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Establishment of a pesticide resistance monitoring tool for the two-spotted spider mite, *Tetranychus urticae*

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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Abstract

Two-spotted spider mite (TSSM), or *Tetranychus urticae* (Koch), is a major agriculture pest known for its rapid development of resistance to pesticides. The analysis of spider mites' pesticide resistance demonstrated that resistance patterns and frequencies vary between *T. urticae* strains collected from different geographic locations and host plants. This research aims at characterization of pesticide resistance patterns in mite populations present in greenhouses in the Southwestern Ontario by identifying genetic and metabolic markers of their pesticide resistance. The establishment of these markers lays the basis for development of a pesticide resistance diagnostic tool that will enable prediction of population resistance status based on the resistance-associated markers. The ultimate aim of the project is to enable identification of genetic patterns to be used for recommendations on pesticide use to growers.

In the Fall of 2018, 19 TSSM populations from different Ontario greenhouses and different crops (tomato, eggplant, cucumber, pepper) were collected. First, the bioassay protocols were optimized and then applied to the collected populations to determine their resistance status. Following that, genotyping and RT-qPCR were performed to check for known genetic markers: single nucleotide polymorphisms associated with abamectin, bifenazate, and etoxazole resistance and metabolic markers of detoxification associated with abamectin resistance, respectively. Lastly, the correlation between the genetic/metabolic markers and resistance status was analyzed, and multiple pesticide resistance was assessed. As a result, the pesticide resistance diagnostic tool was developed for abamectin. More informative markers are needed for bifenazate diagnostic tool, and further research is needed for etoxazole. Cross-resistance to abamectin and bifenazate was also observed in three out of 19 greenhouse populations. This work demonstrates that Canadian populations differ greatly from the populations found in other countries. In addition, the high frequency of resistance-associated alleles in tested populations alerts on a need to develop pesticide resistance diagnostic tools to help growers in mite pesticide resistance management.

Keywords: *Tetranychus urticae*, pesticide resistance, SNP, detoxification

Summary for Lay Audience

Tetranychus urticae (Koch), or two-spotted spider mite, is one of the most damaging agricultural pests. It is able to feed on over 1,100 plant species, 150 of which are crops. Since TSSM favours hot dry climate, the ongoing global warming contributes to its spread, and it is predicted that TSSM populations will dramatically increase and migrate further north posing even greater challenge to the sustainability of Canadian agriculture. Rapid pesticide resistance development is another extraordinary ability of TSSM contributing to its pest status. Greenhouses are particularly susceptible to spider mites' infestations because they are isolated, monocultured, with longer growing seasons, and they are subjected to the extensive pesticide use. However, local spider mite populations appear to have different resistance status for different pesticides; thus, it is hard to predict what pesticide would be efficient against a particular spider mite population. Therefore, there is a need for a pesticide resistance predictive tool which would support decision making for what pesticide to use. Such a tool will increase the quality of the crop, decrease the cost of the production and help extend the utility of current pesticides.

This research looked at various genetic markers associated with abamectin, bifenazate, and etoxazole resistance, and aimed to find a correlation between the markers and resistance status of 19 spider mite populations collected from various vegetable greenhouses across Ontario in the Fall of 2018. Moreover, the resistance to more than one pesticide was also assessed. In conclusion, the pesticide resistance tool was established for abamectin, yet further research is needed for bifenazate and etoxazole. Cross-resistance has been observed between abamectin and bifenazate.

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List of Abbreviations

$\mu\text{mol m}^{-2} \text{s}^{-1}$	μmol of photons per second and square meter
AAFC	Agriculture and Agri-Food Canada
ATP	adenosine triphosphate
bp	base pairs
C^{14} -GlcNAc	C^{14} - <i>N</i> -Acetylglucosamine
cDNA	complementary deoxyribonucleic acid
CHS1	chitin synthase I
CI	confidence intervals
Ct	cycle threshold
CYP	cytochrome P450
cysLGIC	cys-loop ligand-gated ion channel
cyt b	cytochrome b
GABA	γ -aminobutyric acid
GHF	greenhouse farming
GH	greenhouse
GluCl	glutamate-gated chloride channel
GST	glutathione-S-transferases
HisCl	histamine-gated chloride channel
IPM	integrated pest management
IRAC	insecticide resistance action committee
KW	KimWipe
LC_{90}	lethal concentration 90%
n	sample size
nAChR	nicotinic acetylcholine receptor
PCR	polymerase chain reaction
pH	potential of hydrogen
pHCl	pH-sensitive chloride channel
ppm	parts per million
R	resistant
RI	resistance index

RNA	ribonucleic acid
RNAi	RNA interference
RT-qPCR	quantitative reverse transcription PCR
S	sensitive
SE	standard error
SNP	single nucleotide polymorphism
TSSM	two-spotted spider mite
TU-LND	<i>Tetranychus urticae</i> - London

1.0 Introduction

1.1 Agriculture in Canada

Canada is the second largest country, in terms of land mass, in the world; however, only about 7% of the whole of Canadian land is suitable and being used for agriculture today, covering about 64 million hectares (Hein 2020). Nonetheless, agriculture and agri-food are main players in the Canadian economy. Not only do these industries provide food and products for domestic use and export, they also employ a large percentage of people. In 2018, agriculture and agri-food contributed \$140 billion to Canadian Gross Domestic Product. Moreover, agriculture and agri-food sector employed 2.3 million people in 2018 providing 1 in 8 jobs in Canada (Government of Canada 2020).

Primary agriculture involves activities performed within the boundaries of a farm, nursery, or greenhouse. There are five chief primary agricultural sectors in Canada, based on farm cash receipts: 1) grains and oilseeds, 2) livestock, 3) dairy, 4) horticulture, and 5) poultry and eggs. Being the fourth largest agricultural sector, horticulture accounts for about 9% of Canadian agriculture and is the most diverse sector (Canadian Federation of Agriculture 2007). Horticulture in Canada involves growing over 120 varieties of fruits and vegetables as well as various ornamental plants (floriculture, nursery, Christmas tree, turf sod industries), honey, and maple products representing over \$7 billion in profit (Canadian Horticulture Council 2018). In 2019, the farm gate value from fruit and vegetable production was estimated to be over \$5.3 billion (Agriculture and Agri-Food Canada 2020a-d). Canada displays one of the highest consumption rates of fresh vegetables per capita in the world (Our World Data 2017). However, Canada's cold climate and short growing season limits the ability to produce field grown vegetables all year round. Thus, greenhouse farming (GHF) is a solution to overcoming these obstacles. GHF represents self-contained controlled environments equipped with systems allowing the manipulation of heat, artificial lighting, and water and nutrients supplied to nourish plants (Agriculture and Agri-Food Canada 2020c). Therefore, GHF allows for an extended growing season, increased yield of crops with sustained, predictable availability of food and food products. Additionally, it offers opportunities for improved disease and pest management. The GH vegetable production is the largest and fastest growing sector of Canadian horticulture. The profit from GH vegetable production in 2019 was \$1.6 billion (Canadian Horticultural Council 2019). At this

moment, Ontario is the leading province of GH vegetable production supplying 69% of total GH crops in Canada. Tomato, cucumbers, peppers, lettuce, eggplants, and fresh fine herbs are among the top profitable products in GH farming. Tomato, as the most prominent GH crop, accounts for 37% of the total GH vegetable production yielding around \$590,000 annually (Agriculture and Agri-Food Canada 2020c).

1.1.1 Challenges to Canadian agriculture

One of the basic human needs is an access to a sufficient quantity of nutritious food. Food security is essential for human existence not just from the perspective of health and well-being but also from a socioeconomic perspective. However, as we strive to reduce food insecurity, such factors as the rapid growth of human population and climate change complicate the task. Cole *et al.* (2018) projected that in the next 30 years, the world population will increase from 7.87 billion to 9.7 billion people leading to 70% increase in food demand. Likewise, it is projected that climate change may result in reduction of crop production, changes in market and supply chain infrastructure as well as increase in food prices. This poses a challenge to food security and forces the development of new agriculture practices to facilitate food production and attain food security globally. Climate change is only one of the factors affecting crop sustainability. Other abiotic factors depend on the season and include the lack of growth and nutrients and extreme temperatures and irradiance (Oerke 2005). As for biotic stressors, damaging organisms such as weeds, microorganism and fungal pathogens can reduce crop yields substantially (Oerke 2005). Animal pests include insects, mites, nematodes, slugs or snails, rodents, birds, or mammals. Even though there are no data associated with crop losses in Canada, Jaques *et al.* (1994) drew a parallel with USA data and estimated Canadian losses to be hundreds of millions of dollars.

There is a vast variety of different pest management protocols. They fall under four main categories: physical, biological, cultural, and chemical methods (Ministry of Agriculture Food & Rural Affairs, 2012). Physical strategies involve manual removal of pests or utilization of traps. Biological methods involve the use of natural predators of pests (Sabelis and Van de Baan 1983). Cultural methods are the oldest methods for pest population management. Some of the examples of it are crop isolation, manipulation of timing of seeding and planting, and management of surrounding environments (Costello and Daane 1998). Lastly, chemical control is the most

common method of pest management utilizing pesticides or other toxic compounds against pests (James and Price 2004; Van Leeuwen *et al.* 2015).

Ironically, the strength of GHF design in its ability to create optimal climate conditions for plants and to grow plants out-of-season is also its major weakness. Greenhouses are more vulnerable to pest infestations in comparison to field farming because optimal plant growth conditions and extended growing season also create optimal stable pest development environments. Moreover, natural enemies are often not present in GH because the GHF structures are isolated. As such, greenhouses are subjected to the frequent use of pesticides. This causes pesticides to become ineffective due to quicker pesticide resistance and multiresistance development (Cranham and Helle 1985). Multiresistance is especially problematic because it narrows the range of effective pesticides by causing pests to develop simultaneous resistance to pesticides of different chemical classes and modes of actions (Osakabe *et al.* 2009). Ineffective use of pesticides, subsequently, leads to crop losses due to unsustainable pest damage.

1.2 Plant-herbivore interaction

Plants and herbivorous arthropods that feed on them have been co-existing for over 400 million years (Fürstenberg-Hägg *et al.* 2013). Their conflicting incentives have evolved in a tremendously complex relationship of avoidance of each other's defensive and offensive capabilities. This evolutionary "arms race" has affected all biological levels, from the biochemical to population genetics of both players (Fürstenberg-Hägg *et al.* 2013) leading to diversification of both (Després *et al.* 2007; Howe and Jander 2008; Mitchell *et al.* 2016). From the plants' perspective, the main goal is a rapid and accurate recognition of attack signals and initiation of immune responses defending against or tolerating their enemy's attack as well as reducing their enemy's fitness (Nunez-Farfan *et al.* 2007; Rioja *et al.* 2017; van der Meijden *et al.* 1988). Herbivores, on the other hand, have to overcome and adapt to plant defenses, ensuring successful feeding and survival (Ehrlich and Raven 1964). Plants have defenses that can be categorized as either constitutive or induced. Constitutive defenses are always present and can be in the form of physical barriers such as cuticle, trichomes or bark, or defense compounds present at basal levels (Wybouw *et al.* 2015). Induced defenses are triggered in response to perception of herbivory. Constitutive and induced defenses are not mutually exclusive; a defence trait can be constitutively present and then induced to a higher degree upon perception of attack. Induced defenses respond to signals that are either

associated with herbivores feeding (such as recognition of compounds in saliva of herbivores) (Mithofer and Boland 2008) or are associated with plant damage that arises during herbivore feeding (Boller and Felix 2009). These cues are then transmitted within the plant to initiate either the direct and/or indirect defense. Direct defense involves triggering the immune response and synthesis of allelochemicals (secondary compounds involved in protection) such as terpenoids, alkaloids, anthocyanins, phenols, and quinones (Hanley *et al.* 2007). Indirect defense mechanisms are mediated by attracting natural enemies of herbivorous pests (Arimura *et al.* 2009). Plant defense secondary compounds are generally quite efficient in deterring of most herbivorous arthropod pests.

Herbivores, in turn, have evolved counteradaptations to plant allelochemicals such as avoidance, detoxification, or sequestration of toxic plant compounds. If a plant can successfully defend itself and prevent any herbivory damage, such an interaction is called incompatible, and the plant is considered a non-host plant to this particular herbivore (Rioja *et al.* 2017). Opposite to this, if an herbivore is successful at overcoming plant's defenses, this interaction is called compatible, and this plant is considered a host plant to an herbivore (Rioja *et al.* 2017). Depending on how successful herbivores are in overcoming plant defenses and how large their host range is, they can be classified as generalists (or polyphagous), oligophagous herbivores, or specialists. Generalists can feed on many host plants from various families. Oligophagous herbivores feed on several plant species, usually found in the same family. Lastly, specialists can feed on one of a few plant species within the same genus (Barrett and Heil 2012; Bernays and Graham 1988; Futuyma and Gould 1979). This study focuses on the extreme generalist, the two-spotted spider mite, *Tetranychus urticae*.

1.3 *Tetranychus urticae*

Tetranychus urticae Koch. (Acari: Tetranychidae) is referred to as a two-spotted spider mite (TSSM) due to the presence of two dark spots located on its back. Adult females can be green-brown or orange-red in color, are oval in shape and about half a millimeter long. TSSM utilizes a sucking mode of feeding by using a stylet to pierce through the plant epidermis without damaging it and feeding on individual mesophyll cell contents (Park and Lee 2002; Bensoussan *et al.* 2016). As a result, plants develop yellow chlorotic spots that form as a consequence of mite inflicted damage during feeding and local plant response to that feeding (Bensoussan *et al.* 2016). The life

cycle of TSSM consists of five stages: egg, larval, protonymph, deutonymph, and sexually dimorphic adults; females are larger in size and have more rounded shape and males are smaller with a tapered posterior end of the body (Figure 1.1). It takes 10 days on average to develop from an egg to an adult form but only seven days under favourable conditions (~27 °C and low humidity ~60%) (Shih *et al.* 1976). A female can lay up to nine eggs a day with the fertile period lasting for 16 days on average, resulting in 100-150 eggs per female (Laing 1969; Shih *et al.* 1976). The egg stage lasts about 4 days and results into larvae hatching. Larvae are recognizable by their three pairs of legs unlike other stages where TSSM has four pairs of legs. Larvae are the most vulnerable stage of TSSM development. Then, larvae undergo a quiescent stage, or chrysalis, and in a day or two emerge as protonymphs. The protonymphs molt into deutonymphs. Deutonymphs undergo another quiescent stage – teliochrysalys, resulting in an adult TSSM (Shih *et al.* 1976). The sex ratio is about three females to one male (Helle and Sabelis 1985). Given such a high reproduction potential of female TSSM, the skewed sex ratio facilitates faster population expansion. TSSM dispersion occurs by 1) active movement such as walking (Hussey and Parr 1963), 2) by transport by another organism (Yano 2004), and 3) with air currents on silk that they produce (Osakabe *et al.* 2008).

TSSM is one of the major agricultural pests globally. Several features contribute to TSSM's status of a super pest. First, TSSM is an extreme generalist herbivore with an extremely wide host plant range. TSSM feeds on over 1,100 plant species from more than 140 different plant families; over 150 of these plant species are economically important crops including vegetables, fruits, ornamentals and field crops (Migeon and Dorkeld 2010). Such a wide host range implies that TSSM has a very robust xenobiotic detoxification potential. Second, TSSM has a very short life cycle and very high reproductive potential, as discussed above. Furthermore, TSSM is globally distributed, preferring hot dry climatic conditions. Therefore, TSSM outbreaks are further facilitated by climate change. TSSM development and generation time are inversely correlated with increasing temperatures (Bounfour and Tanigoshi 2001). Drought conditions contribute to the 3-fold increase in oviposition (egg laying) (Ximenez de Embun *et al.* 2016). Thus, with global warming, it is expected that TSSM populations will move further north, broadening their geographical distribution and negatively affecting social and economic perspectives due to its pest status. Moreover, TSSM has an arrhenotokous (or haplo-diploid) mating system where diploid females develop from fertilized eggs, and haploid males develop from unfertilized eggs (Oliver

1971; Hebert 1981; Helle and Sabelis 1985; Van Pottelberge *et al.* 2008). Such a reproduction system facilitates quicker evolution and resistance spread in a population (Van Leeuwen *et al.* 2012). Thus, TSSM's biology greatly contributes to its status of a super pest making it particularly difficult to control and maintain under economic threshold in agriculture, especially greenhouse settings.



