DETERMINATION OF THE RESISTANCE STATUS OF *TETRANYCHUS URTICAE* POPULATIONS COLLECTED FROM VEGETABLE CROPS IN THE MEDITERRANEAN REGION TO SOME NEUROTOXIC ACARICIDES AND SCREENING FOR TARGET SITE MUTATIONS

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Omar Masoud Elsayed MASOUD

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ABSTRACT

Master's Thesis

DETERMINATION OF THE RESISTANCE STATUS OF *TETRANYCHUS URTICAE* POPULATIONS COLLECTED FROM VEGETABLE CROPS IN THE MEDITERRANEAN REGION TO SOME NEUROTOXIC ACARICIDES AND SCREENING FOR TARGET SITE MUTATIONS

Omar Masoud Elsayed MASOUD

Ankara University
Graduate School of Natural and Applied Sciences
Department of Plant Protection

Supervisor: Assoc. Prof. Dr. Emre INAK

This study was conducted to investigate resistance mechanisms in the two-spotted spider mite, *Tetranychus urticae*, a widely distributed phytophagous pest that poses a significant threat to many agricultural products. The efficacy of neurotoxic acaricides such as fluralaner, isocycloseram, milbemectin, and broflanilide was determined in *T. urticae* populations collected from vegetable growing areas in the Mediterranean region of Türkiye, and the results were compared with a susceptible reference population, GSS. Additionally, the toxicity of the new generation insecticides broflanilide was investigated against three predatory mites (*Phytoseiulus persimilis*, *Neoseiulus californicus*, and *Amblyseius swirskii*). The results revealed different sensitivity levels among *T. urticae* populations. The highest resistance levels among the tested populations were observed against milbemectin in the Bib and Pat populations. While fluralaner and isocycloseram were found to be highly effective against *T. urticae*, broflanilide was shown to be ineffective against the tested red spider mite populations. The predatory mites exhibited 100% mortality when exposed to the recommended dose of broflanilide. Finally, a potential mutation in the target site genes of the tested active substances was investigated, and no amino acid changes were detected in either the rdl (resistance to dieldrin) or glutamate-gated chloride channels (GluCl1 and GluCl3).

August 2024, 40 pages

Key Words: Neurotoxic Acaricides, acaricide resistance, GABA-gated chloride channel, target site mutations, *Tetranychus urticae*

ÖZET

Yüksek Lisans Tezi

AKDENİZ BÖLGESİ SEBZE YETİŞTİRİCİLİK ALANLARINDAN TOPLANAN *TETRANYCHUS URTICAE* POPÜLASYONLARININ BAZI NÖROTOKSİK AKARİSİTLERE KARŞI DİRENÇ DURUMUNUN BELİRLENMESİ VE HEDEF YERİ MUTASYONLARININ TARANMASI

Omar Masoud Elsayed MASOUD

Ankara Üniversitesi
Fen Bilimleri Enstitüsü
Bitki Koruma Anabilim Dalı

Danışman: Doç. Dr. Emre İNAK

İki noktalı kırmızı örümcek, *Tetranychus urticae* Koch (Acari: Tetranychidae), çok çeşitli konukçu bitkilerde beslenebilen kozmopolit bir türdür. Meyve ağaçları, pamuk, sebzeler ve süs bitkileri de dahil olmak üzere birçok tarımsal üründe önemli verim kayıplarına neden olabilen fitofag bir zararlıdır. Bugüne kadar dünya genelinde 1500'den fazla konukçu bitkide beslenebildiği rapor edilmiştir. Akarlar, larva döneminden ergin döneme kadar tercihen yaprağın alt yüzeyinde beslenirler. Beslenme sonucunda konukçu bitkide, fotosentezde azalma yoluyla verim kayıpları oluşmaktadır. Ayrıca dışkı birikimi, ağ oluşumu ve/veya yaprak dökülmesi bitkinin ticari değerinin yanı sıra görünümünü de etkileyebilmektedir. Bu faktörlerin birleşimi önemli ekonomik kayıplara yol açabilmektedir.

T. urticae'nin kontrolü dünya çapında büyük ölçüde kimyasal pestisitlere dayanmaktadır. Bununla birlikte, kimyasal uygulamalardaki başarısızlıklar, operasyonel faktörler ve direnç gelişimi nedeniyle sıklıkla rapor edilmektedir. Pestisitlere direnç, dünya çapında giderek daha ciddi bir sorun haline gelmektedir. Yaklaşık 500 böcek ve akar türünde bir veya daha fazla pestisit direnci bildirilmiştir. Kırmızı örümcek ve kene türleri, tüm pestisit sınıflarına karşı kolayca direnç geliştirebilmektedir. Akar popülasyonları, sadece birkaç yıl kullanımdan sonra yeni ruhsatlandırılan bir akarisite karşı bile çok yüksek derecede direnç geliştirebilmekte ve aynı etki mekanizması sahip diğer bileşiklere karşı çapraz dirençli hale gelebilmektedir. Direnç gelişimi uzun zamandır küresel bir sorun oluşturmakta ve bu sorun artarak devam etmektedir. Kimyasal kontrolde başarısızlık doğrudan tarımsal üretimde ekonomik kayıplara yol açmaktadır. Bugüne kadar *T. urticae*, ruhsatlı aktif maddelerin neredeyse tamamına karşı direnç geliştirmiştir. Bu nedenle,

ekonomik açıdan önemli birçok türün mevcut genom dizilerinin oluşturulması ve RNAi gibi tekniklerin yaygınlaşması sayesinde son yıllarda direncin moleküler mekanizmasına ait bilgiler önemli ölçüde artış göstermiştir. Özellikle kırmızı örümcek *T. urticae*, direnç mekanizmalarının karmaşıklığını daha iyi anlamak için önemli bir model olarak ortaya çıkmış, diğer önemli Acari türlerindeki adaptasyon mekanizmalarının aydınlatılması için net bir teşvik sağlamıştır.

T. urticae'ye karşı devam eden direnç nedeniyle her zaman yeni etki mekanizmalarına sahip ilaçlara ihtiyaç bulunmaktadır. Bu tezde üç temel hedef belirlenmiştir: 1) Farklı *Tetranychus urticae* popülasyonlarının bazı nörotoksik akarisitlere karşı duyarlılıklarının belirlenmesi ve hassas bir popülasyon ile karşılaştırılması; 2) Bu akarisitlerin hedef etki yerlerinin gen dizilerinin elde edilmesi, aminoasit değişimlerinin tespit edilmesi ve hedef yeri mutasyonlarının belirlenmesi durumunda, bu mutasyonlar ile toksisite testleri arasındaki korelasyonun incelenmesi; 3) Yeni nesil insektisitlerden broflanilide'nin, üç farklı predator akarı (*Phytoseiulus persimilis, Neoseiulus californicus* ve *Amblyseius swirskii*) karşı toksisitesinin araştırılması.

T. urticae popülasyonlarında fluralaner, isocycloseram, milbemektin ve broflanilide gibi nörotoksik akarisitlerin etki durumları belirlenmiştir. Bu akarisitler IRAC etki mekanizması sınıflandırmasında sinir ve kas sistemi etkili pestisitler içerisinde yer almaktadır. Test edilen akarisitlerin IRAC etki mekanizmaları incelenmiş ve toplamda 5 farklı popülasyon Akdeniz bölgesindeki sebze yetiştirme alanlarından toplanarak laboratuvar ortamına getirilmiştir. Bu popülasyonlar, 25°C±2 sıcaklık ve 16:8 fotoperiyot koşullarında fasulye bitkileri üzerinde yetiştirilmiştir. Hassas popülasyon olarak GSS (German Susceptible Strain) kullanılmış ve arazi popülasyonlarından elde edilen sonuçlar, bu hassas popülasyon ile karşılaştırılmıştır.

Biyoassay denemeleri, IRAC metod 012 modifiye edilerek gerçekleştirilmiştir. Deneyler, petri kaplarında ıslatılmış pamuk üzerine yerleştirilen fasulye yaprakları üzerinde yapılmış, yaprakların canlılığını korumak için gerekli önlemler alınmıştır. Fasulye yapraklarının üzerine 20-30 adet ergin kırmızı örümcek konulmuş ve denemeler en az 5 tekrarla gerçekleştirilmiştir. Farklı dozlarda hazırlanan pestisit karışımları, Burkard Scientific ilaçlama kulesi kullanılarak petri kabındaki akarlara püskürtülmüştür. İlaçlama sonrası petri kapları, 25 °C ±2 sıcaklık ve 16:8 fotoperiyot koşullarına sahip iklim odasına alınmış ve ölü-canlı sayımları 24 saat sonra yapılmıştır. Sonuçlar, PoloPC programı kullanılarak probit analizine tabi tutulmuş ve LC_{50} değerleri hesaplanmıştır. Broflanilide için, yapılan ön denemelerde etkinin az olması nedeniyle sadece 500 ve 5000 ppm dozları denenmiş ve ölüm oranları belirlenmiştir. Bir popülasyon *Phytoseiulus persimilis* ve *Neoseiulus californicus, T. urticae* üzerinde fasulye bitkileri ve ıslatılmış pamukla yetiştirilmiş; *Amblyseius swirskii* ise kuru meyve akarları (*Carpoglyphus lactis*) üzerinde yetiştirilmiştir. Bu predatör akarlar için yapılan biyoassaylarda, broflanilide'nin 20 ppm önerilen dozundaki etkisi değerlendirilmiştir.Moleküler analizler ile, ilacın hedef etki

yerinde dirençle ilişkili nokta mutasyonlarının ve yeni mutasyonların tespitini amaçlamıştır. Bu süreçte, öncelikle RNA izolasyonları gerçekleştirilmiştir. Daha sonra, elde edilen RNA'lar cDNA sentezi ve PCR çalışmaları için kullanılmıştır. RNA izolasyonu sonrası kalite ve kantite elektroforez ve nanodrop ile kontrol edilmiştir. Uygunluğu doğrulandıktan sonra, cDNA'lar uygun primerlerle çoğaltılmış ve PCR sonrası elde edilen ürünler elektroforez ile görüntülenmiştir. Elde edilen PCR ürünleri pürifiye edilerek dizi analizine tabi tutulmuş olup, gelen sekans verileri BioEdit v.7.0.5 ve MEGA yazılımları ile analiz edilmiştir.

