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The Relationship between Spirodiclofen Resistance and *Wolbachia* Endosymbiont in *Tetranychus urticae* Koch (Acari: Tetranychidae)

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Abstract:*Tetranychus urticae* Koch (Acari: Tetranychidae) is an important pest that causes economic losses in many varieties of cultivated plants around the world. In this study, it was aimed at determiningthe relationship between spirodiclofen resistance in *T. urticae* and the presence of *Wolbachia*. Therefore, simultaneous selection of spirodiclofen was performed in both *Wolbachia* infected (GSS) and uninfected (GSSN) populations of *T. urticae*. The dry residue method was used to determine lethal concentration (LC) values in *T. urticae*. Bioassay experiments were applied to the larval stage of the mite. The LC value studies were established as 7 doses +1 control and 3 replications. Dead-alive counts were made at the end of the 7th day and resistance ratios were determined. In the last selection of *T. urticae* with *Wolbachia* infection, 23-fold spirodiclofen resistance was determined, and in the last selection without *Wolbachia* infection, 103-fold resistance was determined. The presence of *Wolbachia* was found to be quite low in all *Wolbachia*-uninfected selection populations compared to *Wolbachia*-infected populations. As a result, it is thought that there may be a negative relationship between spirodiclofen resistance and *Wolbachia* endosymbiont in *T. urticae*, and that esterase enzyme may have an effect on the development of resistance within the scope of this relationship.

Keywords: esterase, LC, acaricide, *Tetranychus urticae, Wolbachia*

Tetranychus urticae Koch (Acari: Tetranychidae)'de *Wolbachia* Endosimbiyontu ve Spirodiclofen Direnci Arasındaki İlişki

Öz:*Tetranychus urticae* Koch (Acari: Tetranychidae), dünyada birçok kültür bitkisi çeşidinde ekonomik kayıplara neden olan önemli bir zararlıdır. Bu çalışmada, *T. urticae*' de spirodiclofen direnci ile *Wolbachia* varlığı arasındaki ilişkinin belirlenmesi amaçlanmıştır. Bu nedenle, *T. urticae'* nin hem *Wolbachia* ile enfekteli olan (GSS) hem de enfekteli olmayan (GSSN) popülasyonlarında eş zamanlı spirodiclofen seleksiyonu yapılmıştır. *T. urticae*' de lethal konsantrasyon (LC) değerlerini belirlemek için kuru rezidü yöntemi kullanılmıştır. Biyoassay denemelerde akarın larva dönemine uygulanmıştır. Lethal konsantrasyon çalışmaları 7 doz +1 kontrol ve 3 tekerrürlü olarak yapılmıştır. 7. gün sonunda ölü-canlı sayımı yapılarak direnç oranları belirlenmiştir. *T. urticae'*nin *Wolbachia* ile enfekteli olan son seleksiyonunda 23 kat spirodiclofen direnci belirlenirken, *Wolbachia* ile enfekteli olmayan son seleksiyonda 103 kat direnç belirlenmiştir. *Wolbachia* varlığının, Wolbachia ile enfekte olmayan tüm seleksiyon popülasyonlarında, *Wolbachia* ile enfekte olmuş seleksiyon popülasyonlarına kıyasla oldukça düşük olduğu belirlenmiştir. Sonuç olarak *T. urticae*'de spirodiclofen direnci ile *Wolbachia* endosymbiont arasında negatif bir ilişki olabileceği ve bu ilişki kapsamında esteraz enziminin direnç gelişiminde etkili olabileceği düşünülmektedir.

Anahtar sözcükler: esteraz, LC, akarisit, *Tetranychus urticae, Wolbachia*

1. Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is a polyphagous pest that causes damage to many economically produced plants (Helle & Sabelis, 1985). The pest causes economic loss tomore than 150 important cultivated plants in the world (Zhang, 2003). In the control of *T. urticae*, chemical control is preferred by the producers in the first place (Van Leeuwen et al., 2005). The use of acaricide in the control of the pest is quite common, and it was reported that the world acaricide market value was 900 million euros in 2013 (Van Leeuwen et al., 2015). As a result, the pest has developed resistance to more than 80 acaricides in 60 countries (Miresmailli et al., 2006). It has been reported that *T. urticae* is the pest species that develops the most resistance to chemicals in the world (Dermauw et al., 2013).

Spirodiclofen has a specific mode of action and is located within the spirocyclic tetronic acaricide group.