Figure 1.1 Life stages of two-spotted spider mite (*Tetranychus urticae* Koch). From left to right: egg, larvae, protonymph, deutonymph, adult male, and adult female. Photo credit: Zoran Culo.

1.4 Host adaptation and pesticide resistance

Plant allelochemicals are involved in the defense against herbivores, thus, they are often viewed as “bio-pesticides” because they perform the same function as what we would hope to have in synthetic pesticides (Walia *et al.* 2017). It is evident that biochemical effectors behind both plant tolerance and pesticide resistance are either very similar (Despres *et al.* 2007; Heidel-Fisher and Vogel 2015) or the same (Bass *et al.* 2013). For instance, the polyphagous peach-potato aphid *Myzus persicae* has evolved increased tolerance to the alkaloid nicotine, found in some solanaceous species, in a recent host shift to tobacco (*Nicotiana tabacum*) (Bass *et al.* 2013; Devine *et al.* 1996). This same tobacco-adapted aphid also showed reduced sensitivity to neonicotinoids, a large class of synthetic pesticides, that chemically are similar to nicotine (Jeschke and Nauen 2008). Thus, it is believed that plants' allelochemicals can prime herbivores for the resistance to pesticides. The tolerance for plant allelochemicals and pesticide resistance can arise through various mechanisms such as metabolic resistance via detoxification processes, target site

modifications, excretion transport, and sequestration (Despres *et al.* 2007). One of the most studied mechanisms is detoxification of xenobiotics by enzymes of cytochrome P450 superfamily (Berenbaum *et al.* 1992; Berenbaum *et al.* 1996; Feyereisen 2012; Rosenheim *et al.* 1996). In addition, Rosenheim *et al.* (1996) showed that the herbivore pesticide resistance was diminished when feeding on plants with poorer defensive abilities. Given this, it is believed that since generalist herbivores have encountered a larger number of plant allelochemicals, they are better at adaptation to both plant allelochemicals and synthetic pesticides.

The wide range of hosts that TSSM has successfully adapted to implies that TSSM evolved an ability to successfully counteract a diversity of plant allelochemicals. However, different TSSM populations do not perform equally well across all their potential host plants. Instead, host range depends on a set of mechanisms used for the adaptation derived from previous hosts that may or may not be useful (Agrawal *et al.* 2002; Fellous *et al.* 2014; Fry 1989; Rioja *et al.* 2017). Yet, TSSM has evolved a great ability to adapt to novel hosts (Fry 1989; Futuyma and Gould 1979; Magalhaes *et al.* 2007; Wybouw *et al.* 2015). Even though the exact mechanism behind TSSM host adaptability is not known, there is evidence supporting suppression of plant induced responses (Kant *et al.* 2008; Wybouw *et al.* 2015) and xenobiotic detoxification (Dermauw *et al.* 2013; Wybouw *et al.* 2015; Zhurov *et al.* 2014). Along with the ability to quickly adapt and overcome plant secondary metabolites over a few generations, TSSM has also shown an ability to rapidly develop resistance to chemical pesticides (Van Leeuwen *et al.* 2010). Furthermore, Dermauw *et al.* (2013) and Wybouw *et al.* (2015), using transcriptome analysis, demonstrated that tomato-adapted TSSM and multi-acaricide resistant TSSM share the common pattern of gene expression in which the same genes were being upregulated. To support the preadaptation to xenobiotic resistance further, Dermauw *et al.* (2013) found that adaptation to tomato changed the expression levels of many detoxification enzymes as well as resulted in decreased susceptibility to a few acaricides (bifenthrin, fenbutatin oxide, and pyridaben) that belong to different IRAC groups and possess different modes of actions.

1.5 Pesticide use and pesticide resistance

The main approach to controlling many pests is through the use of chemical pesticides (Sparks and Nauen 2015; Van Leeuwen *et al.* 2015). According to Health Canada (2019), a pesticide is “any product, device, organism, substance or thing that is manufactured, represented,

sold or used as a means for directly or indirectly controlling, preventing, destroying, mitigating, attracting or repelling any pest”. All pesticides used in Canada undergo rigorous scientific examination to determine if they meet health and safety standards and establish instructions and safety precautions (Government of Canada 2021). All pesticides used in Canada must be registered with Health Canada and are regulated by Health Canada’s Pest Management Regulatory Agency. Some types of pesticides include herbicides (against weeds), insecticides (against insects), fungicides (against fungi), nematicides (against nematodes), and rodenticides (against rodents). Insecticide use is tightly regulated by Insecticide Resistance Action Committee (IRAC). IRAC is “an international association of crop protection companies serving as the Specialist Technical Group within focused on ensuring the long-term efficacy of insect, mite and tick control products through effective resistance management for sustainable agriculture and improved public health” (Sparks and Nauen 2015). All pesticides are classified according to IRAC based on their modes of actions. Most of insecticides target nervous system and muscles of pests, and recently new pesticides target pests’ growth and development and mitochondrial respiration (Dekeyser 2005; Marcic 2012; Sparks and Nauen 2015; Van Leeuwen *et al.* 2015). Pesticides are also distinguished based on what developmental stage they target. There are ovicidal pesticides targeting egg stages, chemicals toxic to motile stages of pests, and chemicals that combine both properties (Nauen *et al.* 2001). Sublethal concentrations also need to be considered because pesticide sublethal effects affect life parameters of mites such as survival rate, fecundity, developmental time (Marcic 2007; Marcic *et al.* 2010, Landeros *et al.* 2002; Li *et al.* 2017).

A critical factor influencing pest control management is pesticide resistance development in target pests, rendering commercially available pesticides useless against resistant populations. Pesticide resistance includes any changes in penetration, activation, metabolism, and transport of a pesticide. These modes of resistance will be discussed later in the text. Similar to plant-herbivore arms race, there is an arms race between pests and novel pesticide production. Some pests are faster at the development of resistance to pesticides than others. However, given increasing expenses and stricter regulatory requirements, it is getting harder to develop pesticides with novel modes of actions; thus, actions are needed on retaining the long-term efficacy of existing pesticides by means of developing strategies that help prolong their utility (e.g., rotation-based resistance management programs) (Sparks 2013; Sparks and Nauen 2015). Therefore, chemical diversity of pesticides and strategies of preserving their long-term efficacy are key to successful pest control

management. At present, there are over 25 different modes of actions of pesticides used in resistance management from at least 55 different chemical classes.

There are four main pesticide resistance mechanisms in herbivorous arthropods: target site mutations, increased metabolism of toxic compound, physical and behavioural changes. The majority of investigated and reported cases of TSSM acaricide resistance revealed two chief resistance mechanisms. The first one is the decreased sensitivity of an acaricidal target-site through a mutation in the target site (Feyereisen 1995). Another common resistance mechanism is metabolic resistance by means of increased acaricide detoxification before it reaches the target site to diminish the amounts of toxin that will reach the target site (Feyereisen 2005; Enayati *et al.* 2005; Li *et al.* 2007). Target-site mutations are often presented as Single Nucleotide Polymorphisms (SNPs). They are usually located in conserved regions of genes that are vital for gene function and ultimately TSSM survival upon pesticide exposure. Metabolic resistance is often associated with the overexpression of detoxification genes such as esterases, glutathione-S-transferases (GSTs) and cytochrome P450s (CYPs) (Feyereisen 2005; Hemingway *et al.* 2004; Scott 1999; Enayati *et al.*, 2005; Liu *et al.*, 2006; Kwon *et al.*, 2012; Demaeght *et al.*, 2013; Riga *et al.*, 2014). There are three phases of detoxification as identified by Després *et al.* (2007): 1) modification of the toxin such that it becomes soluble in water and more reactive; 2) the conjugation of the toxin with spider mite metabolites, generating a less toxic form; and 3) the excretion of metabolite. CYPs and esterases perform the first phase, while GSTs contribute to the second phase. The last phase is carried out by membrane-bound transporters such as ATP-binding cassette and solute carriers (Kennedy and Tierney 2013). Other resistance mechanisms include physical changes, such as thicker cuticle, and behavioural changes, such as avoiding pesticide treated areas of the plant (Adesanya *et al.* 2019). The target-site mutation and pesticide detoxification are mechanisms of resistance that are compatible with development of molecular markers of resistance.

Growers use 30% mortality following a pesticide application as an arbitrary threshold that determines if an acaricide is effective against a local TSSM population. Therefore, in this work, 30% mortality threshold was also used: if a population displays less than 30% mortality, it is considered resistant, while populations with mortality over 30% are considered to be susceptible.

1.6 Acaricides

Acaricides are the pesticides used as the main strategy of control against TSSM and other members of Acari pests. TSSM are champions in pesticide resistance development. On average, it takes only 2-4 years for TSSM to develop resistance to a novel pesticide (Knowles 1997; Van Leeuwen *et al.* 2008). This contributes to making TSSM one of the most economically important pests globally. As mentioned earlier, greenhouses are especially vulnerable to spider mite infestations. One of the main contributing reasons is the frequent use of pesticides. Not only does it give an opportunity for TSSM to develop resistance faster by constant selection of resistance traits but also it can destroy populations of natural TSSM enemies used as biocontrol agents in production settings (Ruberson *et al.* 1998; Ambikadevi and Samarjit 1997; Isman 2000; Shi *et al.* 2005; Van Leeuwen *et al.* 2010; Sparks and Nauen 2015). As of today, TSSM has developed resistance to nearly all registered acaricides including over 96 active ingredients of pesticides from various classes (IRAC, 2021).

1.7 Acaricides used in this project

Acaricides used in this project were Avid, Floramite, and TetraSan with active ingredients abamectin, bifenazate, and etoxazole, respectively. These three pesticides are registered for use in Canada by Health Canada. I will refer to them based on their active ingredient throughout this work.

1.7.1 Abamectin (Avid)

Abamectin is the active ingredient of Avid, an acaricide first registered in Canada in 1996. It is used against adult stages of *Tetranychus urticae* on potatoes, pome fruit, strawberries, caneberries, GH ornamentals, GH peppers, cucumbers, and tomatoes (Chaput 2009).

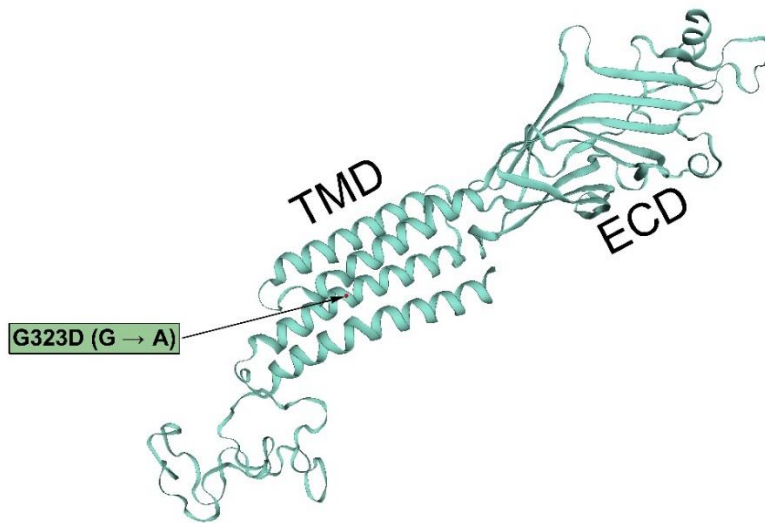
Abamectin belongs to a family of macrocyclic lactones, IRAC group 6 of glutamate-gated chloride channel activators (GluCl) (Burg and Stapley 1989; Fisher and Mrozik 1989). Abamectin is a neurotoxic pesticide that has a particularly high affinity to glutamate-gated ion channel, a member of cys-loop ligand-gated ion channels (cysLGICs). cysLGIC is a superfamily of neurotransmitting receptors that includes the glutamate-gated chloride channels (GluCls), the γ -aminobutyric acid (GABA)-gated channels, pH-sensitive chloride channels (pHCl), nicotinic acetylcholine receptors (nAChRs), and the histamine-gated chloride channels (HisCls) (Dermaw

et al. 2012). They are characterized by five homologous subunits with each consisting of four hydrophobic α -helical transmembrane domains, a large N-terminal extracellular domain, an intracellular loop between the third and fourth transmembrane domains, and a short extracellular C terminus that forms a central ion channel lining (Figure 1.2) (Ghosh *et al.* 2012; Hibbs *et al.* 2011; Horenstein *et al.* 2001; Ozoe *et al.* 2013). These types of receptors are numerous in the nervous systems of invertebrates such as arthropods and play vital biological functions such as synaptic inhibition, pH regulation, cellular excitability, organic solute transport, locomotion, feeding regulation, and sensory input mediation (Cully *et al.* 1994; Ortells and Lunt, 1995). GluCl is the main target of abamectin (Figure 1.2). It is found only in invertebrates such as nematodes and insect pests targeted by macrocyclic lactones, including *T. urticae* (Kehoe *et al.* 2009). Abamectin binds GluCl allosterically and irreversibly opens the chloride channel, allowing the chloride ions to pass through, leading to hyperpolarization, paralysis, and death (Brown *et al.* 2017; Mounsey *et al.* 2007). TSSM has six orthologous GluCl genes (*Tu_GluCl_1* through *Tu_GluCl_6*) as opposed to other arthropods that normally have just one (Dermauw *et al.* 2012). This expansion hints at a TSSM's need for the functional diversity.

Abamectin has been used for over 30 years with first cases of TSSM's resistance reported about 25 years ago (Beers *et al.* 1998; Campos *et al.* 1995). Abamectin resistant populations have been found globally including the US, Columbia, Brazil, the Netherlands, and South Korea (Beers *et al.* 1998; Campos *et al.* 1995, 1996; Cho *et al.* 1995; Koh *et al.* 2009; Lee *et al.* 2003; Stumpf and Nauen 2002; Sato *et al.* 2005). There were also studies reporting multiresistance of abamectin with other acaricides. These included not only acaricides of the same mode of action such as milbemectin (GluCl channel activating acaricide) cross-resistance in a Brazilian population (Sato *et al.* 2005), but also acaricides of other chemical classes and modes of actions: chlorpyrifos (inhibitor of acetylcholinesterase), fenpyroximate (inhibitor of the mitochondrial complex I electron transport), propargite (inhibitor of mitochondrial ATP synthase), and clofentezine (mite growth inhibitor affecting chitin synthase 1) in populations from Turkey (Yorulmaz and Ay 2009).

It is reported that TSSM uses a combination of target site mutation and increased metabolism for the abamectin resistance development. Target site mutations are found in *GluCl1* (G323D) and *GluCl3* (G326E and I321T) (Figure 1.2; Dermauw *et al.* 2012; Kwon *et al.* 2010b; Mermans *et al.* 2017; Riga *et al.* 2014). Metabolic resistance has been linked to the overexpression of detoxification genes including *CYP392A16*, *CYP392D10*, and *CYP392D8* (Riga *et al.* 2014).

Tu GluCl1



Tu GluCl3

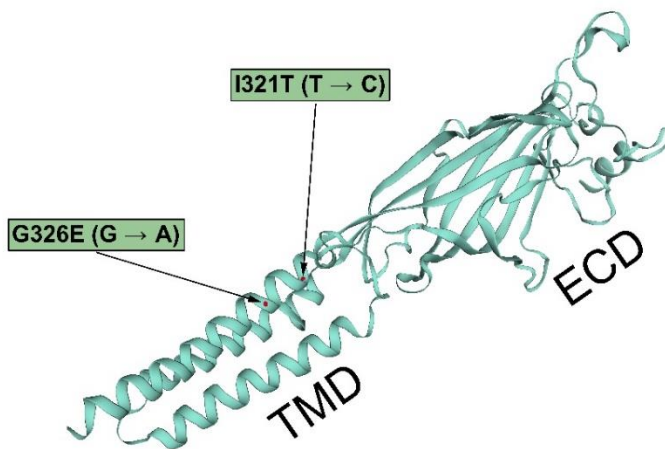


Figure 1.2 Protein models of glutamate-gated chloride channels *Tu* GluCl1 and *Tu* GluCl3. Top, the ribbon structure of *T. urticae* GluCl1 bearing abamectin resistance-associated G323D mutation on transmembrane domain 3. Bottom, the ribbon structure of *T. urticae* GluCl3 carrying abamectin resistance-associated G326E and I321T mutations on transmembrane domain 3. Both models were generated using SWISS-MODEL (Waterhouse *et al.* 2018).