Sonuçlar, *T. urticae* popülasyonları arasında farklı duyarlılık seviyeleri ortaya koymuştur. Test edilen popülasyonlar arasında en yüksek direnç seviyeleri, Bib ve Pat popülasyonlarında milbemectine karşı gözlemlenmiştir. Fluralaner ve isocycloseram ise *T. urticae*'ye karşı oldukça etkili bulunurken, broflanilide aktif maddesinin test edilen kırmızı örümcekler popülasyonlarına karşı etkisiz olduğu gösterilmiştir. Predatör akarlar ise, broflanilide'ın tavsiye edilen dozuna maruz kaldıklarında %100 ölüm oranları göstermiştir. Son olarak, test edilen aktif maddelerin hedef etki genlerinde olası bir mutasyon olup olmadığı araştırılmış olup, GABA-kapılı ve Glutamat-kapılı klorid kanallarında herhangi bir amino asit değişimi tespit edilmemiştir.

Ağustos 2024, 40 sayfa

Anahtar Kelimeler: nörotoksik akarisitler, akarisit direnci, GABA-kapılı klorid kanalı, hedef yeri mutasyonları, *Tetranychus urticae*

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Omar Masoud Elsayed MASOUD
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SYMBOLS AND ABBREVIATIONS

LD_{50}	Lethal Concentration for 50% of the population
LC_{50}	Lethal Concentration for 90% of the population
RR_{50}	Resistance Ratio at 50% mortality
ppm	Parts Per Million
°C	Degrees Celsius (temperature)
µL	Microliters (volume)
rpm	Revolutions per minute (centrifuge speed)
cm^2	Square centimeters (area)
mg/L	Milligrams per liter (concentration)
TSSM	Two-Spotted Spider Mite
Tu, *T. urticae*	*Tetranychus urticae*
P. persimilis	*Phytoseiulus persimilis*
N. californicus	*Neoseiulus californicus*
A. swirskii	*Amblyseius swirskii*
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
cDNA	Complementary DNA
bp	Base Pairs
WB1	Wash Buffer 1
WB2	Wash Buffer 2
F	Forward Primer
R	Reverse Primer
İRAC	Insecticide Resistance Action Committee
ACC	Acetyl-CoA carboxylases
AChE	Acetylcholinesterase
GABA:	Gamma-Aminobutyric Acid
GABAR	Gamma-Aminobutyric Acid Receptor
GluCl	Glutamate-gated Chloride Channels
nAChR	Nicotinic acetylcholine receptor
cysLGIC	Cys-loop Ligand-gated Ion Channel
QTL	A quantitative trait locus
IPM	Integrated Pest Management
Rdl	Resistant to dieldrin
UGT	UDP-Glucuronosyltransferase
MoA	Mode of Action
SE	Standard Error
df	Degrees of Freedom
Chi^2	Chi-Squared
a.i./L	Active ingredient per liter
P450	Cytochrome P450

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1. INTRODUCTION

1.1 *Tetranychus urticae*

The two-spotted spider mite "*Tetranychus urticae*" (Figure 1.1), is a globally distributed pest that attacks many plant species (Helle & Sabelis, 1985; Spider Mites Web, Figure 1.2).

This phytophagous mite significantly impacts agriculturally important plants, such as fruits, cotton, vegetables, and ornamental plants, often resulting in major yield losses (Stumpf & Nauen, 2001; Van Leeuwen & Tirry, 2007). The host range of *T. urticae* is quite extensive, encompassing 1513 different plant species worldwide. This includes weeds, fruits, vegetables, and ornamental plants (Migeon & Dorkeld, 2010).

Throughout their development from larvae to adults, these mites prefer feeding on the underside of leaves (Attia et al., 2013). Their feeding activities not only reduce yields by impeding photosynthesis in host plants but also lead to fecal accumulation, webbing, and leaf abscission. These effects decrease the commercial value and visual appeal of the plants, causing significant economic losses (Attia et al., 2013).

Figure 1.1 *Tetranychus urticae* (www.koppert.com)

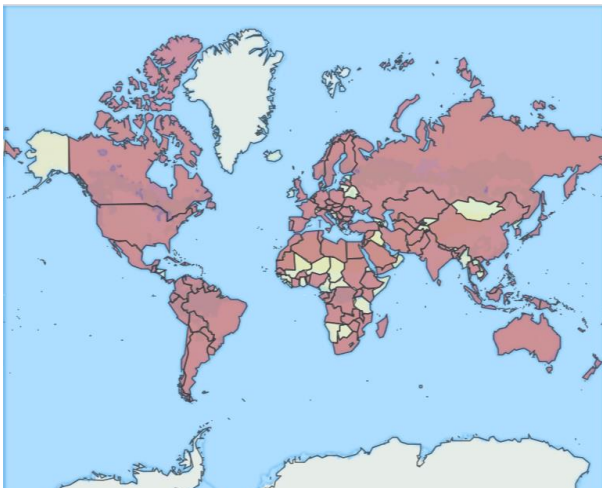


Figure 1.2 World Distribution Map of *Tetranychus urticae* (Spider Mites Web)

1.2 Acaricide

1.2.1 Chemical acaricides

Before the mid-20th century, Spider mites and other mites that feed on plants were previously seen as insignificant pests in agriculture. They were naturally kept in control by predatory mites and insects. However, following World War II, agricultural practices advanced significantly, including the widespread utilization of chemical insecticides and fertilizers, developed irrigation systems, and other methods that made crops more attractive to spider mites, leading to population explosions. The situation worsened with the use of broad-spectrum neurotoxic insecticides, which were more harmful to natural predators of the mites than to the mites themselves. This led to resistant mite populations due to the strong selection pressure from these insecticides. In response, the 1950s saw a shift from broad-spectrum insecticides to specific acaricides, resulting in the development of various generations of synthetic acaricides targeting different biochemical and physiological pathways. The first specific acaricides, like bridged diphenyls, were developed in the 1950s and 1960s, followed by organotins, formamidines, quinoxalines, and propargite in the mid-1970s, In the 1980s, clofentezine and hexythiazox were introduced as mite growth inhibitors. Additionally, Acaricide such avermectins, benzoylureas and pyrethroids were introduced in the 1970s and 1980s. The early 1990s

ushered in the modern era of synthetic acaricides with the creation of compounds targeting mite respiration (Marcic, 2012).

These chemical agents are essential for safeguarding agricultural, horticultural,and ornamental plants from the harmful effects of phytophagous mites, which are well-known for feeding on plant tissues and significantly diminishing crop yield and quality. In plant protection, acaricides are critical, applied strategically to manage infestations and mitigate significant threats to agricultural productivity and profitability. By targeting various stages of the mite life cycle, acaricides interfere with feeding, reproduction, and population dynamics, thereby effectively minimizing their potential for widespread crop damage (Capinera et al., 2008).

Beyond their agricultural applications, acaricides are also crucial in veterinary medicine, especially for managing mite infestations in livestock and pets. These applications are vital for preventing the spread of mite-borne pathogens, protecting animal health, and reducing the risk of disease transmission to other vulnerable hosts (Coles & Dryden, 2014;
Abbas et al., 2014).

Acaricides have a multifaceted role in modern agriculture and veterinary medicine. They play an essential role in IPM strategies, essential for maintaining plants and animals healthy. Their targeted action against destructive mite species highlights their importance in ensuring the sustainable production of food, fiber, and ornamental crops (Capinera et al., 2008).

1.2.2 Acaricide market

The economic impact of mites as agricultural pests is substantial, as demonstrated by acaricide market trends (Van Leeuwen et al., 2015). Examining acaricide usage and sales data reveals the economic scale of issues related to major mite pests across different regions

and agricultural systems. In 2008, the acaricide market analysis revealed that about 80% of the market value was dedicated to managing spider mites. Specifically, *Tetranychus* species, including *T. urticae*, accounted for 372 million euros, which is 62% of the market share (Van Leeuwen et al., 2015).

In 2013, the global market for insecticides was valued at 13.3 billion euros, with the acaricide segment accounting for 900 million euros. Within this segment, ACC inhibitors (IRAC group 23) dominated the market share (Van Leeuwen et al., 2015; Sparks & Nauen, 2015). The significance to recognize that the reported sales figures for acaricides, which often highlight specific compounds used for mite control, may not fully capture the entire market value. This is because the phytophagous mites in various products and areas are also controlled by insecticides like pyrethroids, organophosphates and carbamates.