Spirodiclofen is a selective, non-systemic acaricide, that is one of the tetronic acid derivatives and is widely used in recent times. Spirodiclofen; is effective in all developmental stages of important pest mite species such as *Tetranychus urticae*, *Panonychus ulmi* (Koch) (Acari: Tetranychidae) and *Panonychus citri* (McGregor) (Acari: Tetranychidae) (Wachendorff et al. 2002). Spirodiclofenis used worldwide in the control of phytophagous mites in many cases within integrated pest management programs. This acaricide acts by inhibiting lipid biosynthesis and inhibiting carboxylesterase enzyme activity in pests (Bretschneider et al., 2007).

Wolbachia was first identified in the reproductive tissues of mosquitoes by Herting and *Wolbachia* in 1924 (Hertig & Wolbach, 1924). *Wolbachia* is an endosymbiont proteobacterium that infects invertebrates such as spiders, mites, crustaceans and nematodes, especially insects, and can be transferred from mother to offspring (Stouthamer et al., 1999). In studies conducted, *Wolbachia* was detected in 48 of 63 arthropod species (Aracnida 2, Insecta 61), and in 18 of 20 nematode species (Stouthamer, 1999; Sinkins, 2000). *Wolbachia*, which is found in nearly 80% of arthropods; it causes some reproductive changes such as cytoplasmic incompatibility in its host, death of male individuals, and feminization (Breeuwer et al., 1992; Stouthamer et al., 1999).Although the relationship between mites and *Wolbachia* endosymbiont is still not clearly known, the presence of *Wolbachia* in some phytogaph mite species in the Tetranychidae family has been reported in studies (Vala et al., 2002; Gotoh et al., 2003; Zang et al., 2013; Zele et al., 2018; Pina et al., 2020).

The mechanism underlying the interactions between insecticide resistance and endosymbionts in pests is still not clearly understood. Considering the detoxification abilities and rapid evolution processes of symbionts, it is thought that they may contribute to insecticide resistance in their hosts (Su et al., 2013). It is speculated that endosymbionts may have important roles in modulating host states, detoxifying toxic compounds, and altering their hosts' gene expression. It is thought that detoxifying enzymes such as aromatic ester hydrolase, glucosidase, phosphatase and glutathione transferase can be activated by endosymbionts and this may play a role in insecticide resistance developed in the pest. For example, in *Riptortus pedestris* (Hemiptera: Alydidae), it has been determined that the symbiotic bacterium *Burkholderia* provides protection against organophosphorus pesticides. (Kikuchi et al., 2012). In another study, *Lymantria dispar* (Lepidoptera: Lymantriidae) larvae from *Bacillus thrungiensis* were isolated from the symbiont *Enterobacter* sp. It has been reported that it cannot kill without it (Broderick et al., 2006). Although much is unknown about the interactions between insecticide resistance and symbionts, studies have reported that facultative endosymbionts can cause conditional changes in insecticide resistance in pests. It is thought that there may be a positive, negative or neutral effect between endosymbionts and insecticide resistance developed in the pest, and this situation is also worth investigating.

It is extremely important to know the effect of the *Wolbachia* endosymbiont, which has been identified in the Tetranychidae family, on *T. urticae*, which has the ability to develop rapid resistance to chemicals, in terms of developing new approaches to the control of the pest. Therefore, the possible positive, negative or neutral effect relationship between spirodiclofen resistance and the *Wolbachia* endosymbiont in *T. urticae* was investigated in this study. The development of spirodiclofen resistance was determined in two populations of *T. urticae* with and without *Wolbachia* infection. The relationship between spirodiclofen resistance and the presence and density of *Wolbachia* endosymbionts and the amount of some detoxification enzymes was investigated in both populations.

2. Material and Method

2.1. Origin and reproduction of *Tetranychus urticae*

The susceptible (GSS) population of *T. urticae*, which has been produced in the climate chamber without any pesticide exposure since 2001, was used in the study. As a result of preliminary studies in the GSS population, it was determined that it was infected with *Wolbachia* endosymbiont. The *Wolbachia*-free population obtained as a result of the administration of antibiotics to the GSS population was named as GSSN. The GSS and GSSN populations were used as starting populations for spirodiclofen selections. The production of *T. urticae* populations was carried out on bean (*Phaseolus vulgaris* L. var. barbunia) in climate chambers with 26±2ºC temperature, 60±5% proportional humidity and 16 hours lighting conditions. The populations were produced in the Department of Plant Protection, Faculty of Agriculture, Isparta University of Applied Sciences.

2.2. Molecular identification of endosymbionts in *Tetranychus urticae*

In order to determine the endosymbiont presence in the GSS population of *T. urticae*, DNA isolation was performed collectively from 50 female adult mites. Total DNA isolation was performed with the Qiagen DNeasy Blood & Tissue Kit, following the company's instructions. *Wolbachia, Cardinium, Rickettsia* and *Spiroplasma* are endosymbionts identified in the Tetranychidae family (Zele et al. 2018*)*.Therefore, the presence of these endosymbionts in the GSS population was checked. Primers determined in previous studies were used to detect the presence of endosymbiont bacteria (Jeyaprakash & Hoy 2000; Pina et al. 2020).

