukujevci	FE4	RS	SS
Šid - Vašica	FE4	RS	SS
Futog	FE4	RS	SS
Beška	FE4	RS	SS
Podgorica - Sukuruć	FE4	RS	SS
Podgorica – Vranj1	FE4	RS	SS
Podgorica – Vranj3	FE4	RS	SS
Podgorica - Tuzi	FE4	RS	SS

Table 2. Results of molecular tests of resistance performed on aphids from peach (*Myzus persicae persicae*).

Locality	Esterase	kdr	RFLP-PCR
Belgrade - Radmilovac 2	FE4	RS	-
Stara Pazova	FE4	RS	-
Kruševac	FE4	RS	-
Bela Crkva	FE4	RS	-
Belgrade-Nova Galenika	FE4	RS	-
Horgoš	FE4	RS	-
Podgorica 1	FE4	RS	-
Podgorica 2	FE4	**RR**	-
Topola	FE4	RS	SS
Belgrade - Radmilovac	FE4	RS	SS
Belgrade - Brestovik1	FE4	RS	SS
Belgrade - Brestovik2	FE4	RS	SS
Smederevo	FE4	RS	SS
Belgrade - Ritopek	FE4	RS	SS
Belgrade - Ritopek2	FE4	RS	SS
Belgrade - Vinča1	FE4	RS	SS
Belgrade - Vinča2	FE4	RS	SS
Belgrade - Vinča3	FE4	RS	SS
Belgrade - Galenika	FE4	RS	SS
Bela Crkva	FE4	RS	SS

by Blackman et al. (1996, 1998) and by Field and Devonshire (1998) that sensitive aphids are carriers of both genes. All of the other samples had an amplified FE4 gene. According to the data of Spence and Blackman (1997), the existence of genes E4 and FE4 is closely linked with the life cycle of aphids. The FE4 gene is usually present where a sexual generation exists on peach. In Greece, aphids from tobacco in peach-growing regions can overwinter as eggs on peach, but there too a certain number of aphids overwinter anholocyclically (Margaritopoulos et al., 2002).

The PCR - *kdr* test showed that three samples from tobacco did not have the resistance gene, while one of the tested samples from peach had RR resistance. Aphids with the gene in the homozygous state (RR) were from a peach orchard where intensive measures of protection are employed. All of the

other tested aphids had this gene, but in the heterozygous state. Changes in the Na-channels of nerve membranes that lead to this form of resistance affect behavior of the aphid and reduce its capacity for survival (Foster et al., 1996, 1997, 1999), so that a cold winter effectively eliminates highly resistant (RR) aphids. Inasmuch as winters in our country are long and cold, it is understandable why the number of RR aphids is small.

MACE resistance turned out to be the rarest form of resistance. In certain other investigations, ones where resistance was monitored over a longer period of years, this form of resistance proved to be the most unstable. In the 10-year investigations of Foster et al. (2002), it turned out that 68% of the tested samples had this form of resistance in 1996, but the percentage dropped precipitously in the years to follow (only 7% had it in 1997). The cause of this is not known, but studies have shown that it is closely linked with increased production of carboxylesterase and that it occurs most frequently in R2 and R3 aphids (Anstead et al., 2004). In investigations where biochemical methods were used to establish the level of resistance of *Myzus persicae* from peach and tobacco, not one aphid with the R3 level of resistance was found in our country (Vučetić et al., 2007). That is possibly the reason why this form of resistance is not widespread among the tested populations.

Comparing the samples from tobacco and peach, we can say that no very significant differences exist. Nevertheless, the esterase test showed three samples from tobacco to be sensitive. Also, the *kdr* test revealed three sensitive samples, again from tobacco, one of which exhibited sensitivity to both tests. No sample from peach was sensitive, and one had RR resistance in relation to the *kdr* test. Even though the differences are slight, they indicate that aphids from tobacco are somewhat more sensitive than aphids from peach. In view of the fact that protective measures, especially in large peach orchards, are more intensive than in the case of tobacco, it is understandable why aphids from tobacco are more sensitive to insecticides than are aphids from peach trees.

Such a state of resistance, where one or two mechanisms are dominant and a third does not occur or else occurs very rarely, is normal, since few individuals manifest multiple resistance, i.e., are characterized by development of all resistance mechanisms (Li and Han, 2004).

Molecular methods of establishing resistance have shown us that large percentages of *M. persicae* individuals from both peach and tobacco are carriers of genes responsible for the development of resistance, with the result that both subspecies show reduced sensitivity to the most often employed groups of insecticides. However, populations of *M. persicae* in Serbia and Montenegro still do not belong to the category of extremely resistant aphids.

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УТВРЂИВАЊЕ РЕЗИСТЕНТНОСТИ *MYZUS PERSICAE* (SULZER) МОЛЕКУЛАРНИМ МЕТОДАМА

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У двогодишњим истраживањима резистентности зелене бресквине ваши, *Myzus persicae* (Sulzer) молекуларним методама, установљено је да је код већине тестираних јединки са бресквe и дувана у Србији и Црној Гори, утврђен неки од типова резистентности. Већина тестираних јединки имала је FE4 ген који кодира продукцију FE4 карбоксилестеразе. Ген одговоран за *kdr* (knock-down резистентност) нађен је код већине јединки, али у хетерозиготном стању док је резистентност која се заснива на стварању модификоване ацетилхолинестеразе (MACE) најмање заступљена. Такође, тестови су показали да су ваши са дувана осетљивије на дејство инсектицида од вашију са бресквe. У овим истраживањима коришћена су три теста: PCR-естераза тест, PCR–*kdr* и RFLP–PCR, сваки за по један тип резистентности.