The economic significance of acaricides, along with the difficulties presented by mite pests, is highlighted by this statistical data, underscoring the necessity for effective management strategies to control mite infestations in agricultural environments.

In Turkey, the use of acaricides to manage *Tetranychus urticae* has been on the rise, making the country the tenth-largest global market for these products (Van Leeuwen et al., 2015). From 2006 to 2022, acaricide usage increased significantly from 902 tonnes to 2,462 tonnes (TSI: Turkish Statistical Institute), underscoring the essential role of strong pest management strategies in agriculture.

Approximately 74% of acaricides are deployed on vegetables and fruits, with significant use on crops such as grapes and citrus. However, recent studies have indicated a rising concern over spider mite infestations in crops like corn, cotton, and soybeans (Van Leeuwen et al., 2015).

1.2.3 Neurotoxic acaricides

Neurotoxic acaricides are chemicals that interfere with the normal functioning of the nervous system through exposure to either natural or synthetic toxic agents. This interference can result in damage or death of neurons, which are essential for transmitting and processing signals within the brain and other components of the nervous system. Understanding the target sites of acaricides is crucial for tackling the increasing problem of resistance. Knowing these target sites allows for the classification of acaricides based on their interactions with specific sites, helping to identify potential cross-resistance risks, as outlined by the insecticide Resistance Action Committee (IRAC). Additionally, understanding the molecular targets of acaricides aids in targeted screening efforts, including the identification of regional resistance alleles, which are critical for developing effective resistance management strategies, such as planning rotational spray programs. Table 1.1 presents a detailed the acaricides mode of action and target sites, as classified by the IRAC for Nervous and Muscle Acting Acaricides.

Table 1.1 IRAC Mode of Action Classification

IRAC Group	**MoA/Target Site**	**Chemical Structure**
1	Acetylcholinesterase (AChE) Inhibitors	Carbamate
		Organophosphate
Acetylcholinesterase is enzyme released at nerve-muscle junctions to promote muscle relaxation, as it is responsible for breaking down the neurotransmitter acetylcholine. Inhibition of acetylcholinesterase leads to the accumulation of acetylcholine, causing overstimulation. The continuous presence of acetylcholine results in ongoing nerve impulse transmission and prolonged muscle contractions.		
2	GABA-Gated Chloride Channel Antagonists	Organochlorine
		Isoxazoline
		Pyrazole
By blocking the chloride channels activated by GABA, neurotoxic acaricides cause overstimulation, leading to paralysis and death in insects. GABA, the primary inhibitory neurotransmitter in insects, plays a crucial role in regulating neural activity. Tissues that depend heavily on aerobic respiration, like the central nervous system and the heart are particularly vulnerable to this disruption.		

Table 1.1 IRAC Mode of Action Classification (continue)

IRAC Group	MoA/Target Site	Chemical Structure
3	Sodium Channel Modulators	Pyrethroids (bifenthrin)
These substances cause sodium channels to remain open, resulting in overstimulation and, in some instances, nerve blockage. This continuous activation triggers repeated neuronal discharges (both sensory and motor) and extended negative after-potentials, leading to nervous system hyperactivity. Such conditions can cause paralysis or death, similar to the effects of DDT. The transmission of action potentials along nerve axons relies on sodium channels		
6	Chloride Channel Activators	Avermectin Milbemycins
By triggering glutamate-gated chloride channels in the neurons, these substances induce cellular hyperpolarization and disrupt signal transmission. Glutamate is a key inhibitory neurotransmitter in insects, and its involvement in this process helps to explain the resulting effects on cellular activity.		
19	Octopamine Receptor Agonists	Formamidine
When octopamine receptors are activated, it causes overstimulation. Octopamine, the insect counterpart of adrenaline, serves as a neurohormone for the fight-or-flight response.		
32	Allosteric modulators of nicotinic acetylcholine receptors at Site II	GS-omega/kappa-Hxtx-Hv1a peptide
Allosteric modulators of nicotinic acetylcholine receptors (nAChRs) stimulate nAChRs at Site II, resulting in excessive nervous system activity. In insects, acetylcholine acts as the main excitatory neurotransmitter in their central nervous system.		
33	Modulators of Calcium-Activated Potassium Channels (KCa2)	Acynonapyr
Inhibiting KCa2 channels causes hyperexcitability and convulsions. These channels, which are triggered by increased calcium levels within cells, are essential for controlling action potentials and maintaining neuronal stability.		

1.2.4 Fluralaner

Fluralaner, a state-of-the-art isoxazoline insecticide, specifically targets the γ-aminobutyric acid receptor (GABAR) with distinct binding sites and mechanisms of action, showing absence of cross-resistance with other insecticides that target GABAR, such as fiproles and cyclodienes. It is highly effective against various parasitic mites, including those affecting animals like *Canis lupus dingo* and red-handed tamarins with demodectic mites, as well as poultry red mites in birds and northern fowl mites in laying hens. Impressively, fluralaner has proven to be extremely effective against the

phytophagous mite *Tetranychus urticae* (TSSM), achieving complete mortality at a concentration of 3.1 mg/L, even in strains resistant to fipronil, which typically requires around 500 mg/L for control. Understanding fluralaner's toxicological impact on TSSM is vital for developing strong mite management programs (Leviticus et al., 2020).

1.2.5 Broflanilide

The newly introduced insecticide broflanilide targets the γ-aminobutyric acid receptor (GABAR) in insects. It is listed under MoA group 30 by IRAC as a GABA-gated chloride channel allosteric modulator. It is effective against the larvae of various chewing pests, including termites, ants, cockroaches, flies, and species in the Lepidoptera and Coleoptera orders. It has proven particularly effective against the cotton leafworm (*Spodoptera litura*) and other Insects that are resistant to traditional insecticides. Since its global release in 2020 under the trade names Vedira and Tenebenal, broflanilide has been successfully used in pest control. This pest has become resistant to older noncompetitive antagonists like dieldrin, α-endosulfan, and fipronil, demonstrating broflanilide's potential to effectively combat resistance issues (Shen et al., 2021).

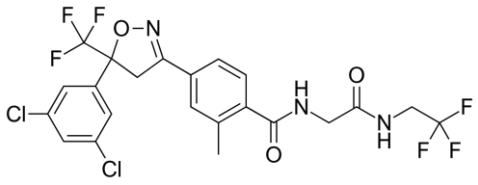
1.2.6 Isocycloseram

Isoxazolines and meta-diamides are unique chemical classes with particular modes of action that involve inhibiting the GABA receptor, although they target different sites than fiproles and organochlorines (Ozoe et al., 2010). While isoxazolines have been extensively utilized in veterinary medicine, the recent introduction of agrochemical products from these groups, such as fluxametamide and isocycloseram by Syngenta Crop Protection, represents a notable advancement in pest management. Specifically, isocycloseram functions as a uncompetitive antagonist at a unique site on the insects GABA receptor, which differs from the sites targeted by cyclodienes and fiproles but is related to the binding sites of other isoxazolines and meta-diamides. (Blythe et al., 2022).

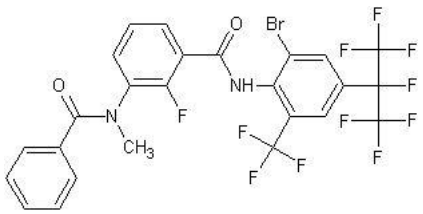
These advancements have led to a reclassification of acaricides based on their primary biochemical targets. According to IRAC, a new Group 30 has been established for GABA-gated chloride channel receptor modulators. This new group reflects the unique yet interrelated activities of isoxazolines and meta-diamides, with examples including fluxametamide and broflanilide. Additionally, IRAC has classified isocycloseram as a novel insecticide within this new classification (Blythe et al., 2022).

1.2.7 Milbemectin

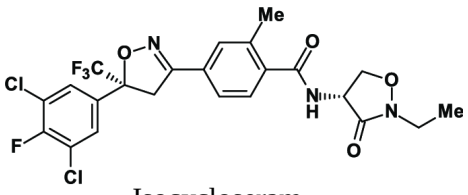
Milbemectin, which includes the macrocyclic compounds milbemycin A3 and milbemycin A4, is classified alongside the acaricide abamectin. Discovered by Sankyo in the 1970s and commercially launched in 1991, milbemectin is primarily used as an acaricide but also proves effective against certain homopteran and lepidopteran pests. It acts as both a contact and stomach poison, being most effective against the immature mobile stages of mites, with reduced activity against eggs and adults. Its mechanism of action includes hyperpolarizing cells and interrupting signal transmission by opening glutamate-gated chloride channels (Zhao et al., 2011).



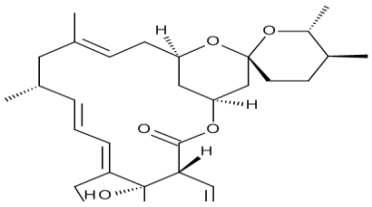
Fluralaner



Broflanilide



Isocycloseram



Milbemectin

Figure 1.3 Chemical structures of acaricides

1.3 Acaricide Resistance

The IRAC defines resistance as “a heritable change in the sensitivity of a pest population, resulting in the repeated failure of a product to achieve the anticipated level of control when used according to the label recommendations for that pest species” (https://irac-online.org).