1.7.2 Bifenazate (Floramite)

Bifenazate is the active ingredient of an acaricide Floramite. Bifenazate was commercialized in 1999 (Dekeyser *et al.* 1996; Dekeyser and McDonald 1994) and registered in Canada in 2005. It is used worldwide against all stages of TSSM on fruiting vegetables such as bell and chilli peppers, and tomato (Health Canada, 2014). Bifenazate is a hydrazine carbazate derivative and belongs to IRAC group 20D of mitochondrial complex III electron transport inhibitors. The mode of action of bifenazate is through inhibition of the mitochondrial electron transport chain, and thereby oxidative phosphorylation and ATP production (Van Leeuwen *et al.* 2008; Van Nieuwenhuysse *et al.* 2009). Bifenazate targets a highly conserved region, the cd1 helix, that aligns the enzyme pocket of cytochrome b Q₀ site of mitochondrial complex III (Figure 1.3). Cytochrome b is responsible for the transfer of electrons from reduced ubiquinone to cytochrome c. Thus, by inhibiting this pathway, bifenazate disrupts the production of ATP in the oxidative phosphorylation pathway. Cytochrome b is encoded by mitochondrial DNA. Bifenazate is a pro-acaricide; that is, it needs to be activated by esterase hydrolysis *in vivo* in order to be active (Van Leeuwen *et al.* 2006).

The mode of bifenazate resistance in TSSM includes target site mutations (Van Leeuwen *et al.* 2008; Van Nieuwenhuysse *et al.* 2009). Van Leeuwen *et al.* (2008) compared bifenazate sensitive and resistant populations and found substitution mutations G126S, I136T, S141F, and P262T in the cd1 helix of the Q₀ pocket of cytochrome b. To confer high bifenazate resistance, it was shown that a combination of G126S and I136T or G126S and S141F is needed (Van Leeuwen *et al.* 2008). G126S has not been observed to confer resistance on its own. Riga *et al.* (2017) reported that the combination of G126S and S141F display high resistance level, while P262T does not show. On the other hand, Sugimoto and Osakabe (2019) only found G126S in bifenazate resistant populations. Later, Shi *et al.* (2019) found a novel A269V mutation in the ef helix of cytochrome bc1 (non-conserved site) in Chinese populations; the strength of the resistance was proportional with the increase of the SNP frequency. Fotoukkaiaii *et al.* (2020) reported another SNP I144T associated with bifenazate resistance. Thus, it appears that variable amino acid substitutions in cytochromes b can contribute to bifenazate resistance and that the strength of their effects depends on either genetic or physiological factors.

There are reported cases of bifentazate resistance in the connection of mite resistance to other acaricides of various modes of actions. Van Leeuwen *et al.* (2006) reported the multi-resistance between chlorfenapyr (a pesticide that acts as an uncoupler of oxidative phosphorylation via disruption of the proton gradient) and bifentazate. There are also reported cases of cross-resistance between acaricides of the same mode of action (mitochondrial complex III electron transport inhibition): bifentazate and fluacrypyrim, and bifentazate and acequinocyl (Van Nieuwenhuysse *et al.* 2009).

Tu Cytb

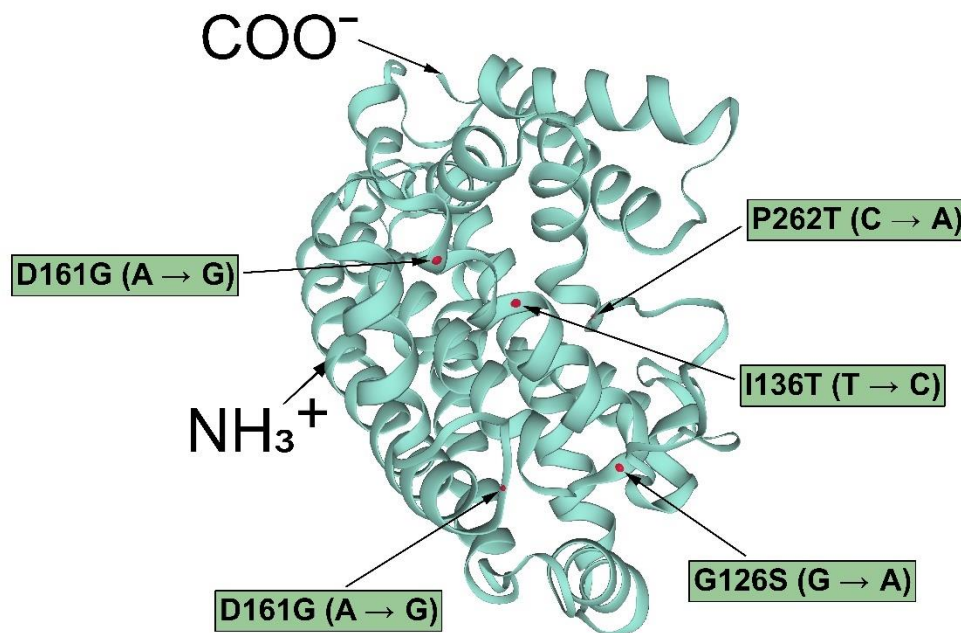


Figure 1.3 Protein model of cytochrome b. The ribbon structure of *T. urticae* cd1 helix in cytochrome b of mitochondrial complex III bearing bifentazate resistance-associated G126S, D161G, S141F, P262T, I136T SNPs. The model was generated using SWISS-MODEL (Waterhouse *et al.* 2018).

1.7.3 Etoxazole (TetraSan)

Etoxazole is the active ingredient of acaricide TetraSan. Etoxazole has been registered in Canada since 2015 and is the newest registered acaricide. It is registered for use in GHF only, protecting GH tomatoes and ornamentals. Mite resistance to etoxazole has been reported in Greece, Cyprus, Netherlands, Italy, Turkey, Japan, Kenya, Belgium (Ilias *et al.* 2014), Japan (Osakabe *et al.* 2017), Turkey (İnak *et al.* 2019), and Australia (Herron *et al.* 2018; Ilias *et al.* 2014).

Etoxazole is an oxazoline analogue that belongs to IRAC group 10B of mite growth inhibitor. It affects chitin synthase I (CHS1), a transmembrane protein involved in chitin biosynthesis (Figure 1.4) (Van Leeuwen *et al.* 2012). Chitin is found in parts of the arthropod exoskeleton, and its biosynthesis is crucial for TSSM's progression through development stages. Etoxazole prevents the incorporation of GlcNAc into the integument. Thus, it is efficient at egg and immature stages by disrupting chitin biosynthesis and preventing egg hatching and juvenile molting. Etoxazole does not directly affect TSSM at adult stages, but their fertility is compromised, and deposited eggs fail to develop into larvae (Van Leeuwen *et al.* 2012).

TSSM uses target site mutation as a mode of resistance to etoxazole. So far, only one nonsynonymous mutation I1017F (isoleucine to phenylalanine) in the chitin synthase 1 (*CHS1*) gene has been reported. This substitution occurs in a C-terminal transmembrane domain of CHS1, which is a highly conserved region. A high correlation between I1017F SNP and etoxazole resistance has been reported (Demaeght *et al.* 2014; Ilias *et al.* 2014; Riga *et al.* 2017; Van Leeuwen *et al.* 2012). Cross-resistance between etoxazole, clofentezine, and hexythiazox, all with the same mode of action (chitin synthase 1 inhibitors), has also been reported (Demaeght *et al.* 2014).

Tu CHS1

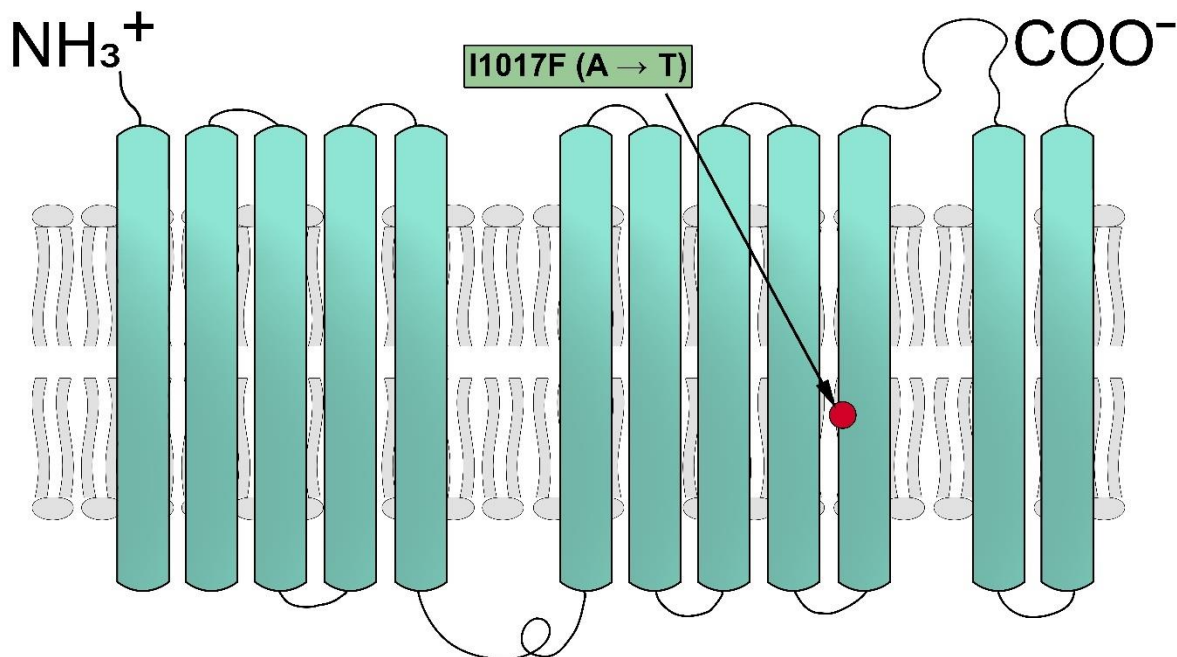


Figure 1.4 Protein model of chitin synthase 1. The structure of *T. urticae* CHS1 bearing etoxazole resistance-associated I1017F SNP on transmembrane domain 5.

1.8 Thesis objectives

The analysis of acaricide resistance in TSSM populations collected from various geographical locations suggests that resistance patterns and frequencies differ between *T. urticae* strains depending on their geographical location and host plants (Kwon *et al.* 2010a; Sato *et al.* 2005; Van Leeuwen *et al.* 2010; Xu *et al.* 2018; Xue *et al.* 2020). Additionally, the acaricide resistance status differs among local populations, and growers lack an effective method of determining which pesticide to apply against the local TSSM strain. Moreover, pesticide resistance can be determined by a recessive gene and/or has a low frequency in a TSSM population, and, thus, the resistance can be overlooked even in laboratory settings by performing toxicity bioassays.

The goal of this work is to characterize genetic markers, find whether there is a correlation between markers and pesticide resistance, and determine markers' suitability to be applied to the

development of the pesticide resistance diagnostic tool. This pesticide resistance diagnostic tool will enable prediction of population resistance status based on the pattern of resistance-associated genetic markers. If successful, it will help growers in decision-making. It will also extend the utility of current pesticides and will prevent the excessive use of pesticides. This is the first work that investigates the pattern of abamectin, bifenazate, and etoxazole resistance and cross-resistance in Canadian (Ontarian) TSSM populations.

My specific objectives were:

a) to optimize protocols required for determination of the discriminative dose (lethal concentration causing 90% mortality of susceptible reference population) and mortality ratio of adulticidal and ovicidal pesticides;

b) to apply optimized protocols on samples collected in Canadian greenhouses to determine their LC_{90} for abamectin, bifenazate, and etoxazole;

c) to genotype TSSM greenhouse populations for known genetic markers of abamectin, bifenazate and etoxazole resistance;

d) to characterize metabolic resistance in mite population samples using RT-qPCR;

2.0 Materials and methods

2.1 Plant materials used

California Red Kidney bean plants (*Phaseolus vulgaris* L.) were used to rear the two-spotted mite (TSSM). The plants were grown in the soil mix (Pro-Mix BX Mycorrhizae) and were maintained in a plant growth chamber at 26°C and relative humidity around 60%. Photoperiod was set to 16:8 h (light:dark) with 120-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity using cool-white fluorescent lights (PHILIPS very high output F96T12/CW/VHO/EW).

2.2 Pesticides used

Adulticidal pesticides Avid and Floramite (active components: abamectin and bifenazate, respectively) and ovicidal pesticide TetraSan (active component: etoxazole) were obtained from Syngenta, Chemtura, and Valent, respectively, in the form of pesticide product formulations provided by Ian Scott, AAFC, London, ON.

2.3 Populations information and timeline schematics:

Nineteen TSSM populations were collected from tomato, cucumber, pepper, and eggplant vegetable greenhouses across Ontario by IPM (Integrated Pest Management) specialists. Pest control histories included the use of various pesticides, oil, soap, and biological pesticides utilizing bacteria or fungi as an active ingredient. Some of the crops underwent a combination of the aforementioned methods of control, and some of the crops were subjected to one of the methods. The TSSM populations were collected in September and October of 2018 (Table 2.1). Once the populations were received by the Grbic laboratory, they were established on beans and placed in boxes with ventilated lids. The population size fluctuated but were kept at 600 adults on average. Population maintenance was done weekly with the removal of old and addition of fresh bean leaves. Initial samples were collected for genetic and metabolic analyses four months post initial population collection (tissue collection '2019'; see Materials and methods 2.3.1-2.3.3 timelines). A second sample was collected 15 months post initial collection; thus, the populations were unchallenged and reared on beans during the 15-month period (tissue collection '2020'; see Figures 2.1-2.3).

Table 2.1 Spider mite populations used in this project.