2.3. Acaricide

In the study, a commercial preparation (Envidor SC 240) (Bayer) with Spirodiclofen active ingredient, which is in the 23rd group in the IRAC mechanism of action list, was used.

2.4. Antibiotic Administration

In the preliminary studies, it was determined that the GSS population was only infected with *Wolbachia* endosymbiont. In the study, Tetracycline antibiotic was applied to the GSS population in order to obtain the *T. urticae* population without *Wolbachia* endosymbiont. Cottons soaked in 0.05% (w/v) antibiotic were placed in 9 cm petri dishes. Bean leaf discs were placed on cotton. After 24 hours, 50 *T. urticae* larvae in the same period were transferred to beans. One day later, newly hatched larvae were placed on new leaf discs and distilled water was added daily to keep the cotton wet. Thus, the mites have been bred under antibiotics for a generation (Gotoh et al., 1995). Approximately one month later (after at least 3 generations), whether this population was infected with *Wolbachia* was determined by PCR studies. As a result of antibiotic administration, a population free of *Wolbachia* endosymbiont was obtained in the GSS population of *T. urticae*. This population was named the GSSN population. Thus, simultaneous selection of spirodiclofen was started in *T. urticae* with *Wolbachia* infected and *Wolbachia* uninfected two populations.

2.5. Spirodiclofen Selection Tests

The GSS and GSSN populations of *T. urticae* were used as starting populations for spirodiclofen selections. For selection processes, first of all, LC_{50} for spirodiclofen was determined in both mite populations. Spiodiclofen was applied to the larval stage of *T. urticae* in all LC_{50} trials for both mite populations. Trials were established as 1 control + 7 doses, 3 replications for each dose. When determining the application dose of Spirodiclofen, it was taken into account that there was no death less than 90% in the first dose and no more than 10% in the control group. For each replication, 25 mite larvae were added to the Petri dish. With the spirodiclofen concentrations prepared by diluting 50% for each dose, spraying was carried out with the help of spraying tower (Burkard Scientific, England) at 1 bar pressure, 2 mL of pesticide was applied to the leaf surface. Dead-alive counts were made at the end of the 7th day and LC_{50} and LC_{60} values were determined. The LC_{60} values determined for the selection dose of spirodiclofen were used for both mite populations. Spiodiclofen resistances were determined by dividing the LC_{50} values determined for each selection population with the LC_{50} values of the initial populations. Probit analysis was used to calculate LC_{50} values using POLOPlus software (LeOra, 2002). Selection studies in GSS and GSSN populations of *T. urticae* were continued until the 10 th selection.

2.6. Frequency Density of Wolbachia endosymbiont in Selection Populations

Wolbachia presence and frequency were determined at two selection intervals while selecting with spirodiclofen in GSS and GSSN populations of *T. urticae*. First of all, the presence of *Wolbachia* was determined from collective mites (50 units), and if detected, *Wolbachia* frequency density was determined by using 10-20 adult female mites. Thus, the relationship between the increase in spirodiclofen resistance in *T. urticae* and the frequency of *Wolbachia* was tried to be revealed. At the end and beginning of the selection, if *Wolbachia* is detected from the mites isolated individually, the nucleotide sequence was determined, up to a maximum of 10. In this way, in addition to *Wolbachia* frequency, possible changes in the sequence were detected in spirodiclofen-resistant and non-resistant individuals.

2.7. Enzyme Activities

α-naphtylacetate as substrates and the method developed by Stumpf and Nauen (2002) were used to determine the esterase activity kinetically. 20 adult females were homogenized in 100 µl sodium phosphate buffer (0.1M, pH 7.5). 25 µl of supernatant + 25 µl of phosphate buffer (0.2 M, pH: 6) was added

to the cells of the microplate. The study was initiated by adding 200 µl of substrate solution to the cells. The substrate solution was obtained by dissolving 30 mg of fastblue RR salt in 50 ml of 0.2 M sodium phosphate buffer and adding 500 µl of 100 Mm α - naphthylacetate to this mixture. Enzyme activity was read at 23°C, 450 nm for 10 minutes.