Pesticide resistance is becoming a growing global concern, with around 500 insect and mite species documented as resistant. In phytophagous mites, especially spider mites, there is a significant trend of rising acaricide resistance. These populations can rapidly develop high resistance levels, sometimes in the early years after the introduction of a novel acaricide, and may also exhibit cross-resistance to other acaricides with the same MoA (De Rouck et al. 2023).

The Arthropod Pesticide Resistance Database (APRD), a collaborative project supported by IRAC, the U.S. Department of Agriculture, Michigan State University, provides detailed information on insect and mite resistance, which affects public health, veterinary medicine, and agriculture. Since its establishment in 1914, the database has recorded 18,380 documented instances of resistance involving 83 species from the Acari subclass. Furthermore, the APRD ranks the top 10 most resistant arthropods' worldwide, with *Tetranychus urticae* placed second. (Table 1.2) (https://www.pesticideresistance.org/search.php)

Table 1.2 The list of arthropod species with the highest resistance, according to the number of active ingredients (ai) and the recorded instances of resistance for each species (APRD)

Species	Order: Family	type	No. a.i	instances
Plutella xylostella	Lepidoptera: Plutellidae	Agricultural	101	1055
Tetranychus urticae	Acari: Tetranychidae	Agricultural	96	558
Myzus persicae	Homoptera: Aphididae	Agricultural	87	514
Bemisia tabaci	Homoptera: Aleyrodidae	Agricultural	69	770
Musca domestica	Diptera:Muscidae	Medicine	66	463
Leptinotarsa decemlineata	Coleoptera: chrysomelidae	Agricultural	56	306
Helicoverpa armigera	Lepidoptera: Noctuidae	Agricultural	55	892
Aphis gossypii	Homoptera: Aphididae	Agricultural	52	431
Hipicephalus microplus	Acari: Ixodidae	Medicine	50	562
Panonychus ulmi	Acari: Tetranychidae	Agricultural	48	203

Extensive research on acaricide resistance in spider mites has resulted in major investigations into their mechanisms of genetic, physiological, and biochemical, enhancing our understanding of the factors driving this evolutionary phenomenon. Resistance in *Tetranychus urticae* generally arises from an increased quality and quantity of both target site resistance and detoxification enzymes, reflecting patterns seen in other arthropods (Cranham & Helle, 1985; Van Leeuwen et al, 2009 and 2010).

Ongoing research on acaricide resistance mechanisms is focused on uncovering their molecular structure. Point mutations, which are nucleotide substitutions that lead to amino acid changes at the target site, have been linked to reduce sensitivity and resistance in *T. urticae* and other spider mites. Reduced sensitivity of the enzyme acetylcholinesterase (AChE), which is essential for the transmission of nerve impulses, is a typical defense mechanism against organophosphates. In the early 1960s, target site resistance was initially detected in *T. urticae*, an arthropod species. (Van Leeuwen & Tirry, 2007).

As a family of supergenes, cysLGIC is a prime target for a wide variety of acaricides. Despite their importance, there is a lack of research on their characterization, evolutionary relationships, and physiological functions in arachnids, such as *T. urticae*. These arthropoda contain a variety of cysLGICs, including nAChRs (nicotinic acetylcholine

receptors), GABA-gated channels, GluCls (glutamate-gated chloride channels), HisCls (histamine-gated chloride channels), and pHCl (pH-sensitive chloride channels). CysLGICs are characterized by their consistent structure, which includes four transmembrane segments and a prominent N-terminal extracellular domain that contains a conserved cysteine-bridge motif. These channels are essential for neurotransmitter binding and ion flow, playing crucial roles in synaptic inhibition, excitability of cells, pH control, and transport of organic solutes across the arthropod nervous system. Spinosad exhibits a strong and selective interaction with nAChRs. In contrast, Cyclodienes (e.g., dieldrin) and phenylpyrazoles (e.g., fipronil) target GABA receptors. Avermectins and ivermectins, which are powerful macrocyclic lactones, target GluCls, which are crucial for nerve function in invertebrates (Dermauw et al., 2012).

A GluCl gene in *T. urticae*, notably, includes variants like Tu_GluCl1, which has shown a G323D point mutation significantly associated with reduced sensitivity to avermectin, suggesting a 17-fold increase in resistance (Kwon et al., 2010). Further, In *T. urticae*, the cysLGIC gene family has unveiled extensive insights into its genetic complexity. This analysis, the largest of its kind among arthropods, highlighted a surprising abundance of resistant to dieldrin and Glutamate-gated chloride channel genes, alongside an array of other cysLGIC subunits. Further discovery of the G326E mutation in Tu_GluCl3 notably expanded our understanding of acaricide resistance mechanisms, potentially influencing future IPM strategies (Dermauw et al., 2012).

Subsequent studies have revealed that the I321T mutation in the GluCl3 gene significantly contributes to abamectin resistance in *T. urticae* (Xue et al., 2021). Additionally, research into the genetic factors underlying abamectin resistance in *T. urticae* has utilized experimental evolution and bulked segregant analyses of two distinct populations, uncovering several quantitative trait loci (QTLs) linked to resistance. Notably, three QTLs were identified, encompassing genes related to the GluCl channel as well as DNA helicases and chemosensory receptors, which have been newly identified as resistance sites. Moreover, the discovery of a non-functional GluCl2 variant in one resistant population suggests that gene knockout maybe a mechanism of resistance (Villacis-Perez et al., 2023).

Additionally, ionotropic γ-aminobutyric acid receptors (GABARs) significantly influence neurotransmission within both vertebrates and invertebrates by regulating chloride currents. These receptors, targeted by synthetic insecticides and natural compounds like picrotoxinin, exhibit changes in susceptibility due to mutations in rdl in *Drosophila melanogaster*. This mutation, alongside similar modifications observed in other cyclodiene-resistant arthropods, highlights the adaptive changes contributing to pesticide resistance (Kobayashi et al., 2020).

The identification of distinct characteristics in the GABAR subunit TuRDL from *T. urticae*, including unique residues that modulate sensitivity to non-competitive antagonist (NCAs) like endosulfan and fipronil, underscores the complexity of resistance mechanisms. These findings emphasize the need for continuous research to fully understand and manage resistance effectively within pest populations (Mermans et al., 2023).

Furthermore, next-generation insecticides like fluralaner and isocycloseram target GABAR at a different site compared to fipronil. As a result, common resistance mutations found in traditional GABA inhibitors do not compromise the effectiveness of these new GABA inhibitors (Blythe et al., 2022).

1.4 Predatory Mites

Relying exclusively on synthetic acaricides for controlling plant-feeding mites has proven to be an unsustainable strategy. In contrast, biological control using predatory mites, particularly those from the Phytoseiidae family, has emerged as an effective alternative, especially in protected crops, although it does have certain limitations. Predatory mites from the Phytoseiidae family, which belong to the Mesostigmata order, play a crucial role in managing *T. urticae* populations. Since 1986, the number of documented species within this family has increased significantly, from about 1,500 species across 79 genera to approximately 2,692 species across 84 genera in 2012 (Vásquez et al., 2023).

These mites are acknowledged worldwide for their natural capacity to regulate populations of phytophagous arthropods on both cultivated and wild plants. Their effectiveness is not limited to spider mites but also includes some pests belonging to family Thripidae and Aleyrodidae in both greenhouse and field (Fathipour & Maleknia, 2016). The use of predatory species in pest management has been effective in many areas. These species include *Phytoseiulus persimilis, Neoseiulus californicus,* and *Amblyseius swirskii* (Liu et al., 2019).

Current plant protection strategies prioritize combining integrated pest management (IPM) strategies such as chemical, biological, cultural, and other control methods, to ensure their effective. Acaricides used in IPM systems must be selective to target mite pests effectively while preserving beneficial predators. To maintain the balance and effectiveness of IPM techniques, it is crucial to evaluate the impact of acaricides on beneficial arthropods (Marcic, 2012).

1.4.1 *Neoseiulus californicus*

N. californicus displays attributes of both specialist predatory mite (type II) and generalist predatory mite (type III) (McMurtry & Croft, 1997; McMurtry et al., 2013). Its primary diet consists of Tetranychidae; However, it is capable of predation other mites, small insects when its main prey is limited, which enhances its versatility as an agent in biological control programs worldwide (Rhodes & Liburd, 2005).

1.4.2 *Amblyseius swirskii*

A. swirskii, classified as a generalist predatory mite (type III) (McMurtry & Croft, 1997; McMurtry et al., 2013), is well-adapted to the Eastern Mediterranean region. It effectively decreases populations of whiteflies, thrips, eriophyid mites, broad mites, and spider mites across various crops, thereby increasing its importance in IPM strategies (Calvo et al., 2015).

1.4.3 *Phytoseiulus persimilis*

P. persimilis, classified as a predatory mite (type I) (McMurtry & Croft, 1997; McMurtry et al., 2013), shows an extremely specialized feeding behavior targeting Tetranychus species, especially *T. urticae*. This specificity has established it as a key component of greenhouse biocontrol since 1968, with adults able to consume large numbers of *T. urticae* eggs, females, and juveniles each day (Escudero & Ferragut, 2005).

Ultimately, the swift development of resistance in *Tetranychus urticae*, driven by its short life cycle and high reproductive rate, requires strong and innovative methods for effective resistance management. The species' extensive host range and generalist feeding habits add to the complexity of control, highlighting the critical need for integrated pest management practices that combine both chemical and non-chemical approaches (İnak et al., 2019).