SeqID	Producer ID	Crop	Date collected	Bean establishment date	Lab rearing plant
1	Farm 'A'	Tomato	10/9/2018	10/10/2018	bean
2	Farm 'B'	Tomato	10/9/2018	10/10/2018	bean
3	Farm 'A'	Pepper	10/9/2018	10/10/2018	bean
4	Farm 'D'	Tomato	10/16/2018	10/17/2018	bean
5	Farm 'E'	Tomato	10/16/2018	10/17/2018	bean
6	Farm 'T'	Tomato	10/16/2018	10/17/2018	bean
7	Farm 'T'	Tomato	10/16/2018	10/17/2018	bean
8	Farm 'T'	Tomato	10/16/2018	10/17/2018	bean
9	Farm 'F'	Tomato	10/17/2018	10/17/2018	bean
10	Farm 'F'	Tomato	10/17/2018	10/17/2018	bean
11	Farm 'C'	Tomato	10/25/2018	10/26/2018	bean
12	Farm 'C'	Cucumber	10/25/2018	10/26/2018	bean
13	Farm 'G'	Tomato	10/25/2018	10/26/2018	bean
14	Farm 'D'	Tomato	10/24/2018	10/26/2018	bean
15	Farm 'D'	Eggplant	10/24/2018	10/26/2018	bean
16	Farm 'A'	Tomato	9/26/2018	9/27/2018	bean
17	Farm 'B'	Tomato	9/26/2018	9/27/2018	bean
18	Farm 'H'	Tomato	9/26/2018	9/27/2018	bean
19	Farm 'T'	Tomato	9/26/2018	9/27/2018	bean
G	Lab	Apple	>15 years ago	Apr-19	bean
S	Lab	Apple	ND	Apr-19	bean
TA	Lab	ND	>7 years ago	Apr-19	tomato
A	Lab, field	Tomato	2014	Apr-19	tomato
B	Lab, field	Tomato	2014	Apr-19	tomato
C	Lab, field	Tomato	2014	Apr-19	tomato
D	Lab, field	Tomato	2014	Apr-19	tomato
E	Lab, field	Peanut	2014	Apr-19	tomato
COL	Lab	Bean	ND	Apr-19	arabidopsis
CYP	Lab	Bean	ND	Apr-19	arabidopsis
FACCE	Lab, field	Tomato	ND	Apr-19	tomato

GH collected populations

Laboratory populations

2.3.1 Abamectin timeline

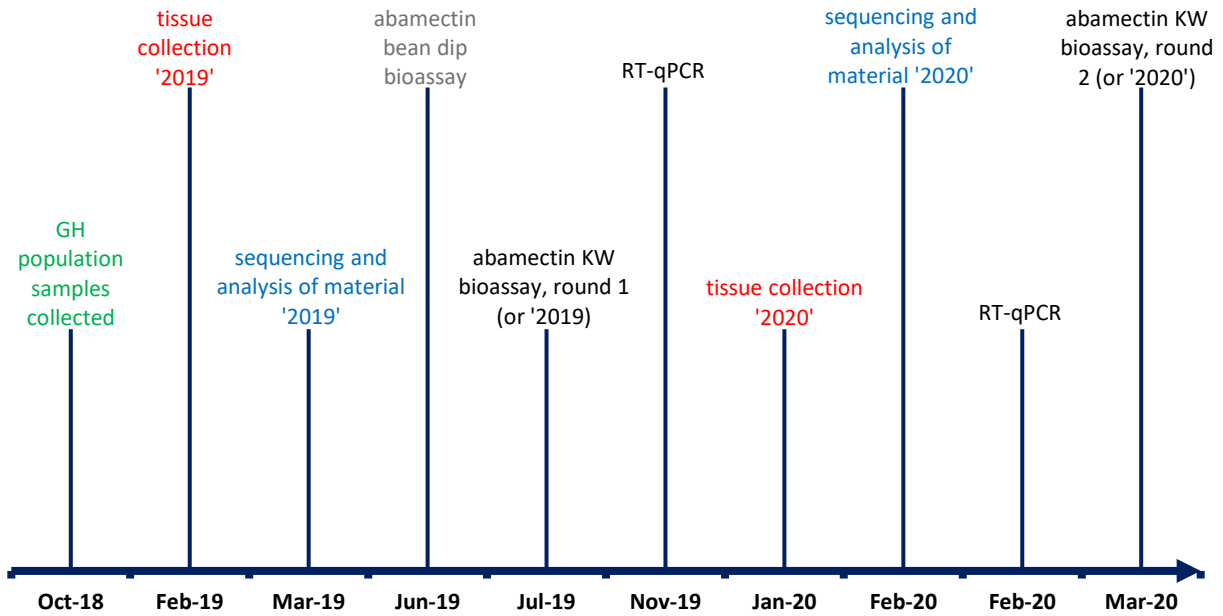


Figure 2.1 Timeline of abamectin resistance analysis in GH populations.

GH population samples were collected by IPM specialists in September/October 2018 from various vegetable greenhouses across Ontario and established in the lab on beans immediately upon receiving (see Table 2.1). In February 2019, four months later, the first round of TSSM tissue samples were collected (referred to as '2019'). The mite material '2019' was sequenced and analyzed the following month, and RT-qPCR analysis was performed in November 2019. Toxicity bioassays started 8 months post-initial collection. Bean dip bioassays were performed in June 2019, followed by KimWipe (KW) bioassays (see Materials and Methods 2.5.1.1-2.5.1.2). The second round of tissue collection took place in January 2020, 14 months post-initial collection during which the populations were reared on beans without selection pressure (referred to as '2020'). The material '2020' was subjected to sequencing, analysis and RT-qPCR the following month. The second round of KW toxicity bioassays took place in March 2020; that is, 17 months post-initial collection during which the populations were reared in the lab on beans without selection pressure.

2.3.2 Bifenazate timeline

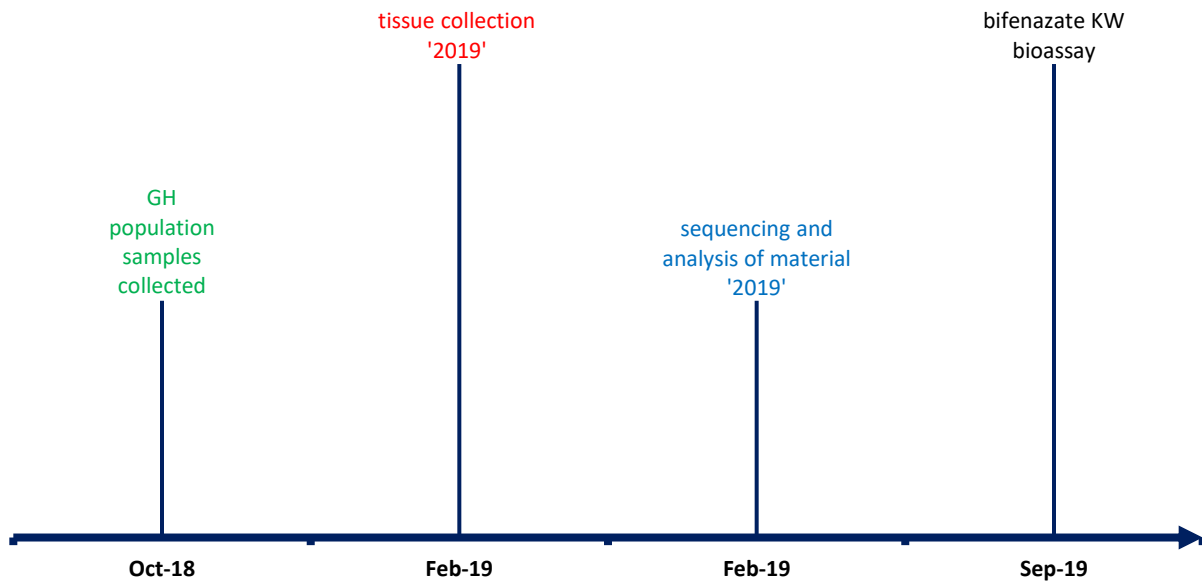


Figure 2.2 Timeline of bifenazate resistance analysis in GH populations.

GH population samples were collected by IPM specialists in September/October 2018 from various vegetable greenhouses across Ontario and established in the lab on beans immediately upon receiving (Table 2.1). In February 2019, four months later, the first round of TSSM tissue samples were collected (referred to as '2019'). The mite material '2019' was sequenced and analyzed the following month. Toxicity KW bioassays started 11 months post-initial collection, in September 2019 (see Materials and Methods 2.5.1.2).

2.3.3 Etoxazole timeline

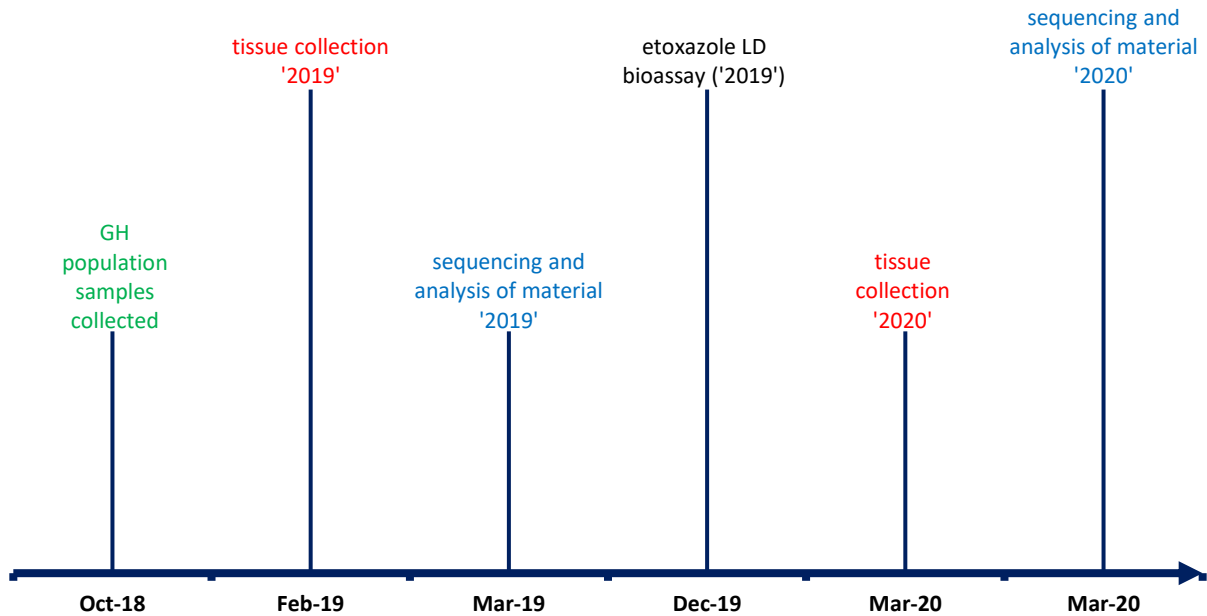


Figure 2.3 Timeline of etoxazole resistance analysis in GH populations.

GH population samples were collected by IPM specialists in September/October 2018 from various vegetable greenhouses across Ontario and established in the lab on beans immediately upon receiving (Table 2.1). In February 2019, four months later, the first round of TSSM tissue samples were collected (referred to as '2019'). The mite material '2019' was sequenced and analyzed the following month. Toxicity Leaf Disk (LD) dip bioassay was performed 14 months post-initial collection, in December 2019 (see Materials and Methods 2.5.1.3). The second round of tissue collection was performed in March 2020, that is 17 months post-initial collection, during which the populations were reared on beans without selection pressure (referred to as '2020'). The mite tissue collection was followed by sequencing and analysis in the same month.

2.4 Workflow

The workflow consisted of two parallel processes (Figure 2.4): 1) characterization of pesticide resistance status of populations via toxicity bioassays with the prior establishment of discriminative dose (LC_{90} – was determined by the mortality of 90% of susceptible reference TU-LND population); and 2) identification of genetic markers. Genetic markers were determined via

genotyping while RT-qPCR was used for gene overexpression analysis. The final data from both processes were analyzed statistically and conclusions were drawn.

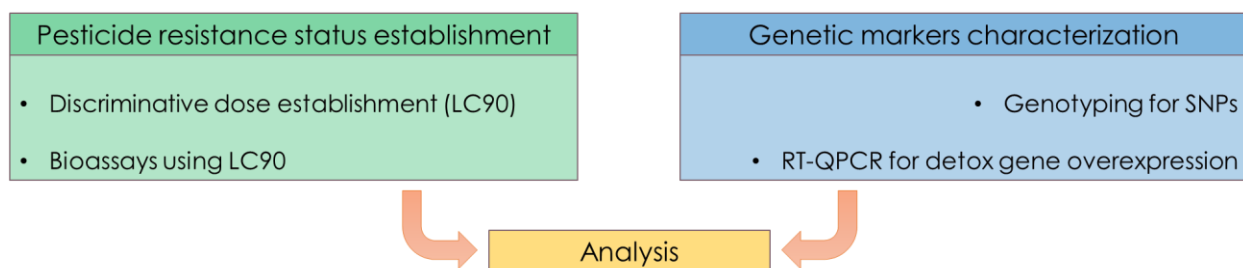


Figure 2.4 The general workflow for genetic markers characterization.

2.5 Discriminative dose establishment for dose response and toxicity bioassays

2.5.1 Concentrations used for discriminating dose establishment

The discriminating dose for bioassays was defined for the purposes of this study as the LC₉₀ of the susceptible London (TU-LND) population. LC₉₀ was determined using toxicity bioassays over a range of active compound concentrations. The active compound dose response series concentrations were: 0.0081, 0.081, 0.81, 8.1, and 81 ppm for abamectin; 0.0113, 0.113, 0.226, 0.45, 0.75, 1.13, and 2.26 ppm for bifenthrin; 0.00005, 0.0005, 0.005, 0.05, 0.5, and 5 ppm for etoxazole. Each pesticide concentration was tested in three replicates (per one trial) in three independent trials.

2.5.1.1 Conventional leaf disk-based bioassay (adulticidal)

Adulticidal conventional leaf disk-based bioassay was used for abamectin toxicity bioassay based on Suzuki *et al.* (2017). Four bean disks 1.5 cm in diameter were treated with discriminating dose of abamectin (LC₉₀ = 0.456 ppm, obtained from the trials in Ian Scott's lab, AAFC). TU-LND treated with LC₉₀ served as positive control, while water treated TU-LND served as negative control. Each disk was dipped into 25 mL of a treatment solution for 5 seconds. The solution was changed after every 4 disks to avoid dilution. Disk dipping was followed by air drying on a metal rack in a fume hood for 20-30 minutes. After the disks were dry (but not desiccated), they were placed on wet cotton pads placed on filter paper with its edges submerged in water in a tray (Figure 2.5a). Each disk was inoculated with 5 adult female TU-LND spider mites (20 spider mites per population). Following the application of mites, trays were covered with ventilated lids and placed in a chamber (24°C, 60% humidity, photoperiod 16:8 h, light:dark) for two days (Figure 2.5b).

Mortality was then recorded using the following qualifications: alive, when the movement of a spider mite was normal or impaired such as very slow, shaky gait or twitching only; and dead, when there was no movement observed when touched with a brush. The toxicity bioassay was performed in three independent trials.

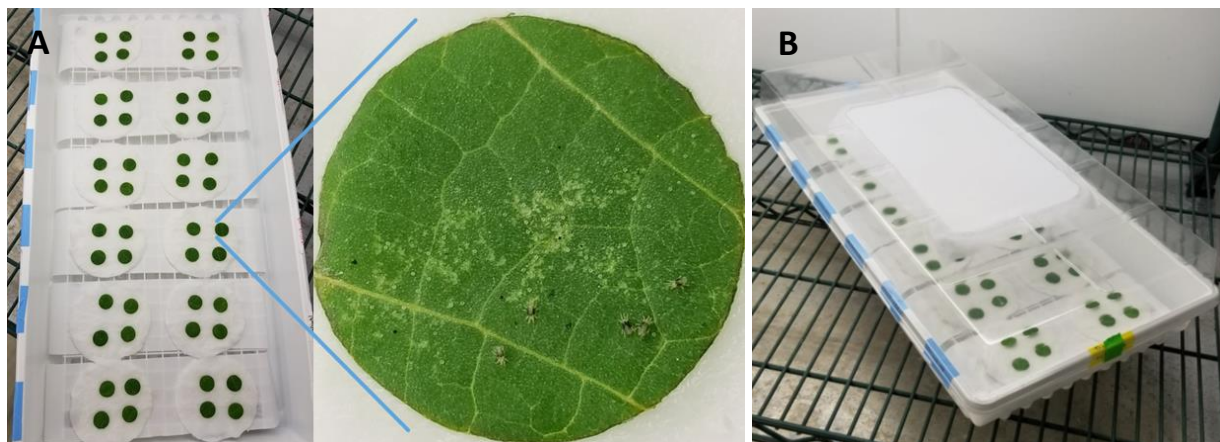


Figure 2.5 Leaf disk-based bioassay setup used for adulticidal pesticide delivery. (A) TSSM infested bean disks are placed on a wet cotton pad that is placed on wet filter paper. The edges of filter paper are submerged in water. Eight bean disks are infested with five female adult TSSM each. (B) Tray with infested bean disks covered with a ventilated lid are placed in an incubation chamber at 24°C, 60% humidity, photoperiod 16:8 h, light:dark for two days.

2.5.1.2 KimWipe-based accelerated bioassay (adulticidal)

Adulticidal KimWipe-based accelerated bioassay (KW) was performed for abamectin and bifenthrin discriminating dose establishment using TU-LND reference strain. A KimWipe square (0.5 cm²) was placed in a small petri plate. Eighteen microliters of each concentration of a pesticide solution or water was added to the KimWipe square. Eighty adult female TU-LND spider mites were placed on the KimWipe and were gently positioned with their dorsal surface up using a fine brush (Figure 2.6a). The spider mites on the KimWipe were covered with another Kimwipes square (0.6 cm²), and 18 µL of pesticide solution or water were added to saturate (Figure 2.6b). Water treated TU-LND served as the negative control. Petri plates were sealed with parafilm and left to incubate overnight at 24°C (Figure 2.6c). The following morning (~20 hours later), the petri plates were opened, KimWipes with mites on them were separated and transferred on a cut bean leaf isolated with wet Kimwipes paper to contain the mites on a bean surface arena (Figure 2.6d). Once mites dried and recovered (~20-30 minutes), mortality was assessed as a ratio of alive to dead

spider mites. Three KimWipe setups were used for each concentration resulting in 240 mites/concentration. The assay was performed in three independent trials ($n = 3 \times 240$).