The method developed by Stumpf and Nauen (2002) was used to determine the GST enzyme kinetically. 30 adult females were crushed in 300 µl Tris HCL buffer (0.05M, pH:7.5). The total volume of 100 µl supernatant, 100 µl 1-chloro-2,4-dinitrobenzene (CDNB) and 100 µl reducedglutathione (GSH) was placed in the microplate cells. CDNB was prepared in 0.1% ethanol and 0.4 mM CDNB was found in the cells at the final concentration. The change in absorbance was read at 340 nm, 25 °C and 5 min.

In the determination of cytochrome P450 monooxygenase enzyme, p-nitroanisole (PNOD) as substrates and Rose et al. (1995) method was adapted and used. 50 female individuals were crushed with a plastic crusher at a homogenization buffer of 100 µl (0.05 MTris-HCl +1.15% KCl + 1mm EDTA pH (7.7). The mixture was cubed at 30 °C for 5 min by adding 45µl homogenization buffer + 45 µl supernatant+100µl 2mM PNOD to the microplate cells. The reaction was initiated by adding 10 µl 9.6 mM NADPH to the microplate cells. The enzyme activity of P450 was measured in a Versamax kinetic microplate reader (MolecularDevices) at 405 nm 30 ×C for 15 min.

In biochemical studies, control cells were read as non-homogeneous. Enzyme readings were performed four times. All enzyme activities were analyzed in the Softmax PRO software program and the results were given as mOD min^{-1} mg^{-1} protein. Bradford (1976)'s total protein determination method was used to determine the total protein amounts of the samples and Bovine Serum Albumin (BSA) was taken as standard. The enzyme assay results were analyzed using one-way analysis of variance (ANOVA) and the Tukey test to determine differences between populations.

3. Results

3.1. Molecular ıdentification of endosymbionts in *Tetranychus urticae*

The presence of 4 endosymbiont bacteria commonly found in arthropods in the GSS population of *T. urticae,* which was used as the origin, was investigated. Among the bacteria *Wolbachia* (1), *Cardinium* (2), *Rickettsia* (3) and *Spiroplasma* (4), only *Wolbachia* was detected in the GSS population (Figure 1).

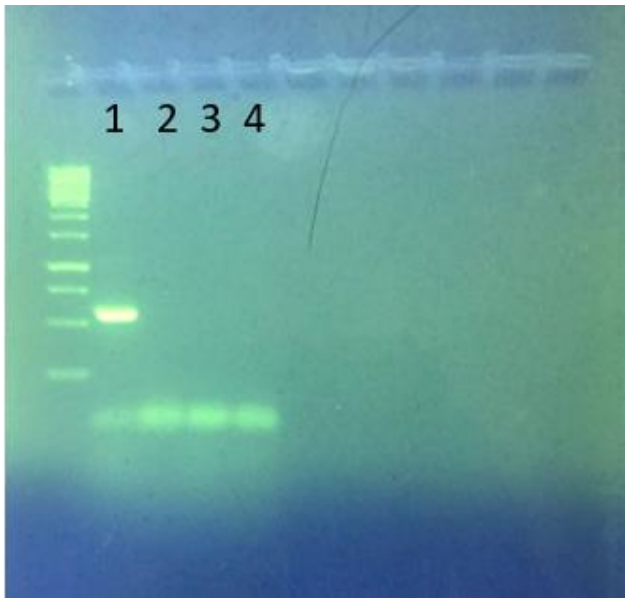

Fig. 1 Endosymbionts of *Tetranychus urticae* in the GSS population

Şekil 1*. Tetranychus urticae'nin GSS popülasyonunda endosimbiyontlar*

3.2. Spirodiclofen selection

The LC values determined as a result of spirodiclofen selection in the GSS population of *T. urticae* and the resistance ratios according to the LC_{50} value are given in Table 1. The resistance ratios determined against spirodiclofen in selection populations vary between 0.8 and 27 times. As a result, 27.0-fold resistance was determined in the S10 population obtained as a result of pre-selection pressure with spirodiclofen.

The LC values determined as a result of spirodiclofen selection in the GSSN population of *T. urticae* and the resistance ratios according to the LC_{50} value are given in Table 2. As a result of selection studies, resistance rates determined against spirodiclofen in selection populations vary between 2 and 103 times. As a result, 103.05-fold resistance was determined in the SN10 population obtained as a result of pre-selection pressure with Spirodiclofen.

3.3. Determination of *Wolbachia* endosymbiont by molecular method

The presence and frequency of *Wolbachia* were determined with both selection intervals in GSS, GSSN and selection populations of *T. urticae* (Table 3). When the table is examined, it is seen that the frequency of *Wolbachia* is higher in all non-antibiotic-treated GSS and selection populations than in antibiotic-treated GSSN and selection populations. As a result of the

studies, although there was a slight decrease in *Wolbachia* frequency in the population without antibiotics after spirodiclofen administration, it was observed that the frequency of *Wolbachia* increased again in advanced populations such as S8. As a result of antibiotic applications, *Wolbachia* bacteria were successfully eliminated in the majority of the population. It was observed that the frequency of *Wolbachia* in the population in subsequent generations did not increase significantly.