2. MATERIAL AND METHODS

2.1 Mite populations

2.1.1 *Tetranychus urticae* populations

Five *T. urticae* populations were collected from vegetable farming areas from Mediterranean region of Türkiye (Table 2.1). The German Susceptible Strain (GSS), a susceptible mite population, was kindly provided by Prof. Dr. Thomas Van Leeuwen (Gent University, Belgium) and was used as a susceptible laboratory reference population.

The *T. urticae* populations were cultivated on bean plants at 25°C ± 2 and 16L:8D photoperiod condition (Figure 2.1).

Figure 2.1 *Tetranychus urticae* populations

Table 2.1 Host plants and origin of *T. urticae* populations

Population code	Origin	Host plant	Date
Kod	Antalya	Tomato	February/2023
Mer	Mersin	Eggplant	April/2023
Bib	Antalya	Pepper	May/2023
Pat	Antalya	Eggplant	May/2023
Papa	Antalya	Cotton	August/2023
Gss	Germany	Tomato	September/2023

2.1.2 *Phytoseiulus persimilis* population

The laboratory population of *P. persimilis* was kindly provided by İpek Yaşar (Çanakkale Onsekiz Mart University). *P. persimilis* population were reared using *T. urticae* on bean plants into the cage under a photoperiod of 16L:8D photoperiod condition, and at a temperature of 25°C ± 2 (Figure 2.2).

2.1.3 *Neoseiulus californicus* population

The laboratory population of *N. californicus* was kindly provided by Dr. Narin Gök (Directorate of Plant Protection Central Research Institute). *N. californicus* population were reared on *T. urticae* on bean plants into the cage and bean leaf on wet cotton under 16L:8D photoperiod condition, and at a temperature of 25°C ± 2 (Figure 2.2).

2.1.4 *Amblyseius swirskii* population

The laboratory population of *A. swirskii* was kindly provided by Prof. Dr. Alper Nabi Kumral (Bursa Uludağ University). *A. swirskii* population were reared on dried fruit mites, *Carpoglyphus lactis* (Astigmata, Carpoglyphidae) under a photoperiod of 16 hours of light and 8 hours of darkness, and at a temperature of 25°C ± 2.

Figure 2.2 *Phytoseiulus persimilis* and *Neoseiulus californicus* populations

2.2 Acaricides

The pesticides used in the study were fluralaner (10 g/L), isocycloseram (200 g/L), milbemectin (9.3 g/L), and broflanilide (150 g/L). These pesticides were classified under the IRAC MoA classification as pesticides affecting the nervous and muscular systems, and they belonged to groups 2, 6, and 30. The IRAC mode of action and the mechanisms of the tested acaricides are presented in Table 2.2.

Table 2.2 Target sites of action for the acaricides utilized in the study

Acaricide	Commercial name	IRAC Group	Target Site in Nervous system
milbemectin	Milbeknock	6	Glutamate-gated chloride channels
fluralaner	Exzolt	30	GABA-gated chloride channels
isocycloseram	Simodis	30	GABA-gated chloride channels
broflanilide	Cimegra	30	GABA-gated chloride channels

2.3 Bioassays

The bioassays were conducted by modifying IRAC Method 012. The procedure included.

2.3.1 *Tetranychus urticae* bioassays

- **Preparation**: Cotton discs were placed in 90 mm Petri dishes and wetted, with 9 cm^2 bean leaf squares placed on top with tissue paper edges (Figure 2.3).
- **Transfer**: A minimum of 20-30 adult *Tetranychus urticae* were transferred onto the leaves using a brush with a fine tip (1mm size).
- **Application**: A minimum of five different doses, determined from preliminary tests, were applied using a spray tower (Burkard Scientific, England) at 1 bar pressure with 2 ml of an acaricide and water mixture (1.95±0.05 mg of acaricide deposit/cm^2). Control Petri dishes were treated with only distilled water, ensuring control mortality did not exceed 10% (Figure 2.3).
- **Post-application**: The Petri dishes placed at a climate room set at 25±2°C, with a 16L: 8D photoperiod condition.
- **Observation**: Counting alive and dead mites 24 hours post- application. Mites that exhibited no movement greater than twice their body length when stimulated were classified as dead.
- **Analysis**: The results were interpreted with PoloPC (LeOra Software, Berkeley, CA) for probit analysis, calculating LC_{50} and LC_{90} values and generating dose-mortality graphs.

Preliminary bioassays showed that broflanilide had almost no effect on *T.urticae* when applied at recommended field rate (20 ppm), only high concentrations, 500 and 5000 mg/L, were further tested and percent mortalities were recorded after correction using Abbott's formula.

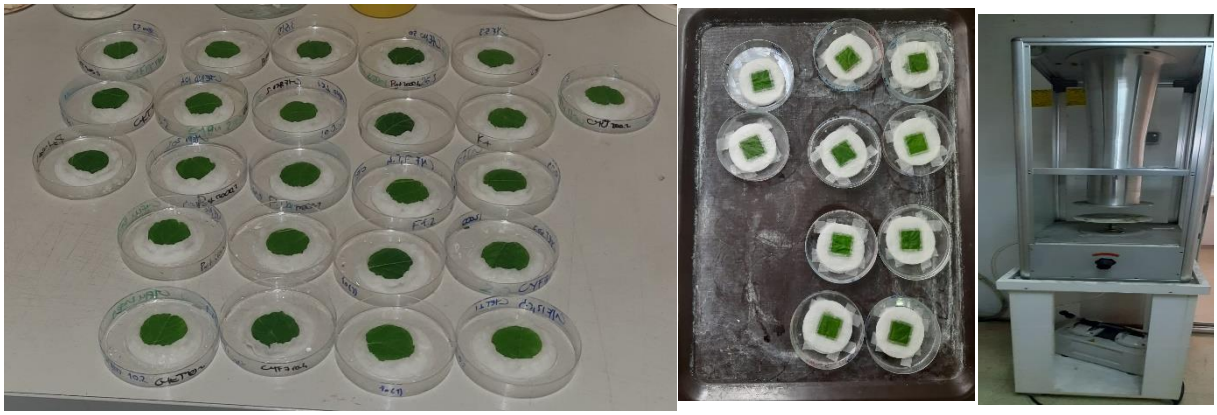


Figure 2.3 Bioassay for *Tetranychus Urticae*

For the predatory mites, only the recommended dose was tested, and the percentage mortality was determined for broflanilide (20 ppm) with the following steps in the bioassay:

2.3.2 *Phytoseiulus persimilis* bioassays

- **Preparation and Application:** Using at least 4 replicates of the recommendation dose, applied with a spray tower at 1 bar pressure and 2 ml of Acaricide + water mixture
  (2 ± 0.05 mg acaricide deposit/cm^2). Control Petri dishes received only distilled water, with control mortality never exceeding 10%. A three-piece enclosed glass system was used to keep the predatory mites from escaping. First, the bottom piece was sprayed with acaricide. Then, 9 cm^2 squares of bean leaves were placed on the sprayed surface. Next, a middle piece with a circular opening was placed over the bean leaf to create an enclosed space. The middle piece was also treated with acaricide. Finally, the top part of the glass system was covered and left to air-dry at room temperature. This step was essential to prevent the accumulation of moisture, which could obstruct external visibility during mite counting and observation. External desk clips were applied to ensure a close between the bottom and middle pieces. (Figure 2.4).

- **Transfer**: At least 10-15 adult *Phytoseiulus persimilis* were transferred onto the leaves with a fine-tipped brush (1mm size), along with some *Tetranychus urticae* to

serve as a food source. Subsequently, the upper piece of the tripartite system was covered and securely closed using external desk clips, completing the enclosure. This ensured the containment and controlled environment necessary for the experiment.

- **Post-application**: The Petri dishes placed at a climate room set at 25±2°C, with a 16L:8D photoperiod condition.
- **Observation and analysis**: Twenty-four hours after the application, the mites were assessed for survival. Mites that exhibited no movement greater than twice their body length when stimulated were classified as dead. The mortality rate was calculated and adjusted using Abbott's formula.

Figure 2.4 Bioassay for *Phytoseiulus persimilis*

2.3.3 *Neoseiulus californicus and Amblyseius swirskii* bioassays

- **Preparation and Application:** Using at least 4 replicates of the recommendation dose, applied with a spray tower at 1 bar pressure and 2 ml of Acaricide + water mixture (2 ± 0.05 mg acaricide deposit/cm^2). Control petri dishes received only distilled water, with control mortality never exceeding 10%. 60 mm Petri dishes with glued edges In order to inhibit the escape of predatory mites, they were treated with acaricide and allowed to dry naturally at room temperature. This precaution was essential to avoid acaricide drops that might impede the movement of predatory mites (Figure 2.5).

- **Transfer**: A minimum of 10-15 adult *N. californicus* and *A. swirskii* were transferred into the petri dishes with a fine-tipped brush (1mm size), along with some *Tetranychus urticae* for *N. californicus* and *Carpoglyphus lactis* for *A. swirskii* to serve as their food source.
- **Post-application**: The Petri dishes placed at a climate room set at 25±2°C, with a photoperiod of 16 hours of light and 8 hours of darkness.
- **Observation and analysis**: Twenty-four hours after the application, the mites were assessed for survival. Mites that exhibited no movement greater than twice their body length when stimulated were classified as dead. The mortality rate was calculated and adjusted using Abbott's formula.