Once the discriminating doses for abamectin and bifenthrin were established, the same protocol with a few modified steps was used for abamectin and bifenthrin toxicity bioassays. The changes are following: the KimWipe squares were saturated with 36 μL of pesticide solution at LC_{90} concentration (0.342 ppm for abamectin and 1.573 ppm for bifenthrin) in two steps; TU-LND treated with LC_{90} served as the positive control, while water treated TU-LND served as the negative control; the assay was performed in three independent trials with one KimWipe setup of 80 mites/population/trial.

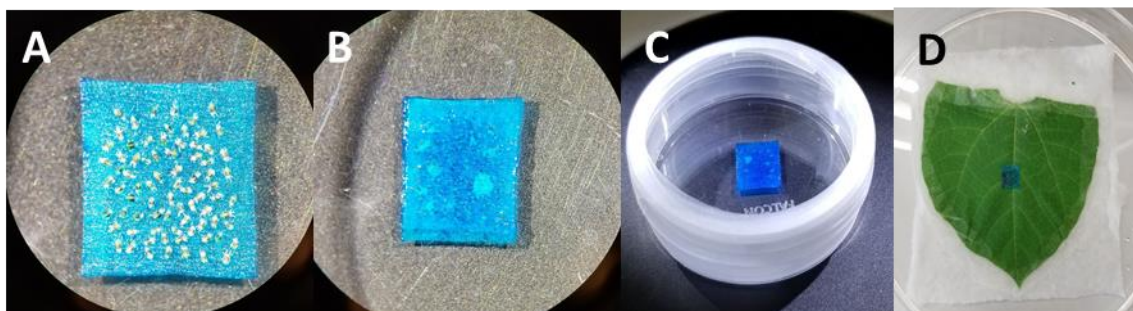


Figure 2.6 KimWipe-based bioassay setup used for adulticide pesticide delivery to adult TSSM. (A) Eighty adult female TSSM are placed with dorsal surface up on a KimWipe square saturated with pesticide solution. (B) KimWipe square infested with TSSM is covered with another KimWipe square saturated with pesticide solution. (C) Petri plate with TSSM infested KimWipe squares is sealed with parafilm and left to incubate overnight. (D) KimWipe square with mites on top are left to dry on bean leaf. Viable mites will move from the KimWipe to the leaf.

2.5.1.3 Leaf disk-based bioassay (ovicidal)

Ovicidal leaf disk-based bioassay was used for etoxazole discriminating dose establishment using TU-LND reference strain. Four bean disks, 1.5 cm in diameter, were placed in a small petri plate filled with 0.7% agar for each concentration of a pesticide solution. At day 0, each disk was inoculated with five adult TU-LND female spider mites (60 mites/concentration/trial) and left to oviposit overnight (Figure 2.7). At day 1, the spider mites were removed, and each disk with eggs on it was dipped into 25mL of a treatment solution for 5 seconds. The solution was changed after each 4 disks to avoid dilution. Water was used as a control

treatment. The disks were placed back on agar to dry in a fume hood for 20-30 minutes. Once the disks were dry, the plates were covered with ventilated lids and placed in a chamber (24°C, 60% humidity, photoperiod 16:8 h, light:dark). The viability of eggs was assessed as a ratio of the number of emerged mites on days 5 and 9 to the total number of eggs laid on day 0.

Once the discriminating dose for etoxazole was established, the same protocol with a few modified steps was used for etoxazole toxicity bioassays. The changes are following: at day 0, each disk was inoculated with five female spider mites resulting in 20 mites per population in one trial, letting them oviposit overnight; next day, the mites were removed, and each disk with eggs on it was dipped into 25 mL of an etoxazole treatment solution at LC₉₀ (0.0196 ppm) for 5 seconds; TU-LND treated with LC₉₀ served as positive control, while water treated TU-LND served as negative control. The assay was performed in three independent trials.



Figure 2.7 Leaf disk-based bioassay setup used for ovicidal pesticide delivery. Small petri plate filled with 0.7% agar and four TSSM infested bean disks placed on top.

2.6 Genotyping for SNPs

One hundred adult mites were taken from each of the 31 GH rearing populations and used for total RNA extraction using RNeasy Mini Kit, including DNase treatment, following the protocol of the manufacturer (Qiagen, Venlo, Limburg, Netherlands). Extracted RNA was then quantified using NanoDrop 2000C Spectrophotometer (Thermo Scientific). Then, three micrograms of total RNA were converted into cDNA using Maxima cDNA synthesis kit (Thermo

Fisher Scientific, Waltham, MA), following protocol of the manufacturer. Incubation was done in a T100 Thermal Cycler (Bio-Rad). The target genes then were analyzed by quantitative sequencing to check the presence/absence and frequency of SNPs associated with the resistance (Table 2.2). Synthesized cDNA was used as a template for standard PCR, using primers found in Table 2.2. PCR was run for 10 cycles. A template concentration was 10 ng. The amount of one template per reaction was equivalent to 10 ng of RNA. The PCR products were then confirmed by gel electrophoresis. Then, the PCR products were purified and quantified using NanoDrop 2000C Spectrophotometer (Thermo Scientific). Following that, sequencing reactions were prepared according to Roberts Research Institute protocol: 10 μ L of the template were mixed with 5.0 μ L of 2.0 μ M primers. The mixes were then sent to Roberts Research Institute for Sanger sequencing. Mite genotyping was performed with standard PCR and Sanger sequencing using markers found in Table 2.2. Sequencing data were analyzed using Staden software (Staden *et al.* 2000). Below is an example of sequencing analysis using Staden software based on the fluorescent traces produced by Sanger DNA sequencing (Figures 2.8a,b). As seen in Figure 2.8a, sample of GH-collected population 08 (at the top) was compared with reference London population (in the middle), and the difference obtained by subtracting one from the other is shown at the bottom (Figure 2.8a). Figure 2.8b illustrates an example of a heterozygous mutation found in the greenhouse-collected population 02 at *cytB*. The overlap of peaks indicates the presence of two nucleotides at the same position. The height of the peak indicates an approximate frequency of the nucleotides present.

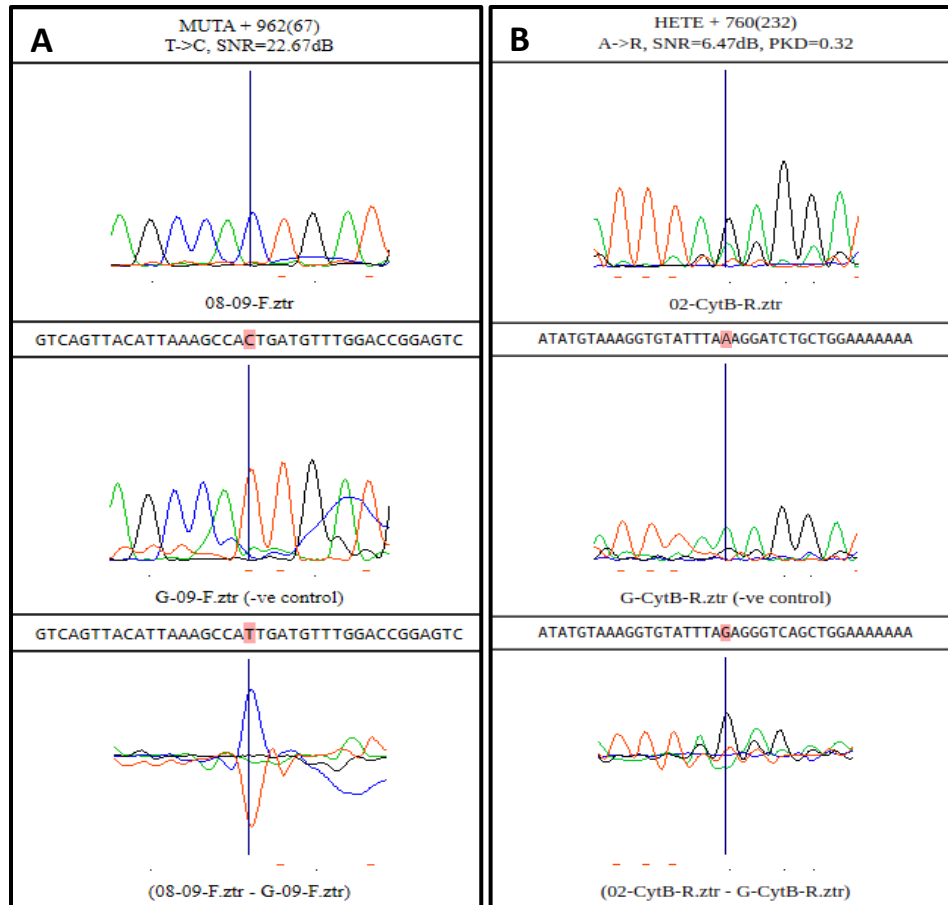


Figure 2.8 Electropherograms displaying (A) homozygous point mutation in *GluC13* gene sequence of a greenhouse-collected population 08 and (B) heterozygous point mutation in *cytB* gene sequence of a greenhouse-collected population 02. The figures were generated with Staden software.

Table 2.2 Primers used for genotyping of single nucleotide polymorphisms to characterize pesticide target site mutation.

Mutation	TeturID	Gene	Primer	Amplified product (bp)	Annealing temperature (°C)
G323D	Tu_GluCI1_dia_F	<i>Tu_GluCI1</i>	TTGGATTGACCCCTAACTCAGCA	263	58
	Tu_GluCI1_dia_R		TTGCACCAACAATTCCCTTGA		
G326E, I321T	Tu_GluCI3_dia_F	<i>Tu_GluCI3</i>	CCGGGTCAGTCTTGGTGTTA	251	58
	Tu_GluCI3_dia_R		CACCACCAAGAACCCTGTTGA		
G126S, P262T, I136T, S141F, D161G	CytB-F	<i>cytB</i>	AATAATTTTACAAAATAAECTCATGC	822	54
	CytB-R		GAAATTTCTGTAAAAGGGTATTC		
I1017F	Tu_CHS1_dia_F	<i>chs1</i>	TGTCCGCTTGTATGCACTACT	822	57
	Tu_CHS1_dia_R		GCCACCAAGTGGGTCAAGAT		

2.7 RT-qPCR for metabolic resistance markers

Total RNA was extracted from 100 adult mites from each of the 31 GH rearing not challenged populations using RNeasy Mini Kit, including DNase treatment (Qiagen, Venlo, Limburg, Netherlands). Three micrograms of total RNA were converted to cDNA using Maxima cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). Three technical replicates were performed per biological replicate, using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA). The mean Ct value of triplicate technical replications was used as the Ct value for the corresponding biological replication. RT-qPCR was performed using an Agilent Mx3005P qPCR instrument (Agilent Technologies, Santa Clara, CA). *CYP392D10*, *CYP392A16* and *CYP392D8* genes were chosen to quantify the level of expression via RT-qPCR. These genes encode cytochrome P450 enzymes known for their detoxification of xenobiotics and endogenous compounds (Riga *et al.* 2014). The reference gene used to normalize cDNA addition to wells was *Rp49* (*tetur18g03590*), a ribosomal protein that has been used in analysis of mite gene expression in previous work (Demaeght *et al.* 2013; Morales *et al.* 2016). *Rp49* gene was found to be transcribed at similar levels in all samples as indicated by Ct values within ± 1 cycle. Primer pairs can be found in Table 2.3. Possible buffer contamination was controlled for with the inclusion of no template controls and no RT controls were used to verify lack of genomic DNA contamination. The cycles ran as following: 600 sec at 95 °C followed by 40 cycles; 30 sec at 95 °C; then 60 sec at 60 °C, followed by melting curve. The amount of one template per reaction was equivalent to 1 ng of RNA; gDNA concentration was 1ng/25 μ L.

Table 2.3 Primers used in RT-qPCR to determine the expression of three CYP genes and characterize metabolic pesticide resistance.

TeturID	Gene	Forward/Reverse primer	Amplified product (bp)	Primer efficiency
Tetur06g04520 (F)	<i>CYP392A16</i>	AAATACCGAGGTCGGACGTA	117	97.60%
Tetur06g04520 (R)		AAGCACTTTTTC AATCTGGTCAC		
Tetur03g05110 (F)	<i>CYP392D10</i>	ATTGGATTTCGAACGTC AACC	109	94%
Tetur03g05110 (R)		GTAAATT AAGAGGAGTGATTGTTGCT		
Tetur03g05070 (F)	<i>CYP392D8</i>	TGAGCTCAGAAACGCGAAT	100	93%
Tetur03g05070 (R)		CTCGATTTTCATGGGTTGCTT		
tetur18g03590 (F)	<i>Rp49</i>	CTTCAAGCGGCATCAGAGC	179	97.60%
tetur18g03590 (R)		CGCATCTGACCCCTTGA ACTTC		

2.8 Statistical analysis

Dose response mortality data and egg hatchability were estimated by performing bioassays over the range of active compound concentrations. Mortality data and hatchability were analyzed with “base” and “drc” R packages using two-parameter log-logistic model for binomial response (Ritz *et al.* 2015).

Toxicity bioassay mortality data and egg hatchability were analyzed and plotted with “base” and “agricolae” R packages (Ritz *et al.* 2015; Felipe de Mendiburu 2019). To assess difference in response of TSSM lines to active compounds at LC₉₀, one-way ANOVA was followed by Tukey’s honestly significant difference (HSD) test at $\alpha = 0.05$.

2.9 Protein model generation

The schematics in Figures 1.2-1.3 were built using SWISS-MODEL (Waterhouse *et al.* 2018) using amino acid sequence obtained from ORCAE (GluCl1, tetur02g04080, and GluCl3, tetur10g03090) and UniProt (cytochrome b, B2C9D9_TETUR) (Sterck *et al.* 2012).

The models for the schematics were chosen based on the highest identity with the amino acid sequences of the proteins in SWISS-MODEL. GluCl1 and GluCl3 schematics were built based on avermectin-sensitive *C. elegans* glutamate-gated chloride channel (GluCl) alpha (SMTL ID: 3ria.1). Cyt b schematics was based on Cytochrome bc1 complex from chicken with designed inhibitor bound (SMTL ID: 4u3f.1).

3.0 Results

3.1 Development of the KimWipe-based accelerated pesticide bioassay

The leaf disk-based bioassay is a conventional method used for the delivery of various chemical compounds to spider mites to study their effects on mites (Douris *et al.* 2017; Brown *et al.* 2017; Liu *et al.* 2019; Papapostolou *et al.* 2020; Xue *et al.* 2020). However, given 31 populations to be tested, the leaf-disk bioassay was determined to be too laborious and time-consuming. Thus, an alternative, more efficient method was needed for determining the pattern of adult TSSM acaricide resistance in 19 local GH and 12 laboratory populations. A KimWipe-based bioassay method was adopted for this purpose based on the work of Suzuki *et al.* (2017) (soaking method) and Abouelmaaty *et al.* (2019) (sandwich method). The soaking method was developed for the delivery of small molecules to spider mites. Using this method, mites are completely submerged in aqueous solution and are allowed to take up the solution for up to 24 hours. The sandwich method requires a droplet of an aqueous solution of a chemical that is placed between a bean disk and a polypropylene sheet. Mites are then placed on this feeding arena. The newly developed KimWipe-based accelerated bioassay combines both methods. Mites are placed between two pieces of KimWipes and are completely soaked in a pesticide solution for 24 hours (see Materials and Methods 2.5.1.2). The abamectin bioassay trials were used to compare leaf-disk dipping (Figure 3.1) and the newly developed double KimWipe accelerated bioassay (Figure 3.3b) using discriminatory dose ($LC_{90} = 0.456$ ppm for LD toxicity bioassay and $LC_{90} = 0.342$ ppm for KW toxicity bioassay). A strong correlation ($R^2 = 0.8679$) was found between these two methods, and, thus, they were considered comparable in their goal of assessing mite mortality (Figure 3.2). Because the KimWipe method is more labor and time efficient, all adulticidal dose response trials and toxicity experiments were performed using this optimized bioassay.