Table 1. LC, df, x^2 values and resistance ratios against spirodiclofen in GSS and selection populations of *Tetranychus urticae*

Çizelge 1. *Tetranychus urticae'nin GSS ve seleksiyon popülasyonlarında LC, df, x^2 değerleri ve spirodiclofen direnç oranları*

Population	n*	Slope±SE	LC_{50} (mg a.i. L^{-1}) (95% CL)	LC_{60} (mg a.i. L^{-1}) (95% CL)	df	x^2	R**
GSS	598	1.180±0.110	0.036 0.028-0.045	0.060 0.045-0.076	6	2.8	-
S1	603	1.611±0.146	0.038 0.024-0.048	0.043 0.033-0.052	6	2.5	1.0
S2	605	1.630±0.129	0.062 0.038-0.093	0.088 0.057-0.139	6	1.8	1.7
S3	582	1.427±0.137	0.151 0.096-0.218	0.228 0.156-0.328	6	2.0	4.2
S4	598	1.506±0.318	0.208 0.158-0.261	0.252 0.199-0.316	5	2.8	5.8
S5	578	1.398±0.134	0.258 0.184-0.295	0.318 0.212-0.395	5	2.5	7.1
S6	591	1.881±0.142	0.326 0.223-0.460	0.441 0.307-0.640	6	1.4	9.0
S7	602	1.486±0.186	0.578 0.196-0.956	0.856 0.384-1.490	6	1.9	16.0
S8	565	2.266±0.159	0.765 0.662-1.121	0.991 0.856-1.154	6	2.9	21.2
S9	584	1.848±0.168	0.919 0.645-1.233	1.260 0.924-1.706	5	2.3	25.5
S10	596	1.480±0.126	0.972 0.777-1.200	1.440 1.166-1.800	6	2.5	27.0

*: Total Number of Individuals
**: Resistance Ratio

Table 2. LC, df, x^2 values and resistance rates against spirodiclofen in GSSN and selection populations of *Tetranychus urticae*

Çizelge 2. *Tetranychus urticae'nin GSSN ve seleksiyon popülasyonlarında LC, df, x^2 değerleri ve spirodiclofen direnç oranları*

Population	n*	Slope±SE	LC_{50} (mg a.i. L^{-1}) (95% CL)	LC_{60} (mg a.i. L^{-1}) (95% CL)	df	x^2	R**
GSSN	598	2.102±0.165	0.019 0.014-0.024	0.024 0.019-0.031	6	1.2	-
SN1	575	1.822±0.151	0.038 0.028-0.050	0.052 0.040-0.069	6	1.8	2.0
SN2	592	1.463±0.335	0.158 0.115-0.206	0.187 0.141-0.249	6	1.6	8.3
SN3	578	1.639±0.140	0.163 0.086-0.172	0.189 0.127-0.230	6	2.2	8.5
SN4	565	1.162±0.113	0.195 0.122-0.213	0.273 0.211-0.355	5	2.5	10.2
SN5	563	1.589±0.215	0.583 0.353-1.020	0.842 0.336-1.521	5	2.8	30.6
SN6	585	1.480±0.130	1.032 0.751-1.423	1.528 1.120-1.956	6	1.5	54.3
SN7	603	1.754±0.140	1.063 0.806-1.384	1.780 1.140-1.968	6	2.8	55.9
SN8	592	1.962±0.167	1.221 1.000-1.464	1.944 1.370-2.275	6	2.4	64.2
SN9	576	1.778±0.140	1.392 1.072-1.773	2.232 1.514-2.496	5	1.7	73.2
SN10	569	1.985±0.180	1.958 1.652-2.320	2.965 2.456-3.201	6	2.5	103.05

*: Total Number of Individuals
**: Resistance Ratio

3.3. Determination of *Wolbachia* endosymbiont by molecular method

The presence and frequency of *Wolbachia* were determined with both selection intervals in GSS, GSSN and selection populations of *T. urticae* (Table 3). When the table is examined, it is seen that the frequency of *Wolbachia* is higher in all non-antibiotic-treated GSS and selection populations than in antibiotic-treated GSSN and selection populations. As a result of the studies, although there was a slight decrease in *Wolbachia* frequency in the population without antibiotics after spirodiclofen administration, it was observed that the frequency of *Wolbachia* increased again in advanced populations such as S8. As a result of antibiotic applications, *Wolbachia* bacteria were successfully eliminated in the majority of the population. It was observed that the frequency of *Wolbachia* in the population in subsequent generations did not increase significantly.