Figure.2.5 Bioassays for *Neoseiulus californicus* and *Amblyseius swirskii*

2.4 Molecular Analysis

Molecular analyses were conducted to detect the presence of point mutations previously identified as related to resistance at the acaricide's target site and to identify new point mutations conferring resistance to *Tetranychus urticae*. For this purpose, RNA isolation was first carried out, followed by PCR studies using the obtained cDNA:

2.4.1 RNA isolation steps

The PURELINK™ Mini Kit protocol has been used for RNA isolation.

- **Sample Preparation**: 100–200 individuals were added to Eppendorf tube (2 ml).
- **Lysis**: A volume of 600 µL lysis buffer was added, and the sample was homogenized. Afterward, it was centrifuged at a speed of 12,000 rpm for 2 minutes.
- **Ethanol Addition**: 400 µL of the supernatant was extracted from the tube and combined
  with 400 µL of 70% ethanol. The mixture was then vortexed for 12 seconds.
- **Transfer to Spin Column**: 700 µL from the mixture was transferred to a spin column.
- **Initial Centrifugation**: The column was centrifuged at 12,000 rpm for 25 seconds, following which the liquid that passed through was discarded.
- **First Wash**: The column was supplemented with 700 µL of Wash Buffer 1 (WB1) and thereafter subjected to centrifugation at a speed of 12,000 rpm for a duration of 30 seconds, following which the liquid that passed through was discarded.
- **Second Wash**: The column was supplemented with 500 µL of Wash Buffer 2 (WB2) and thereafter subjected to centrifugation at a speed of 12,000 rpm for 30 seconds. This wash step was repeated once, following which the liquid that passed through was discarded.
- **Drying the Filter**: The filter was centrifuged dry at 12,000 rpm without any added buffer.
- **Elution**: The filter was transferred to a sterile 1.5 mL tube. Afterwards, 30 µL of ultra-pure water was added. The mixture was left undisturbed for 1 minute and then centrifuged at 12,000 rpm for 2 minutes (Figure 2.6).

Figure 2.6 RNA isolation steps

After isolation, RNA quantity and quality were assessed using electrophoresis and a NanoDrop spectrophotometer (Figure 2.7). Once its integrity was confirmed, the iScript™ cDNA Synthesis Kit (Biorad, USA) was employed to perform cDNA synthesis. Target genes were amplified with primers designed by Dermauw et al. (2012) (Table 2.3). The polymerase chain reaction (PCR) was performed using the following conditions: an initial denaturation step at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 60 seconds. The reaction was concluded with a final extension step at 72°C for 3 minutes.

The quality of the post-PCR products was reassessed via electrophoresis. Purification and sanger sequencing processes were performed by BM company (Ankara, Türkiye). The obtained sequencing data was analyzed using BioEdit v.7.0.5 (Hall 1999) and MEGA (Kumar et al. 2018).

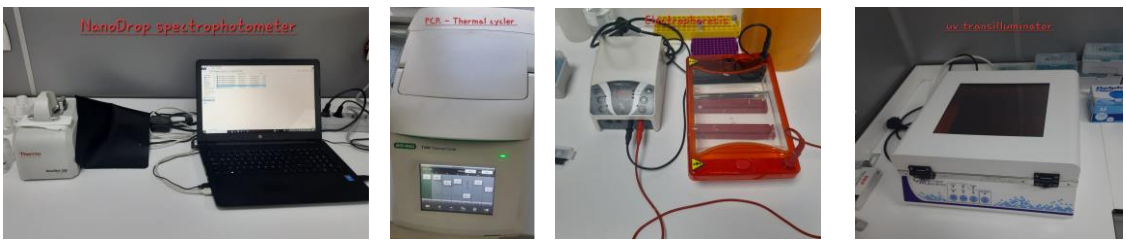


Figure 2.7 Devices used for evaluation of RNA and post-PCR quality control

Table 2.3 The primers employed in the research

Tetur-ID	Primer Name	Sequence (5' - 3')	Annealing temperature (°C)	size (bp)
tetur12g03620	Tu_Rdl1bF Tu_Rdl1bR	CGGTAACTGCAAGTTGTCCA CATTGGATTGGTTGTTGTGC	54	761
tetur36g00580	Tu_Rdl2bF Tu_Rdl2bR	TGGATAGACAAGCCTGCTCA TTCATTTTCTGAGGGCAACC	54	769
tetur36g00590	Tu_Rdl3bF Tu_Rdl3bR	GCCAATGCTTTAAGTACAGGAAA GCCTTGGATTACGTGCATTC	54	910
tetur02g04080	Tu_GluCl1_dia_F Tu_GluCl1_dia_R	TTGGATTGACCCTAACTCAGCA TTGCACCAACAATTCCTTGA	54	263
tetur10g03090	Tu_GluCl3_dia_F Tu_GluCl3_dia_R	CCGGGTCAGTCTTGGTGTTA CACCACCAAGAACCTGTTGA	54	251

[1] https://bioinformatics.psb.ugent.be/orcae/overview/Tetur

2.5 Alignment of GABA-Gated Chloride Channels across Arthropoda

An alignment of GABA-Gated Chloride Channels (rlds) was created using the sequences available in public GenBank. In case of *P. persimilis* and *N. californicus*, available genome data with the accession numbers GCA_037576195 and GCA_028455905, respectively, were mined to uncover gene sequences.

3. RESULTS

3.1 Bioassay results

3.1.1 *Tetranychus urticae*

Within the scope of this study, the acaricides fluralaner, isocycloseram, and milbemectin were applied to *Tetranychus urticae* populations collected from various provinces, and the LC_{50}, LC_{90}, and RR_{50} values at the 24th hour were determined. The analysis of these values provides insights into the susceptibility of each population.

For fluralaner, The LC_{50} values showed the highest concentration at 2.645 mg a.i./L in the KOD population. The LC_{90} values varied between 1.981 mg a.i./L and 6.321 mg a.i./L. Bioassay for fluralaner revealed reduced susceptibility in the KOD population (Table 3.1).

For milbemectin, the LC_{50} values peaked at 3.700 mg a.i./L in the Bib population. The LC_{90} values ranged between 1.209 mg a.i./L and 5.892 mg a.i./L, yet remained effective below the registered dose of 9.3 mg a.i./L (100 ml / 100 L water) (Table 3.2).

For isocycloseram, the LC_{50} values reached a maximum of 0.263 mg a.i./L in the Bib population. The LC_{90} values spanned from 0.101 mg a.i./L to 1.101 mg a.i./L. Bioassay for isocycloseram showed no decreased susceptibility across any populations, with all values significantly below the registered dose of 30 mg a.i./L (15 ml/100 L water) (Table 3.3).

In summary, the susceptibility patterns varied significantly across different treatments and populations. milbemectin showed the highest levels of resistance in the Bib and Pat populations, while isocycloseram generally had very high toxicity on *T. urticae* populations.

Table 3.1 The LC_{50} values of fluralaner for GSS and field-collected *T. urticae* populations

Population	Slope ± SE	LC_{50} (mg a.i./L) (95% CL)	LC_{90} (mg a.i./L) (95% CL)	df	Chi^2	RR_{50} (95% CL)
GSS	2.246±0.195	0.532 (0.457 - 0.622)	1.981 (1.555 - 2.735)	16	9.082	-
Kod	3.387 ± 0.497	2.645 (2.017 - 3.154)	6.321 (5.422 - 7.840)	14	6.228	4.97
Mer	6.420±1.224	2.014 (1.719 - 2.215)	3.190 (2.871 - 3.859)	13	3.709	3.78
Bib	2.837±0.214	0.665 (0.579 - 0.759)	1.881 (1.580 - 2.334)	17	9.456	1.25
Pat	2.705 ± 0.311	0.713 (0.536 - 0.887)	2.124 (1.731 - 2.733)	16	3.946	1.34
Papa	2.884 ± 0.215	0.658 (0.588 - 0.733)	1.831 (1.570 - 2.222)	16	10.226	1.24

Table 3.2 The LC_{50} values of milbemectin for GSS and field-collected *T. urticae* populations

Population	Slope ± SE	LC_{50} (mg a.i./L) (95% CL)	LC_{90} (mg a.i./L) (95% CL)	df	Chi^2	RR_{50} (95% CL)
GSS	1.478±0.117	0.164 (0.122 - 0.213)	1.209 (0.905 - 1.717)	19	12.323	-
Kod	2.612 ± 0.302	1.251 (0.887 - 1.602)	3.870 (3.163 - 4.864)	17	3.296	7.63
Mer	2.286 ± 0.225	0.589 (0.488 - 0.704)	2.143 (1.675 - 2.993)	18	11.747	3.59
Bib	6.340 ± 0.852	3.700 (3.386 - 3.989)	5.892 (5.309 - 6.918)	17	4.557	22.56
Pat	7.275 ± 0.856	3.699 (3.454 - 3.937)	5.550 (5.088 - 6.296)	17	5.699	22.56
Papa	2.135 ± 0.167	0.905 (0.771 - 1.052)	3.602 (2.908 - 4.728)	15	14.024	5.52

Table 3.3 The LC_{50} values of isocycloseram for GSS and field-collected *T. urticae* populations

Population	Slope ± SE	LC_{50} (mg a.i./L) (95% CL)	LC_{90} (mg a.i./L) (95% CL)	df	Chi^2	RR_{50} (95% CL)
GSS	2.133±0.176	0.202 (0.170 - 0.237)	0.804 (0.641 to 1.078)	16	11.696	-
Kod	2.699 ± 0.452	0.185 (0.116 - 0.242)	0.552 (0.456 - 0.709)	17	8.834	0.92
Mer	3.493±0.305	0.044 (0.039 - 0.049)	0.101 (0.087 - 0.124)	20	12.812	0.22
Bib	2.059±0.155	0.263 (0.225 - 0.305)	1.101 (0.882 - 1.458)	17	10.961	1.30
Pat	2.212 ± 0.164	0.188 (0.158 - 0.222)	0.715 (0.585 - 0.913)	16	14.763	0.93
Papa	2.414 ± 0.178	0.157 (0.136 - 0.180)	0.534 (0.443 - 0.674)	16	9.254	0.78

3.1.2 Broflanilide

The mortality data for *T. urticae* populations exposed to broflanilide at different concentrations (control, 500 ppm, and 5000 ppm) reveal significant differences in the response of various populations. (Table 3.4).