Abamectin LD, groups and ranges

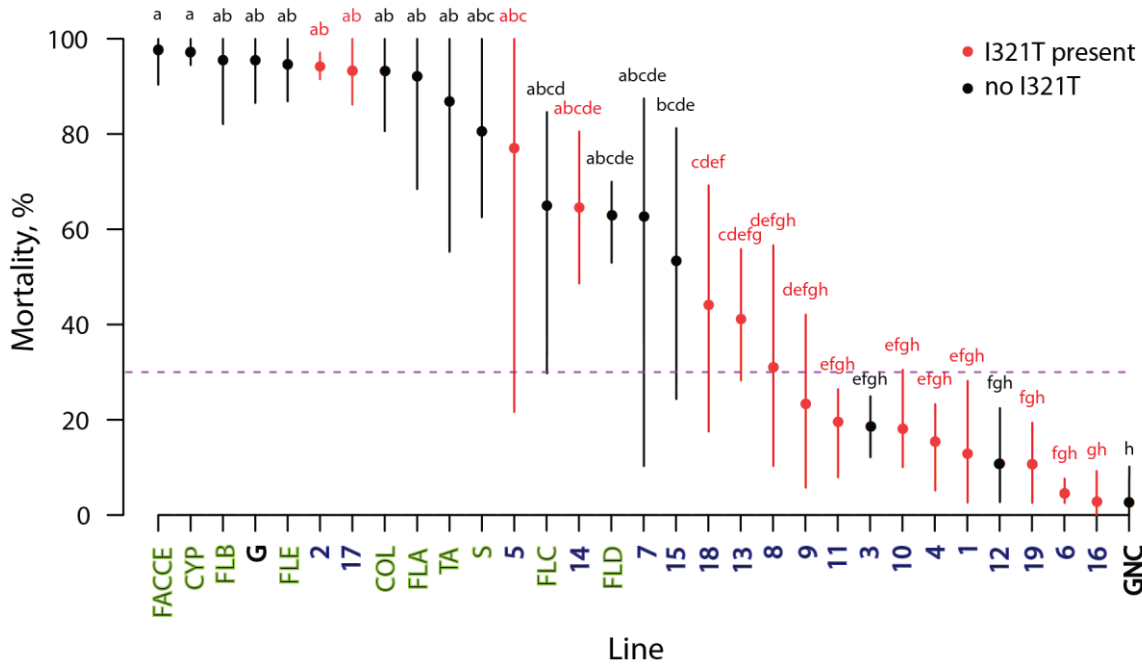


Figure 3.1 The analysis of mite susceptibility to abamectin using conventional leaf disk-based method. Adulticidal toxicity bioassay of mite populations that were reared unchallenged on beans for 8 months after their collection from greenhouses. Greenhouse mite populations (01 through 19) are shown in blue, and laboratory populations are shown in green. Populations were treated with discriminating concentration of abamectin ($LC_{90} = 0.456$ ppm) in three independent trials ($n=20$ /trial). TU-LND (shown in black) was used as negative (GNC) and positive (G) controls. Shown are proportions of mites that died (as a %) \pm 95% confidence intervals (CI). The red dots with CI represent mortality of populations carrying I321T mutation in *GluCl3*. The black dots with CI represent mortality of populations that lack I321T mutation in *GluCl3*. Letters represent significant differences between means using Tukey's HSD test at $\alpha = 0.05$.

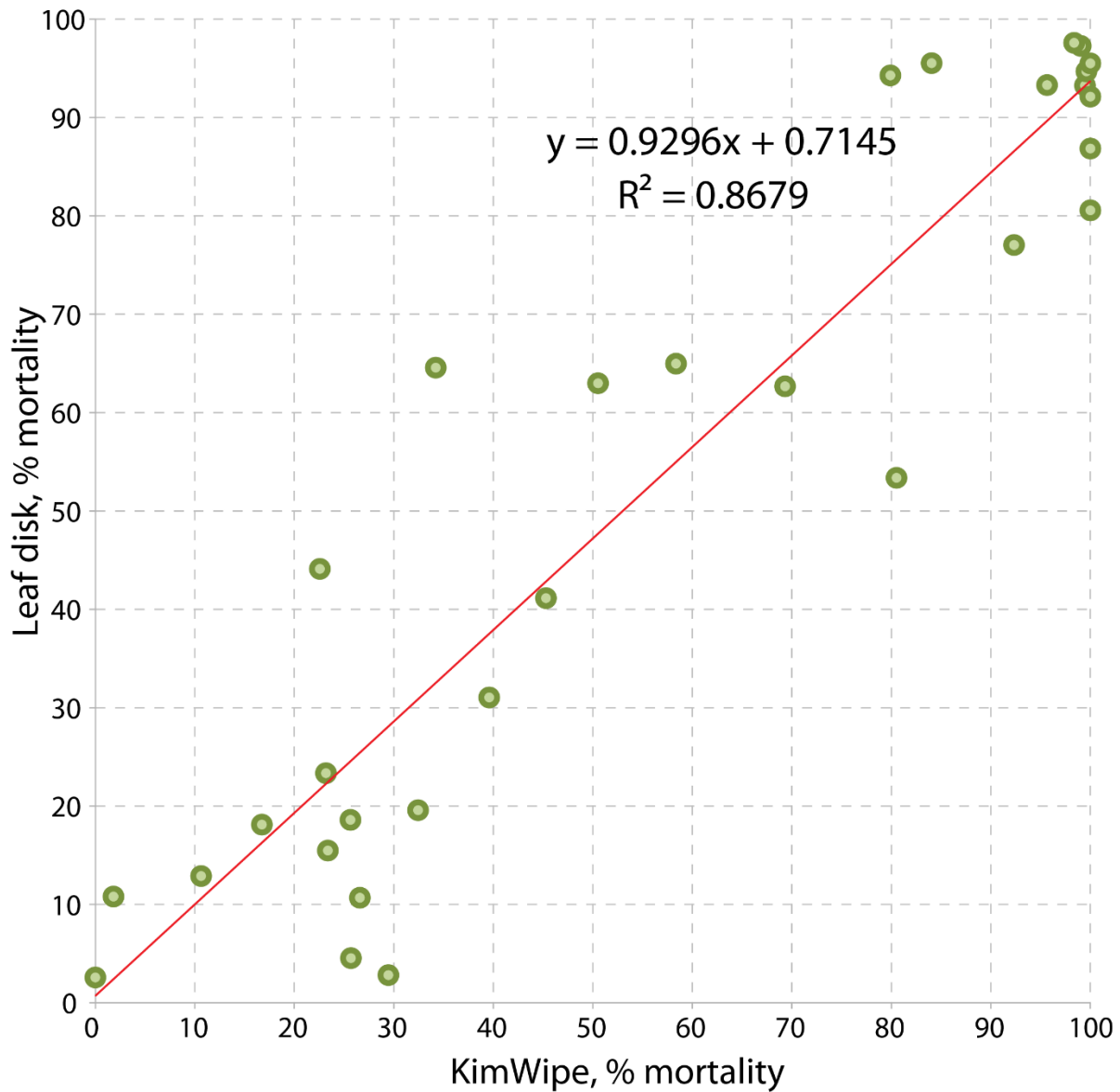


Figure 3.2 Correlation between Leaf disk and KimWipe methods. Mite mortality (%) after application of a conventional leaf disk dip and the novel high throughput KimWipe methods upon the application of abamectin at discriminatory doses ($LC_{90} = 0.456$ ppm for LD bioassay and $LC_{90} = 0.342$ ppm for KW bioassay) in GH and laboratory populations (green dots). Linear regression line was fitted to data (red line).

3.2 Abamectin

3.2.1 Ontario greenhouse-collected *Tetranychus urticae* populations display phenotypical resistance to abamectin

To establish reference data, a laboratory susceptible TU-LND strain was exposed to a range of abamectin concentrations, from 0.0081 to 81 ppm, including the recommended field dose of abamectin (42 ppm). At low abamectin concentrations the mortality of TU-LND mites was not affected. However, at high doses, mite mortality reached 100%, Figure 3.3a, indicating that abamectin concentrations tested are suitable to identify the LC₉₀, a concentration that causes 90% mortality of the reference TU-LND population. The negative control consisting of water treated TU-LND resulted in nearly 0% mortality indicating that the protocol execution did not negatively affect mite fitness (not shown). Using a Probit Analysis of dose response data, the TU-LND LC₉₀ of abamectin was determined to be 0.342 ppm. This concentration was subsequently used as a discriminatory dose to determine the abamectin resistance status of Ontario GH and laboratory populations. Resistance to abamectin was inferred if <30% mortality occurred in response to the LC₉₀ discriminating abamectin concentration. As seen in Figure 3.3b, the mortality of water-treated TU-LND (water control GNC) was 0%, as expected. Consistent with the dose responses shown in Figure 3.3a, the mortality of TU-LND reference populations (G, S) treated with LC₉₀ abamectin concentration was 100%. Ten out of 19 GH populations were highly resistant to abamectin with mortalities below 30%. Five GH populations fell into the intermediary resistance category with mortalities ranging from 30 and 70% and four GH populations were susceptible to abamectin with mortalities greater than 70%. Laboratory strains displayed high susceptibility to abamectin with mortalities above 80%, except for FLC and FLD that fell into intermediate range. In conclusion, Ontario GH populations have high incidence of abamectin resistance.

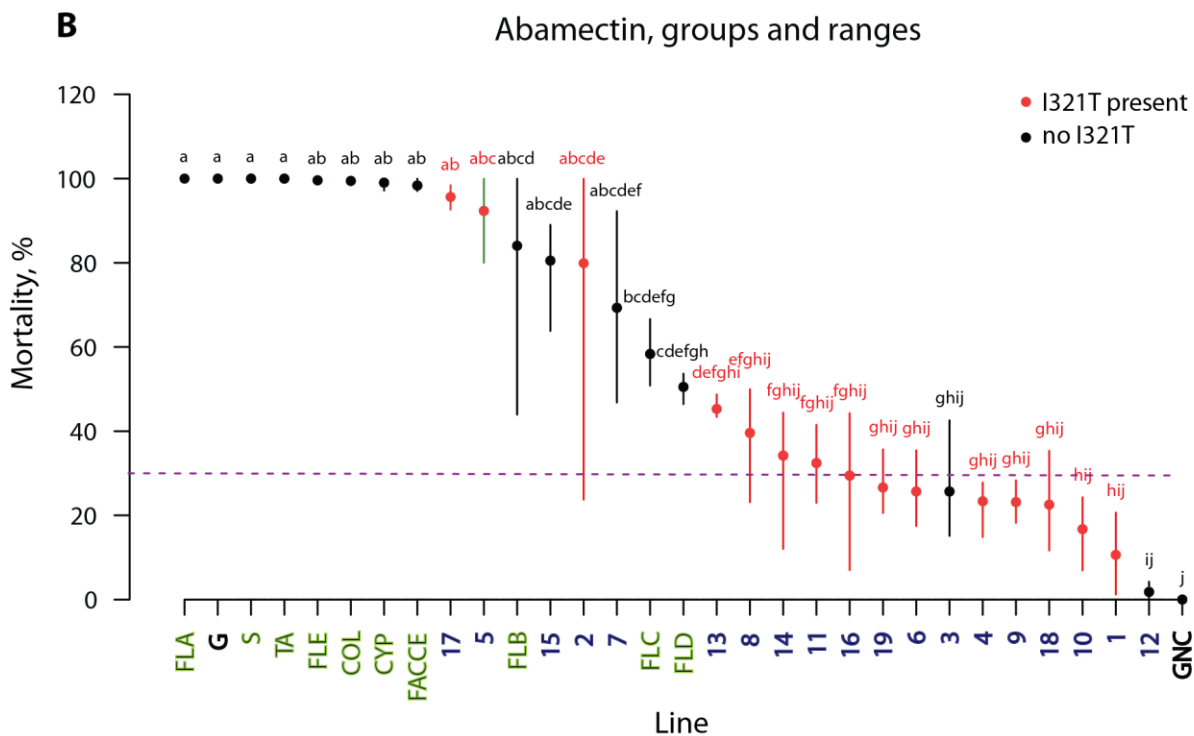
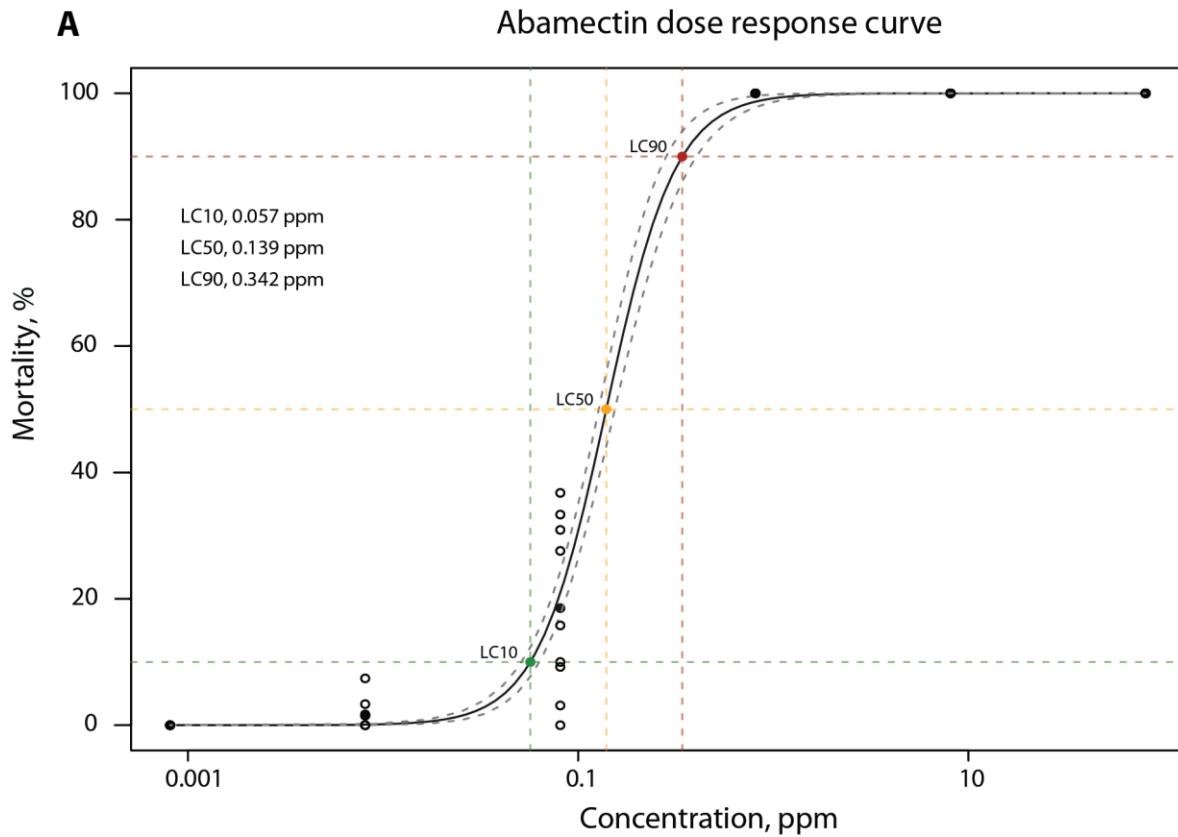


Figure 3.3 The analysis of mite susceptibility to abamectin, ‘2019’. (A) Dose-response curve showing the mortality of sensitive reference population (TU-LND) treated with serial dilution 0.0081, 0.081, 0.81, 8.1, and 81 ppm of abamectin. Shown are proportions of mites that died at tested concentrations (n = 240/trial, performed in three independent trials). (B) Adulticidal toxicity bioassay of mite populations that were reared unchallenged on beans for 9 months after their collection from greenhouses. Greenhouse mite populations (01 through 19) are shown in blue, and laboratory populations are shown in green. Populations were treated with discriminating concentration of abamectin ($LC_{90} = 0.342$ ppm) in three independent trials (n=80/trial). TU-LND (in black) was used as negative (GNC) and positive (G) controls. Shown are proportions of mites that died (as a %) \pm 95% confidence intervals (CI). The red dots with CI represent mortality of populations carrying I321T mutation in *GluCl3*. The black dots with CI represent mortality of populations that lack I321T mutation in *GluCl3*. Letters represent significant differences between means using Tukey’s HSD test at $\alpha = 0.05$.