Table 3. *Wolbachia* frequency variation in GGS, GSSN and selection populations
Çizelge 3. *GGS, GSSN ve seleksiyon popülasyonlarında Wolbachia frekans değişimi*

Selection Populations	Without Antibiotics	Selection Populations	With Antibiotics
GSS	9/10	GSSN	0/10
S2	3/10	SN2	1/10
S4	6/10	SN4	2/10
S6	3/10	SN6	1/10
S8	7/10	SN8	0/10
S10	5/10	SN10	2/10

In addition, the sequences of certain parts of the *Wolbachia* gene sequences in the GSS, S10 and SN10 populations were revealed in *T. urticae*. However, no differences in gene sequences were detected for all three populations (Figure 2).

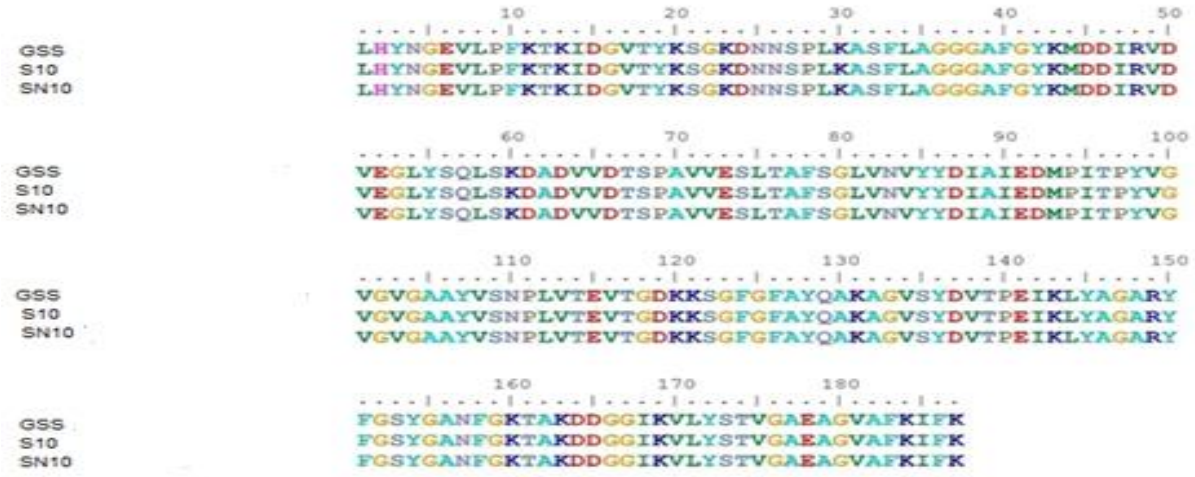

Fig. 2 Comparison of *Wolbachia* amino acid sequences in GSS, S10 and SN10 populations
Şekil 2. *GSS, S10 ve SN10 popülasyonlarında Wolbachia amino asit dizilimlerin karşılaştırılması*

3.4. Enzyme activities

Esterase, GST and P450 enzyme activities in GSS, GSSN and selection populations of *T. urticae* were determined at mOD min^{-1} mg^{-1} protein value, and the results are given in Table 4. Esterase enzyme amounts were found to be statistically similar in GSS and selection populations of *T. urticae*. In the GSSN and selection populations of *T. urticae*, the esterase enzyme amounts in the SN6, SN8 and SN10 populations are higher than the esterase enzyme amounts in the GSSN, SN2 and SN4 populations. When the increase in spirodiclofen resistance in *Wolbachia* uninfected populations and selections was examined, it was determined that the resistance increased significantly in SN6, SN8 and SN10 populations compared to SN4, SN2 and GSSN populations. The GST enzyme was found to be similar in the GSS and selection populations of *T. urticae*, and no statistical difference could be determined. Similarly, no difference was found between GST enzyme amounts in GSSN and selection populations of *T. urticae*. Among the GSS and selection populations of *T. urticae*, the amount of monooxygenase enzyme determined in the S6, S8 and S10 populations was found to be higher than in the GSS, S2 and S4 populations. The lowest amount of monooxygenase enzyme in the GSSN and selection populations of *T. urticae* was determined in the GSSN population and formed a statistical group different

from other populations. The amount of enzyme determined in SN2, SN4, SN6 and SN8 populations was similar. The highest amount of monooxygenase enzyme was determined in the SN10 population, which has 103 times spirodiclofen resistance, and formed a statistical group different from other populations.