The mortality data for *T. urticae* populations exposed to different concentrations of broflanilide (control, 500 ppm, and 5000 ppm) indicate significant variations in responses among the populations. At 500 ppm, the mortality ranged from 11.65% in the GSS population to 62.79% in the Pat population. Conversely, at 5000 ppm, the mortality ranged from 40.65% in the GSS population to 88.75% in the KOD population. (Figure 3.1). The mortality in control groups was always below 10%.

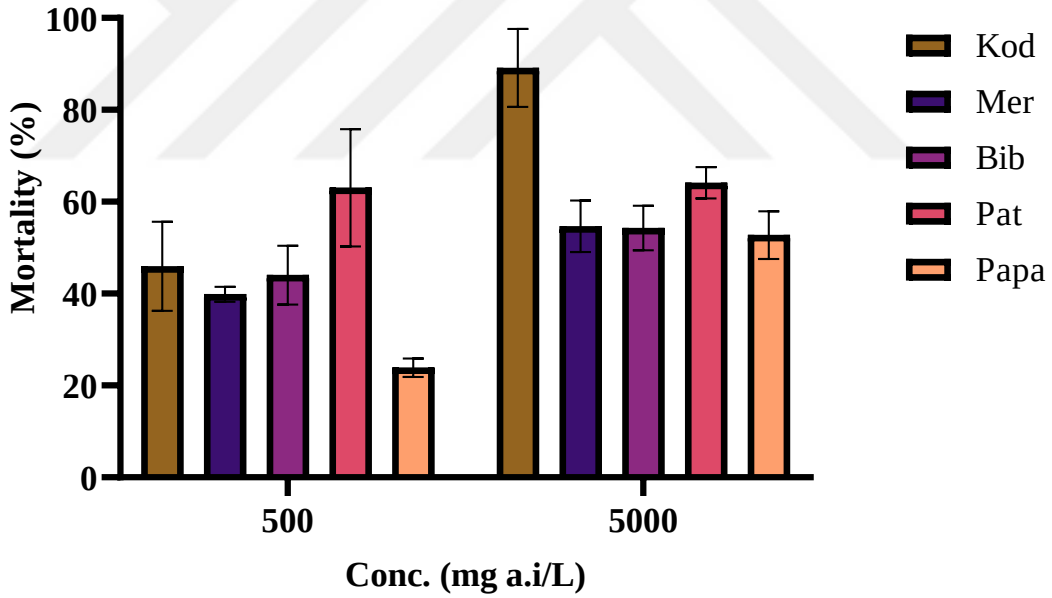


Figure 3.1 The mortality data for broflanilide Comparison Across 500 and 5000 ppm used on *Tetranychus urticae* populations

Table 3.4 The mortality data for broflanilide applied to of *Tetranychus urticae* populations during the 24th hour at (500 ppm and 5000 ppm).

population	Concentration	Total	alive	dead	Mortality (%) ± SE
Gss	500	114	95	19	11.65 ± 1.34
	5000	105	58	47	40.65 ± 4.23
Kod	500	119	58	61	45.93 ± 9.68
	5000	115	12	103	89.13 ± 8.49
Mer	500	125	69	56	39.87 ± 1.63
	5000	125	52	73	54.68 ± 5.61
Bib	500	126	65	61	44.02 ± 6.38
	5000	122	51	71	54.29 ± 4.84
Pat	500	125	42	83	63.04 ± 12.8
	5000	124	40	84	64.15 ± 3.41
Papa	500	151	103	48	23.87 ± 2.01
	5000	146	62	84	52.73 ± 5.21

3.1.3 Predatory mites

The findings indicate that broflanilide, at its recommended dose of 20 PPM, was highly toxic, achieving a 100% mortality rate among all tested predator species: *A. swirskii*, *N. californicus*, and *P. persimilis*. This uniform high mortality highlights the effectiveness of the broflanilide at its recommended dose (Table 3.5). It is noteworthy that mortality in the control groups remained below 10%.

Table 3.5 The mortality data for broflanilide (20 ppm) applied to Predatory mites during the 24th hour

Predator mite	Total	Alive	Dead	Mortality (%)
Amblyseius swirskii	34	0	34	100%
Neoseiulus californicus	41	0	41	100%
Phytoseiulus persimilis	41	0	41	100%

3.2 Screening for Target-site Point Mutations

3.2.1 The Rdl (Resistance to dieldrin) genes

Screening for target-site mutations was performed on cDNA samples from four field populations to identify potential mutations in the Rdl gene. Consistent with the bioassay, no target site mutations were found in the analyzed populations.

3.2.2 Glutamate-gated chloride channels

Screening for target-site mutations was performed on cDNA samples from five populations to detect potential mutations in the GluCl1 and GluCl3 channels. Consistent with the results from the bioassay, no target site mutations were discovered in the populations analyzed.

3.3 Comparison of Rdl Gene Alignments Across Arthropod

The alignment of GABA-Gated Chloride Channels (Rdl) using sequences from GenBank and genome data revealed distinct amino acid differences between *P. persimilis* and *N. californicus* compared to *T. urticae* (Figure 3.2).

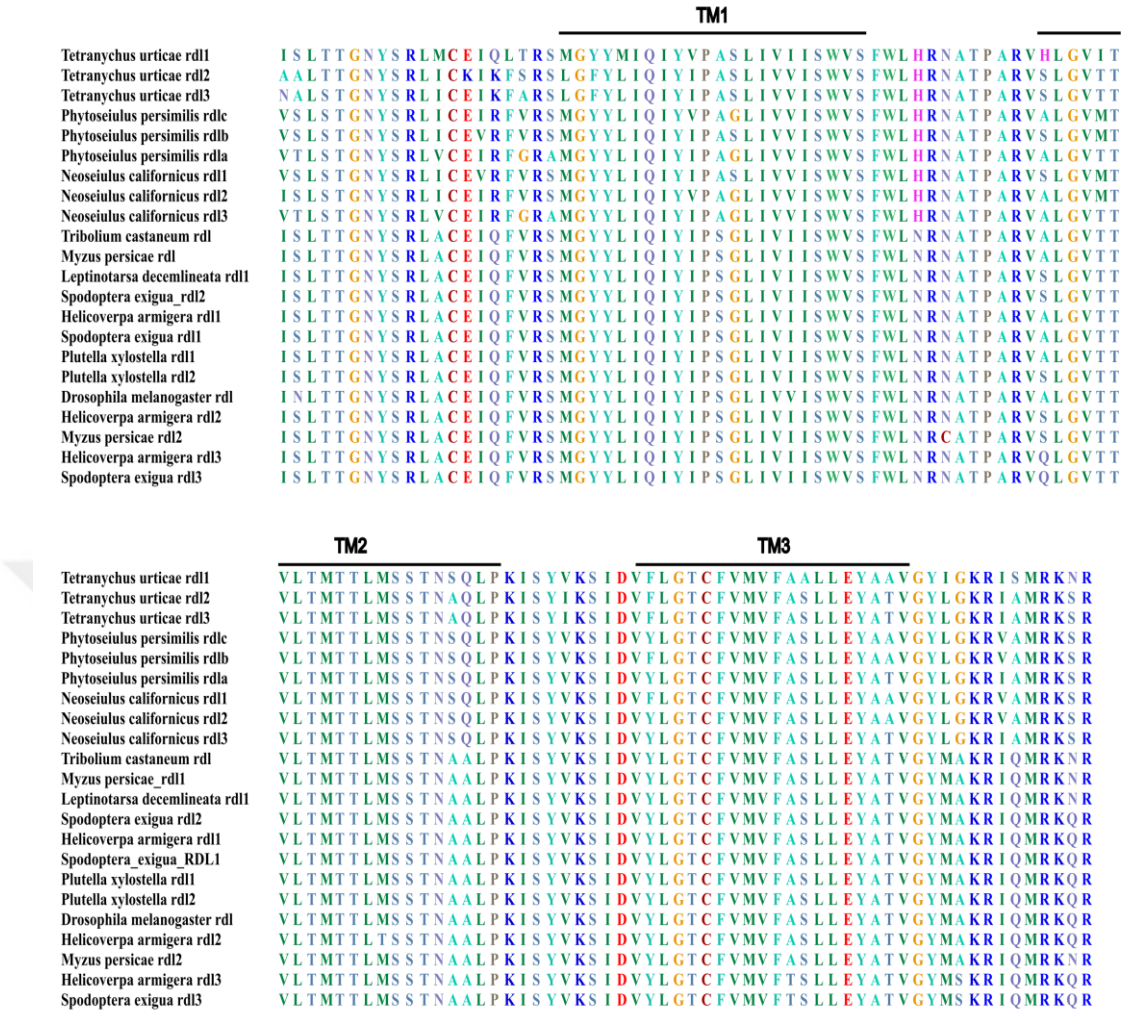


Figure 3.2 Comparison of Rdl gene alignments across Arthropod

4. DISCUSSION

Fluralaner, a new isoxazoline insecticide, targets the GABA receptor by binding to specific sites and mechanisms (Ozoe et al., 2010). It is highly effective against the phytophagous mite *Tetranychus urticae* (TSSM), achieving 100% mortality at a concentration of 3.1 mg a.i./L, even in strains resistant to fipronil (approximately 500 mg/L) (Leviticus et al., 2020).