3.2.2 Pattern of resistance to abamectin in Canadian mite populations

To test the potential involvement of target-site mutations in the abamectin resistance in Ontario TSSM greenhouse populations, GH and laboratory non-selected strains were genotyped and assessed for the presence of the G323D (*GluC11*), G326E (*GluC13*), and I321T (*GluC13*) mutations and their frequencies. As seen in Table 3.1, neither G323D or G326E SNPs were detected in either Ontario GH TSSM or laboratory strains. Their frequency was 0%. However, I321T SNP was identified in 15 out of 19 of GH strains. Strains 17 and 18 were heterozygous for the allele, with frequencies 75% and 90% respectively. All other populations were homozygous for this mutation (100% frequency) (Table 3.1). In summary, SNPs reported in literature, G323D and G326S, were not detected in Ontario GH populations, while the novel I321T SNP was found in Ontario populations at high incidence and frequencies.

Table 3.1 Abamectin resistance-associated SNPs in greenhouse-collected mite populations genotyped 4 months after their collection from greenhouses and laboratory-reared populations. The presence of abamectin resistance-associated SNP (I321T in *GluCl3*, G326E in *GluCl3* and G323D in *GluCl1*) is indicated by ‘R’ (resistant), while the absence is indicated by ‘S’ (susceptible). ‘*’ denotes heterozygosity. SNP frequency is shown as percentage of SNP in the DNA sample obtained from a pool of >50 mites.

Producer	Crop	ID	2019	
			S/R, frequency %	
			I321T	G326E, G323D
Farm 'A'	Tomato	01	R, 100	S, 0
Farm 'B'	Tomato	02	R, 100	S, 0
Farm 'A'	Pepper	03	S, 0	S, 0
Farm 'D'	Tomato	04	R, 100	S, 0
Farm 'E'	Tomato	05	R, 100	S, 0
Farm 'I'	Tomato	06	R, 100	S, 0
Farm 'I'	Tomato	07	S, 0	S, 0
Farm 'I'	Tomato	08	R, 100	S, 0
Farm 'F'	Tomato	09	R, 100	S, 0
Farm 'F'	Tomato	10	R, 100	S, 0
Farm 'C'	Tomato	11	R, 100	S, 0
Farm 'C'	Cucumber	12	S, 0	S, 0
Farm 'G'	Tomato	13	R, 100	S, 0
Farm 'D'	Tomato	14	R, 100	S, 0
Farm 'D'	Eggplant	15	S, 0	S, 0
Farm 'A'	Tomato	16	R, 100	S, 0
Farm 'B'	Tomato	17	R*, 75	S, 0
Farm 'H'	Tomato	18	R*, 90	S, 0
Farm 'I'	Tomato	19	R, 100	S, 0
Lab	Tomato	FLA	S, 0	S, 0
Lab, field	Tomato	FLB	S, 0	S, 0
Lab, field	Tomato	FLC	S, 0	S, 0
Lab, field	Tomato	FLD	S, 0	S, 0
Lab, field	Tomato	FLE	S, 0	S, 0
Lab, field	Tomato	FACCE	S, 0	S, 0
Lab	Bean	G	S, 0	S, 0
Lab	Tomato	TA	S, 0	S, 0

3.2.3 Mite resistance to abamectin is not stable over time

The selection of target site mutations in the face of pesticide selection pressure is often associated with a fitness cost, leading to the loss of the SNP frequency once acaricide selection stops (Nicastro *et al.* 2010; Wang *et al.* 2016). To check if the genetic markers were stable and present in the Ontario GH-collected (01-19) and laboratory populations over time, TSSM populations were maintained on bean without acaricide selection. The GH and laboratory populations were re-genotyped about a year later. SNPs were lost in populations that originally carried them, with frequencies being beyond the detection limit imposed by the methods used in this study (Table 3.2). These data point to the instability of the retention of mite abamectin resistance-associated SNPs over time.

Table 3.2 Comparison of abamectin resistance-associated SNPs in greenhouse-collected mite populations genotyped four months (‘2019’) and 15 months (‘2020’) after their collection from greenhouses and laboratory reared populations. The presence of abamectin resistance-associated SNP (I321T in *GluCl3*, G326E in *GluCl3* and G323D in *GluCl1*) is indicated by ‘R’ (resistant), while the absence is indicated by ‘S’ (susceptible). ‘*’ denotes heterozygosity. SNP frequency is shown in percentages.

Producer	Crop	ID	2019		2020	
			S/R, frequency %		S/R, frequency %	
			I321T	G326E, G323D	I321T	G326E, G323D
Farm 'A'	Tomato	01	R, 100	S, 0	S, 0	S, 0
Farm 'B'	Tomato	02	R, 100	S, 0	S, 0	S, 0
Farm 'A'	Pepper	03	S, 0	S, 0	S, 0	S, 0
Farm 'D'	Tomato	04	R, 100	S, 0	S, 0	S, 0
Farm 'E'	Tomato	05	R, 100	S, 0	S, 0	S, 0
Farm 'I'	Tomato	06	R, 100	S, 0	S, 0	S, 0
Farm 'I'	Tomato	07	S, 0	S, 0	S, 0	S, 0
Farm 'I'	Tomato	08	R, 100	S, 0	S, 0	S, 0
Farm 'F'	Tomato	09	R, 100	S, 0	S, 0	S, 0
Farm 'F'	Tomato	10	R, 100	S, 0	S, 0	S, 0
Farm 'C'	Tomato	11	R, 100	S, 0	S, 0	S, 0
Farm 'C'	Cucumber	12	S, 0	S, 0	S, 0	S, 0
Farm 'G'	Tomato	13	R, 100	S, 0	S, 0	S, 0
Farm 'D'	Tomato	14	R, 100	S, 0	S, 0	S, 0
Farm 'D'	Eggplant	15	S, 0	S, 0	S, 0	S, 0
Farm 'A'	Tomato	16	R, 100	S, 0	S, 0	S, 0
Farm 'B'	Tomato	17	R*, 75	S, 0	S, 0	S, 0
Farm 'H'	Tomato	18	R*, 90	S, 0	S, 0	S, 0
Farm 'I'	Tomato	19	R, 100	S, 0	S, 0	S, 0
Lab	Tomato	FLA	S, 0	S, 0	S, 0	S, 0
Lab, field	Tomato	FLB	S, 0	S, 0	S, 0	S, 0
Lab, field	Tomato	FLC	S, 0	S, 0	S, 0	S, 0
Lab, field	Tomato	FLD	S, 0	S, 0	S, 0	S, 0
Lab, field	Tomato	FLE	S, 0	S, 0	S, 0	S, 0
Lab, field	Tomato	FACCE	S, 0	S, 0	S, 0	S, 0
Lab	Bean	G	S, 0	S, 0	S, 0	S, 0
Lab	Tomato	TA	S, 0	S, 0	S, 0	S, 0

3.2.4 Non-selected TSSM populations lost abamectin resistance

I hypothesized that if SNP I321T is associated with TSSM abamectin resistance, then its loss should lead to restoration of TSSM susceptibility to abamectin. However, this would only be the case if TSSM resistance is not also arising due to the metabolic resistance that could compensate for the SNP loss. To determine the resistance state of TSSM populations at this later timepoint, the KimWipe-based accelerated toxicity bioassay was performed (9 months after the first round). The discriminating dose for the second round of the toxicity bioassays was kept the same ($LC_{90} = 0.342$ ppm), and water was used as negative control. As seen in Figure 3.4, none of the populations displayed high resistance at LC_{90} of abamectin. Out of GH populations that displayed high resistance in the first round of bioassays, strains 01, 03, 06, 12, 16, 18, and 19 displayed higher mortality in the second round by falling into intermediary mortality range, between 30 and 70%. Strains 08, 11, 13, and 14 remained in the intermediary range of mortality, between 30 and 70% mortality. GH strains 04, 09, and 10, that were highly resistant in the first round of the bioassays, became susceptible with the mortalities above 70%. Strain 07, that first displayed intermediary mortality, around 70%, became highly susceptible with the mortality around 90%. Strains 02, 05, 15, and 17 remained highly susceptible with the mortalities above 70%. Out of laboratory strains, only FLC remained in the intermediary mortality range. COL strain had decreased its susceptibility to LC_{90} and changed from being 100% susceptible to having intermediary susceptibility around 70%. The rest of the laboratory populations displayed high susceptibility, above 70% mortality. The comparison of the bioassay results obtained in 2019 and 2020 bioassays (Figure 3.5) showed that resistance levels are either similar or lower in 2020 compared to 2019. Thus, it is evident that after being non-challenged and reared on a neutral host, all TSSM populations have greater susceptibility to abamectin.

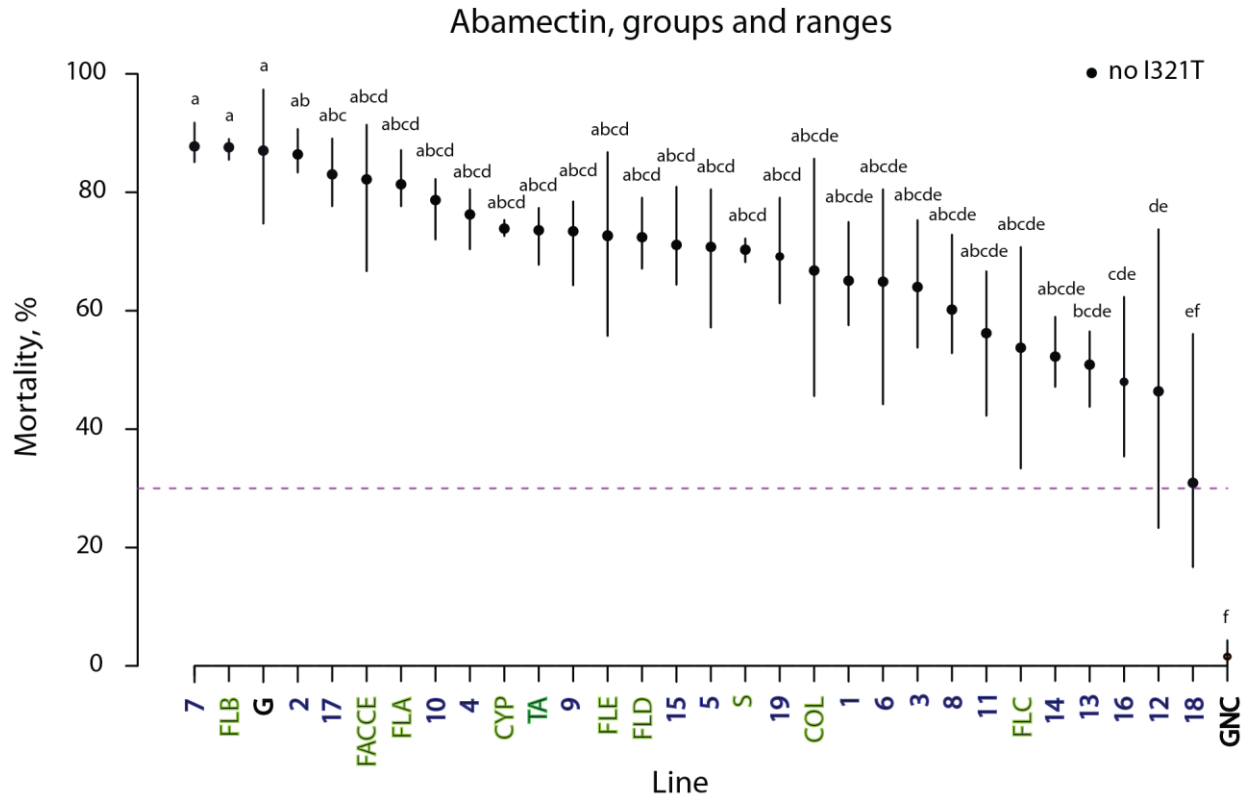


Figure 3.4 The analysis of mite susceptibility to abamectin, ‘2020’. Adulticidal toxicity bioassay of mite populations that were reared unchallenged on beans for 17 months after their collection from greenhouses. Greenhouse mite populations (01 through 19) are shown in blue, and laboratory populations are shown in green. Populations were treated with discriminating concentration of abamectin ($LC_{90} = 0.342$ ppm) in three independent trials ($n=80$ /trial). TU-LND (shown in black) was used as negative (GNC) and positive (G) controls. Shown are proportions of mites that died (as a %) \pm 95% confidence intervals (CI). The black dots with CI represent mortality of populations that lack I321T mutation in *GluCl3*. Letters represent significant differences between means using Tukey’s HSD test at $\alpha = 0.05$.

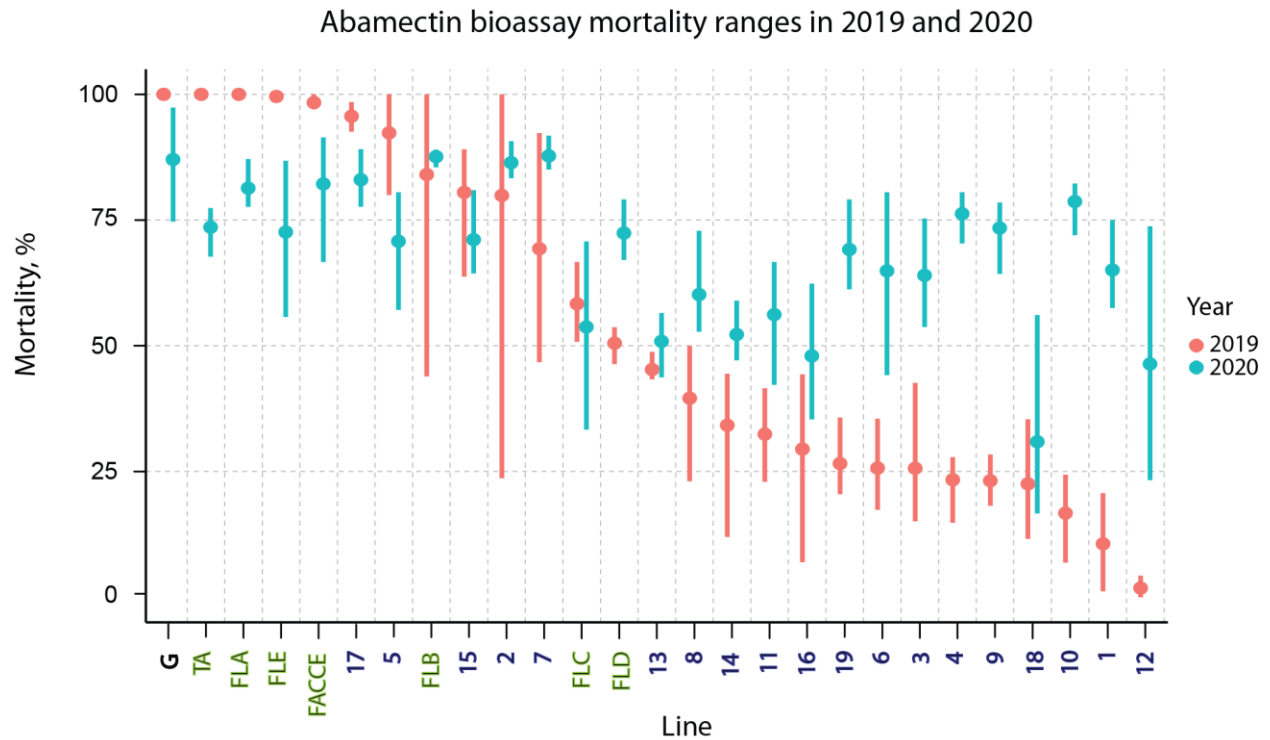


Figure 3.5 Comparison of mite susceptibility to abamectin after 8 months of maintenance in the lab without selection pressure. Adulticidal toxicity bioassays of populations that were reared unchallenged on beans for 9 months (‘2019’) and 17 months (‘2020’) after the initial collection from greenhouses and laboratory populations. Greenhouse populations (01 through 19) are shown in blue, and laboratory populations are shown in green. Red dots represent mortality values of ‘2019’ bioassay, blue dots represent mortality values of ‘2020’ bioassay. All populations were treated with discriminating concentration of abamectin ($LC_{90} = 0.342$ ppm) in three independent trials ($n=80$ /trial). TU-LND (shown in black) was used as positive (G) controls. Shown are proportions of mites that died (as a %) \pm 95% confidence intervals (CI).