Table 4 Esterase, GST and P450 enzyme activities in GSS, GSSN and selection populations of *Tetranychus urticae*

Çizelge 4*. Tetranychus urticae'nin GSS, GSSN ve seleksiyon popülasyonlarında esteraz, GST ve P450 enzim aktiviteleri*

Population	n*	Specific activity mOD min^{-1} mg^{-1} protein (±SE)	Specific activity mOD min^{-1} mg^{-1} protein (±SE)	Specific activity mOD min^{-1} mg ^{1} protein (±SE)
		Esteraz	GST	P450
GSS	4	11.63(±0.001) a**	3.5(±0.003) a**	0.0023(±0.002) b**
S2	4	11.88(±0.002) a	3.2(±0.003) a	0.0032(±0.002) b
S4	4	12.57(±0.002) a	3.8(±0.004) a	0.0035(±0.003) b
S6	4	11.16(±0.005) a	4.0(±0.002) a	0.0049(±0.003) a
S8	4	13.25(±0.004) a	3.6(±0.002) a	0.0053(±0.001) a
S10	4	13.52(±0.002) a	3.8(±0.001) a	0.0055(±0.001) a
GSSN	4	8.69(±0.003) c	2.9(±0.004) a	0.0017(±0.004) c**
SN2	4	8.92(±0.003) c	3.2(±0.004) a	0.0035(±0.004) b
SN4	4	8.72(±0.002) c	3.0(±0.003) a	0.0038(±0.003) b
SN6	4	13.75(±0.001) b	3.5(±0.002) a	0.0050(±0.003) b
SN8	4	14.48(±0.002) b	3.8(±0.002) a	0.0056(±0.002) b
SN10	4	18.90(±0.032) a	4.2(±0.003) a	0.0072(±0.002) a

* Number of recurrences

**The same letters indicate the same group statistically (P<0.05)

4. Discussion and Conclusion

There is no study in the literature to determine the relationship between insecticide/acaricide resistance development in endosymbionts and mites. However, there are studies in which the presence of some endosymbionts, especially in the Tetranychidae family. Vala et al. (2002) determined that *Wolbachia* in *T. urticae* did not affect the lifespan, but caused a change in the survival curves. Gotoh et al. (2003) determined that seven (16.7%) of 42 Tetranychidae species in Japan were infected with *Wolbachia*. Zang et al. (2013) determined that there is *Wolbachia* endosymbiont in Tetranychus species (*T. truncatus, T. phaselus, T. puerariсola* (Acari: Tetranychidae) and *T. urticae*) in China. Zélé et al. (2018) reported that the most common endosymbiont combination in the Tetranychidae family may be *Wolbachia* and *Cardinium*. Pina et al. (2020) reported that *T. truncatus* showed combinations of *Wolbachia* and *Cardinium* or *Spiroplasma* and *Rickettsia*, while *T. evansi, T. ludeni* and *T. urticae* only showed combinations of *Wolbachia* and *Cardinium*. These studies are important because they show that *Wolbachia* endosymbiont is common in *Tetranychidae* species. It is possible that *Wolbachia* symbiont, which is common in Tetranychidae species, is associated with the development of resistance in phytophagous mite species.

Concurrent selection of spirodiclofen was performed in the GSS and GSSN populations. In the GSS and selection populations, spirodiclofen resistance reached a maximum of 27 times. However, although there was a slight decrease in the *Wolbachia* frequency determined at intervals of both selections in the S2 and S6 populations, the frequency of *Wolbachia* was found to be higher in all populations than in the populations treated with antibiotics. In contrast, in GSSN and selection populations, a more rapid development of spirodiclofen resistance was determined after the 5th selection. In *Wolbachia* frequency determination studies performed with both selection intervals, much less *Wolbachia* presence was determined compared to populations that did not receive antibiotics. It was determined that spirodiclofen resistance increased up to 103 times in GSSN and selection populations that were treated with antibiotics. On the other hand, 27-fold spirodiclofen resistance was determined in GSS and selection populations containing *Wolbachia* more intensely. In the literature, high ratios of spirodiclofen resistance development have been reported in laboratory and field populations of *T. urticae* (Rauch & Nauen, 2003; Van Pottelberge et al., 2009a; Van Pottelberge et al., 2009b; Ferreria et al., 2015). However, these studies did not examine whether endosymbionts have an effect on high spirodiclofen resistance in pests. As a result of our study, it can be thought that there may be a negative relationship between the presence and density of *Wolbachia* and the development of spirodiclofen resistance in *T. urticae*.