Our study evaluated the acaricidal activity of fluralaner against multiple populations of *T. urticae*. The LC_{50} and LC_{90} values of fluralaner across different populations provided insights into its effectiveness and the toxicity levels present in each population. Toxicity levels to fluralaner varied notably among the different TSSM populations. The German Susceptible Strain (GSS) showed the lowest LC_{50} value at 0.532 mg a.i./L, demonstrating high susceptibility to fluralaner. In contrast, the KOD population demonstrated the highest LC_{50} value of 2.645 mg a.i./L, reflecting a decreased susceptibility. The corresponding LC_{90} values ranged from 1.981 mg a.i./L in the GSS population to 6.321 mg a.i./L in the KOD population (Table 3.1).

Although our results for the populations of Mer, Bib, Pat, and Papa are close to those conducted by Leviticus et al. (2020), who demonstrated the high toxicity of fluralaner against TSSM females and its superior performance compared to other commercial insecticides. Fluranaler is currently not registered for use in agricultural areas, but it is approved for veterinary purposes. However, the high toxicity of this active compound suggests a promising future in controlling TSSM, which is a difficult pest to manage. Nevertheless, varying phenotypic susceptibility among mite populations underscores the need for regular monitoring of resistance levels following its potential registration.

We assessed the acaricidal toxicity of isocycloseram, a new isoxazoline insecticide, on various populations of *T. urticae*. Isocycloseram targets the GABA receptor at a site different from those affected by fiproles and organochlorines. It functions as an uncompetitive antagonist of the insect GABA receptor, effectively blocking

GABA-mediated neurotransmission, which leads to paralysis and death of the *T. urticae* (Blythe et al., 2022).

The LC_{50} values for isocycloseram ranged from 0.044 mg a.i./L in the Mer population to 0.263 mg a.i./L in the Bib population. Similarly, the LC_{90} values ranged from 0.101 mg a.i./L to 1.101 mg a.i./L, compared to the registered dose of isocycloseram at 30 mg a.i./L. These findings highlight the significant acaricidal potency of isocycloseram. Furthermore, the RR_{50} values indicated low to no resistance in most populations, with the KOD population exhibiting an RR_{50} of 0.92 and the Mer population showing high susceptibility with an RR_{50} of 0.22 (Table 3.3).

Despite being registered in only a limited number of countries, the excellent efficacy of isocycloseram suggests it holds great promise for future spider mite control. Isocycloseram's distinct mode of action renders it a valuable asset in resistance management programs. Although both fluralaner and isocycloseram are classified under the same MoA group, they target the GABA receptor at different binding sites. This distinction is crucial as it reduces the likelihood of cross-resistance, thereby making them suitable candidates for future rotation programs (Ozoe et al., 2010; Blythe et al., 2022).

The LC_{50} and LC_{90} values for milbemectin varied significantly across different TSSM populations. The LC_{50} values ranged from 0.164 mg a.i./L in the GSS population to 3.700 mg a.i./L in the Bib population. Correspondingly, the LC_{90} values were from 1.209 mg a.i./L to 5.892 mg a.i./L. The RR_{50} values indicated high resistance in the Bib and Pat populations, both with an RR_{50} of 22.56. Other populations, such as KOD, Mer, and Papa, exhibited RR_{50} values of 7.63, 3.59, and 5.52, respectively, indicating high levels of resistance (Table 3.2).

These findings are consistent with previous research, which demonstrated notably resistance to milbemectin in various TSSM populations. Sato et al. (2005) observed a strong correlation between resistance to abamectin and milbemectin, showing the presence of cross resistance between these acaricides. This phenomenon of cross

resistance is likely attributed to the similarities in their mechanisms of action, as both pesticides target glutamate-gated chloride channels.

Xue et al. (2020) found elevated levels of resistance to milbemectin in two TSSM populations, which could potentially reduce the effectiveness of milbemectin due to the presence of cross-resistance mechanisms. The study suggested that this potential resistance might be due to mutations in the target-site of GluCl channels and an increased expression of detoxification genes, i.e. UDP glycosyltransferases (UGT) and cytochrome P450 monooxygenases (P450), which play a key role in metabolic resistance. Nevertheless, the majority of other populations showed low levels of resistance, suggesting that the detected cross-resistance might not pose a major operational issue. Examination of LC_{90} values across 32 field populations of *T. urticae* indicates that the recommended field rate of milbemectin (10 mg a.i./L) continues to be effective for managing most *T. urticae* populations in Europe. These findings align closely with our results.

A study conducted in Isparta, Türkiye, by Ulukaya and Ay (2022), using the same susceptible strain GSS, reported RR_{50} (90% CL) values of 2.75, 1.98, and 6.99 which the LC_{90}. These results confirm the high rate of resistance in populations from the Mediterranean region in southern Turkey, where our study was conducted, as compared to the Isparta region in western Turkey. This implies a possible risk of growing resistance as a consequence of the continuous application of acaricides.

Although some TSSM groups exhibit high levels of resistance, the acaricide remains effective in susceptible populations when applied at the recommended dose of 9.3 mg a.i./L. This dose exceeds our highest LC_{90} value of 5.89 mg a.i./L observed in the Bib population. To mitigate the risk of developing resistance in the future, it is advisable to use milbemectin in conjunction with other acaricides. This strategy helps minimize the likelihood of resistance and maintains the long-term efficacy of the treatment.

Broflanilide, a new meta-diamide insecticide, functions as an allosteric modulator of the GABA-gated chloride channel by targeting the Pest GABAR (Sparks et al., 2020). For

broflanilide, preliminary trials showed that broflanilide had limited toxicity against red spider mites. Therefore, this acaricide was treated only at 500 and 5000 ppm doses, and the percentage mortality was determined (Table 3.4).

These findings indicate that while broflanilide can achieve high mortality rates at higher concentrations, its toxicity at lower concentrations is limited and varies among TSSM populations. This variability suggests that broflanilide may not be uniformly effective across all TSSM populations, particularly at lower doses

Contrary to our findings, Shen et al. (2021) reported that broflanilide exhibited high toxicity against TSSM eggs and adult females. They concluded that broflanilide holds promise as a potential acaricide for controlling TSSM in agricultural settings, highlighting its strong acaricidal toxicity and substantial reduction in fecundity among adult females. The reasons for these discrepant results belonging to Chinese and Turkish spider mite populations should be further elucidated in future studies. The alignment of the target-site genes from different arthropods, whether broflanilide is toxic on them or not, shows the potential amino acids that could be the molecular basis of the selectivity of this compound. However, broflanilide is a insecticide, and thus metabolic differentiations as a reason for selectivity should not be overlooked.

Next to phytophagous mite *T. urticae*, we evaluated the mortality rates of *A. swirskii*, *N. californicus*, and *P. persimilis*, when exposed to broflanilide 20 mg a.i./L (20 ppm). Only the recommended dose was treated, and the percentage mortality was determined.

The findings indicate that broflanilide was highly toxic to predatory mites at the recommended dose (Table 3.5). Its use could potentially disrupt biological control programs by eliminating beneficial predatory mites. However, lower doses could be considered when predatory mites are already established or will be intentionally released in a certain production area. Its inclusion in IPM programs should be re-evaluated to balance effective pest control with the preservation of beneficial predatory mite populations. Nevertheless, ecological selectivity could still be achieved in pest management programs despite low physiological selectivity.

The amino acid differences especially at the transmembrane domain 1 and 3 in the Rdl gene, known to be binding site of tested new generation insecticides, (Figure 3.2) between *T. urticae* and predatory mite/lepidopteran and coleopteran insects, may explain the differential susceptibility to broflanilide, if the selectivity is caused by target-site binding. These differences could affect the pesticide's binding or function in GABA-gated chloride channels, leading to reduced effectiveness on *T. urticae* and increased toxicity to predators. However, metabolic-based selectivity cannot be ruled out, as broflanilide is a proinsecticide and needs to be metabolized into its toxic metabolite. If there is a lack of broflanilide-metabolizing enzymes in T. urticae, the parent compound cannot be toxic, as previously documented for bifenazate. (Van Nieuwenhuyse et al., 2012). Therefore, further research on these differences is essential to understanding the molecular basis of selectivity.

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