However, it should be taken into account that the *Wolbachia* frequency density should be determined on a small number of individuals and random sampling may have an effect on the results. Therefore, it is thought that the relationship between *Wolbachia* presence and spirodiclofen resistance in *T. urticae* should be revealed more clearly by increasing the number of samples and repetitions in order to clearly demonstrate this connection in future studies.

Although there is no study on endosymbiont-pesticide resistance in mites, this issue has been investigated in some pests. Symbiont-mediated insecticide resistance/susceptibility varies according to insect species, symbiont species and chemical compound. In a study, the highest susceptibility to acetamiprid, thiametroxam, sporimesifen and pyripoxen was seen in multisymbiont whiteflies such as *Rickettsia-Arsenophonus*, *Rickettsia-Wolbachia* (Ghanim & Kontsedalov, 2009). As a matter of fact, it was emphasized that *Rickettsia* bacteria should be taken into account in the studies to determine insecticide resistance in *Bemisia tabaci* (Hemiptera: Aleyrodidae) populations (Kontsedalov et al. 2008).On the other hand, *Wolbachia* did not change the susceptibility of *Aedes aegypti* (Diptera: Culicidae) to the chemical insecticides bifenthrin, temephos and s-methoprene and *Bacillus thuringiensis* (Bt). In addition, it was determined that *Rickettsia*, coexisting with another symbiont *Arsenophonus*, increased the insecticide resistance against acetamiprid in *B. tabaci*, but did not affect the susceptibility to diafenthiuron. Fenitrothion, an organophosphorus insecticide, can contribute to insecticide resistance by being degraded by *Burkholderia*, a soil-borne symbiont. It has been reported that *S. kochii* in the bean beetle, *Riptortus pedestris* (Hemiptera: Alydidae), has a broad spectrum detoxification capacity and can hydrolyze parathion, an organophosphorus insecticide (DongLiu et al. 2019).

Detoxifying enzymes such as aromatic ester hydrolase, glucosidase, phosphatase and glutathione S-transferase in the pest's body can be activated by symbionts. It has been reported that changes in the activity of detoxification enzymes can lead to changes in the insecticide resistance of symbiont hosts (Kikuchi et al. 2012). In our study, no statistical difference was found between the amount of esterase enzyme in *Wolbachia* infected GSS and selection populations. On the other hand, when the antibiotic-treated GSSN and selection populations were examined, the highest esterase enzyme was determined in the SN10 population. It is observed that esterase amounts of SN6, SN8 and SN10 populations are significantly increased compared to GSSN, SN2 and SN4 populations. However, the rapid increase in spirodiclofen resistance, especially in populations after the SN5 population, suggests that there may be a relationship between resistance and esterase enzyme. At the same time, it is thought that the absence of *Wolbachia* endosymbionts may be effective in the rapid increase in resistance. Because spirodiclofen resistance did not increase much in non-antibiotic-treated GSS and selection populations. It was determined that the GST enzyme activity did not show a statistically significant change in the GSS and GSSN populations of *T. urticae* with and without antibiotic application, and in all selection populations. When P450 monooxygenase enzyme activity was examined, it was determined that the amount of enzyme in S6, S8 and S10 populations was higher in GSS and selection populations compared to other selection populations. Similarly, when the GSSN and selection populations were examined, the highest amount of monooxygenase enzyme was determined in the SN10 population. However, monooxygenase enzyme amounts were found to be higher in the SN2, SN4, SN6 and SN8 populations compared to the GSSN population. However, unlike the monooxygenase enzyme esterase enzyme, it has also increased in selection populations that have been administered antibiotics and those that have not. It has been determined that esterase and P450 enzymes are related to the increase in spirodiclofen resistance in *T. urticae* (Van Pottelberge et al., 2009; Kramer & Nauen, 2011; Badieinia et al., 2020). However, there is no direct study yet to prove that *Wolbachia* can reduce or detoxify the effects of insecticides on pests. However, there are other examples of symbionts that we know can detoxify toxic compounds. Fenitrothion, an organophosphorus insecticide, can cause insecticide resistance by being degraded by a soil-borne symbiont, *Burkholderia* (Hayatsu et al., 2000). In the bean beetle, *R. pedestris*, *S. kochii* endosymbiont can hydrolyze the organophosphorus insecticide parathion (Shen & Downd 1991).

In conclusion, endosymbionts, which show examples of living together in many living species in nature, are also frequently found in pests. Endosymbionts cause some changes in pests, especially on reproduction. However, it is thought that it may also have effects on the development of resistance, which causes difficulties in the control of pests. Knowing the relationship between insecticide

resistance and endosymbiont species and density in pests is important in terms of developing new strategies for control. This study is important in that it is the first study to determine the relationship between acaricide resistance and *Wolbachia* in *T. urticae*.

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