3.2.5 TSSM metabolic resistance to abamectin

RT-qPCR was done to check if Ontario greenhouse-collected populations have increased expression levels of these genes relative to the reference strains. Then, the second RT-qPCR was performed on the same GH collected TSSM samples after being reared on beans without selection for about 15 months post initial collection (material ‘2020’) to determine if the expression levels of three CYPs have changed. The laboratory TU-LND strain was used as a reference.

As seen from Table 3.3, there is no clearly discernable pattern of CYP expression in the initially collected populations and the same populations after a year being reared on unchallenging beans (material '2019' and '2020', respectively). CYP392A16 expression pattern is very similar between 2019 and 2020 analyses. FLA, FLB, FLE, and FACCE laboratory populations showed downregulation of these CYPs, while the rest of the populations display the expression similar to the expression in reference population in both 2019 and 2020 analyses. CYP392D10 expression is elevated in majority of the GH-collected populations in both 2019 and 2020. Laboratory populations display expression levels similar to the reference strain in both data sets. CYP392D8 expression displays elevated expression in GH-collected populations in 2020 but not in 2019. Laboratory populations retain approximately same expression of this CYP in 2019 compared to 2020.

Table 3.3 Expression levels of CYP392A16, CYP392D10, and CYP392D8 in mite populations that were reared unchallenged on beans for 4 months ('2019') and 15 months ('2020'). Mite populations (01 through 19) were collected from greenhouses. The remaining populations were laboratory reared.

Producer	Crop	ID	2019			2020		
			CYP392A16	CYP392D10	CYP392D8	CYP392A16	CYP392D10	CYP392D8
Farm 'A'	tomato	01	1.41	5.89	1.56	0.24	4.46	3.07
Farm 'B'	tomato	02	1.37	4.18	-5.08	0.5	2.59	-16
Farm 'A'	pepper	03	3.11	10.47	ND	0.66	7.27	0.68
Farm 'D'	tomato	04	1.60	5.17	-10.50	0.25	5.61	0.21
Farm 'E'	tomato	05	0.82	4.70	-3.99	-0.25	5.16	0.54
Farm 'I'	tomato	06	1.75	6.28	1.36	-0.3	4.6	1.68
Farm 'I'	tomato	07	-0.37	6.13	ND	-0.09	5.56	-1.16
Farm 'I'	tomato	08	-0.96	1.68	-1.40	0.32	4.37	4.9
Farm 'F'	tomato	09	0.51	5.07	-11.18	-1.13	5.43	-1.32
Farm 'F'	tomato	10	-0.31	3.15	-1.69	-0.42	1.73	-0.99
Farm 'C'	tomato	11	-1.16	1.97	-1.46	0.75	6.95	5.94
Farm 'C'	cucumber	12	1.5	8.4	4.8	0.72	6.42	4.56
Farm 'G'	tomato	13	-0.12	3.41	1.37	-0.09	6.37	5.67
Farm 'D'	tomato	14	1.14	4.56	0.40	0.8	7.47	5.45
Farm 'D'	eggplant	15	-1.08	5.40	-10.52	0.34	4.83	3.58
Farm 'A'	tomato	16	1.04	4.64	2.42	0.07	5.25	4.57
Farm 'B'	tomato	17	0.48	4.43	-1.93	0.17	3.85	-16
Farm 'H'	tomato	18	2.27	8.09	-1.24	0.54	3.77	5.48
Farm 'I'	tomato	19	1.41	5.53	1.78	-0.23	3.93	4.63
Lab	tomato	FACCE	-13.05	2.19	ND	-16	-0.63	-16
Lab	tomato	FLA	-13.44	0.93	ND	-12.95	-1.31	-9.29
Lab	tomato	FLB	-13.02	0.60	ND	-2.73	1.86	2.52
Lab	tomato	FLC	2.05	1.30	4.42	-0.92	3.59	3.66
Lab	tomato	FLD	0.72	0.57	5.30	-0.97	3.24	4.56
Lab	tomato	FLE	-3.64	1.11	0.23	-14.69	-0.94	-16
Lab	bean	G	0.00	0.00	0.00	0	0	0
AAFC	bean	S	NA	NA	NA	NA	NA	NA
Lab	tomato	TA	2.69	1.77	5.46	NA	NA	NA

3.3 Bifenazate

3.3.1 Ontario greenhouse-collected *Tetranychus urticae* populations display partial resistance to bifenazate

The reference, susceptible TU-LND strain was used for testing the range of bifenazate concentrations (0.0113, 0.113, 0.226, 0.45, 0.75, 1.13, and 2.26 ppm; the field dose of bifenazate is 75 ppm). At low bifenazate concentrations the mortality of TU-LND mites was not affected, however, mite mortality reached 100% at high doses (Figure 3.8a). This indicates that bifenazate concentrations tested are suitable to identify the LC₉₀, a concentration that causes 90% mortality of the reference TU-LND population. The negative control consisting of water treated TU-LND resulted in nearly 0% mortality, accounting for natural mortality, thus, the protocol execution did not negatively affect mite fitness. Using the Probit Analysis of dose response data, the TU-LND LC₉₀ to bifenazate was determined to be 1.573 ppm. This concentration was subsequently used as a discriminatory dose to determine the bifenazate resistance status of all populations with 30% threshold that defined resistance status of populations. As seen in Figure 3.8b, the mortality of water-treated TU-LND (water control GNC) was around 0%, as expected, with the mortality stemming from natural causes. Consistent with the dose responses shown in Figure 3.8a, the mortality of TU-LND reference population (G) treated with LC₉₀ bifenazate concentration was around 100%. Only 4 GH-collected populations displayed the resistance to bifenazate with mortalities below 30%. 11 GH-collected populations had their resistance in the intermediary range, between 30 and 70% mortality. Four GH-collected populations were highly susceptible to bifenazate with mortalities above 70%. Three laboratory strains displayed intermediary resistance, while the rest of them were highly susceptible to bifenazate. To conclude, moderate incidence of bifenazate resistance was observed in Ontario GH-collected populations with about 20% of populations being bifenazate resistant.

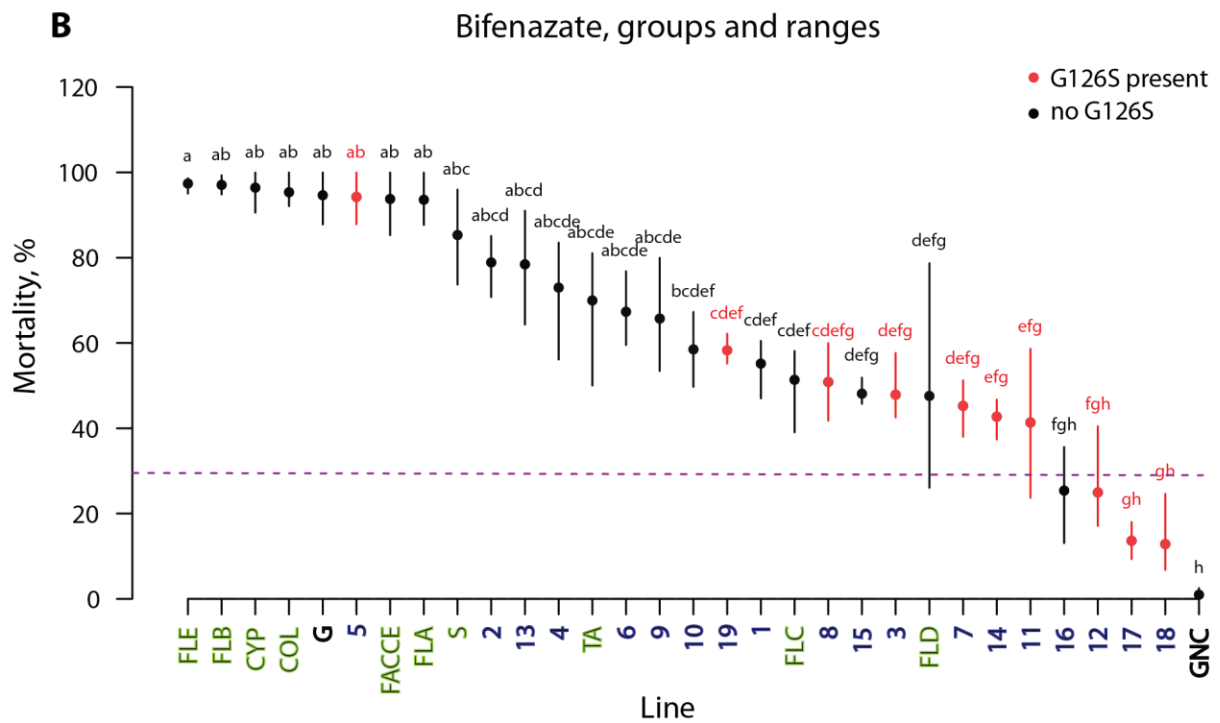
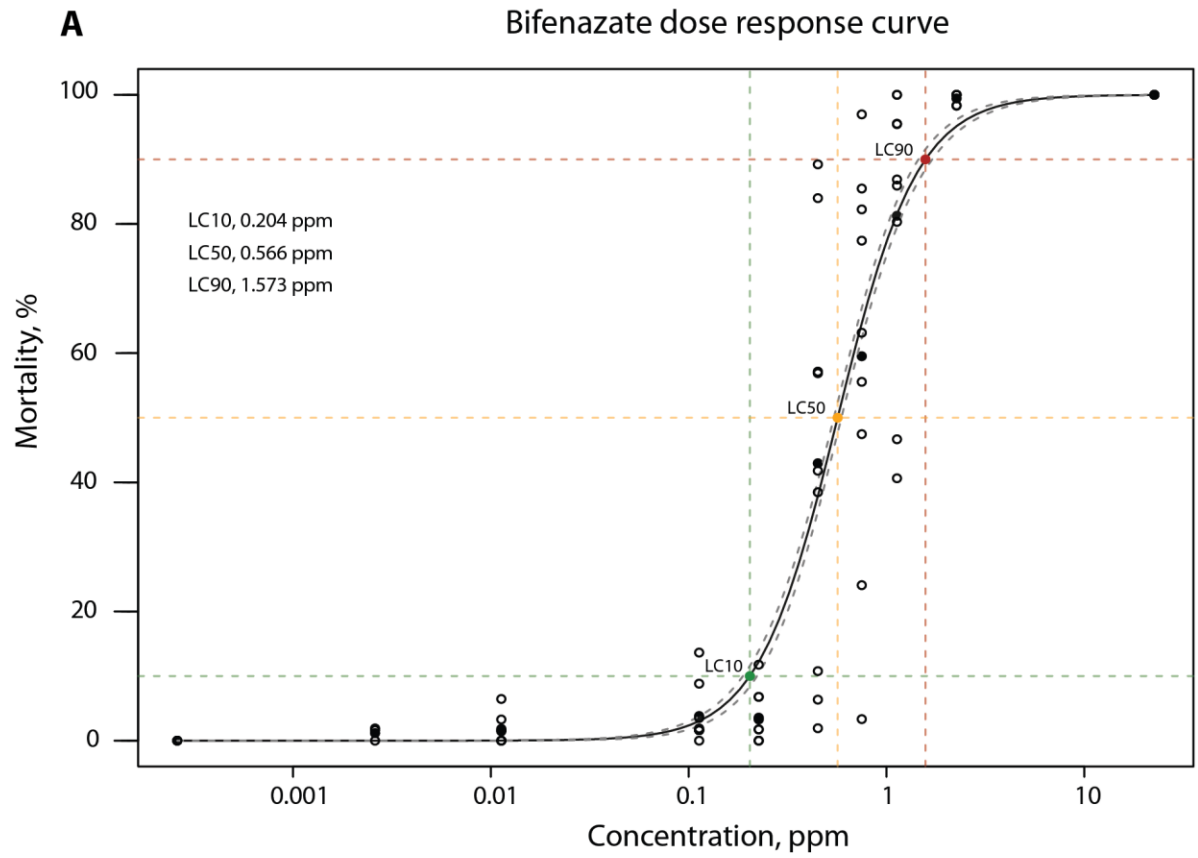


Figure 3.6 The analysis of mite susceptibility to bifenthrin. (A) Dose-response curve showing the mortality of sensitive reference population (TU-LND) treated with serial dilution 0.0113, 0.113, 0.226, 0.45, 0.75, 1.13, and 2.26 ppm of bifenthrin. Shown are proportions of mites that died at tested concentrations (n = 240/trial, performed in 3 independent trials). (B) Adulticidal toxicity bioassay of mite populations that were reared unchallenged on beans for 10 months after their collection from greenhouses. Greenhouse mite populations (01 through 19) are shown in blue, and laboratory populations are shown in green. Populations were treated with discriminating concentration of bifenthrin ($LC_{90} = 1.573$ ppm) in three independent trials (n=80/trial). TU-LND (in black) was used as negative (GNC) and positive (G) controls. Shown are proportions of mites that died (as a %) \pm 95% confidence intervals (CI). The red dots with CI represent mortality of populations carrying G126S mutation in *cytB*. The black dots with CI represent mortality of populations that lack G126S mutation in *cytB*. Letters represent significant differences between means using Tukey's HSD test at $\alpha = 0.05$.

3.3.2 Ontario greenhouse-collected *Tetranychus urticae* populations do not carry SNPs associated with bifentazate resistance

To examine the pattern and frequencies of the reported SNPs in Ontarian populations, GH and laboratory non-selected strains were genotyped and analyzed for the presence of the SNPs and their frequencies. Ontario GH-collected and laboratory populations were screened for the presence of G126S, I136T, S141F, D161G, and P262T mutations in *cytB* (Table 3.4). The genotyping showed the lack of I136T, S141F, D161G, and P262T SNPs in both Ontario GH TSSM and laboratory strains, as mutant alleles were below detectable frequencies. G126S, however, was present in GH strains 03, 05, 07, 08, 11, 12, 14, 17, 18, and 19. Strains 17 and 19 carried heterozygous S/R allele with frequencies 55/45% both, while the other populations were homozygous for this mutation with frequencies of 75% for strains 05 and 18 and 100% for the rest of the resistance strains. In summary, none of the SNPs reported in literature, except for G126S, were detected in Ontario GH populations. The presence of G126S is low.

Table 3.4 Bifenazate resistance-associated SNPs of greenhouse-collected mite populations genotyped four months after the collection from greenhouses and laboratory-reared populations. Following mutations were genotyped: G126S, I136T, S141F, D161G, P262T in mitochondrial complex III at Q_o site in *cytB* gene. The presence of a SNP is indicated by ‘R’ (resistant), while the absence is indicated by ‘S’ (susceptible). ‘S/R’ represents heterozygous strains. SNP frequency is shown in percentages.

Producer	Crop	ID	S/R, frequency %	
			G126S	I136T, S141F, D161G, P262T
Farm 'A'	Tomato	01	S, 0	S, 0
Farm 'B'	Tomato	02	S, 0	S, 0
Farm 'A'	Pepper	03	R, 100	S, 0
Farm 'D'	Tomato	04	S, 0	S, 0
Farm 'E'	Tomato	05	R, 75	S, 0
Farm 'I'	Tomato	06	S, 0	S, 0
Farm 'I'	Tomato	07	R, 100	S, 0
Farm 'I'	Tomato	08	R, 100	S, 0
Farm 'F'	Tomato	09	S, 0	S, 0
Farm 'F'	Tomato	10	S, 0	S, 0
Farm 'C'	Tomato	11	R, 100	S, 0
Farm 'C'	Cucumber	12	R, 100	S, 0
Farm 'G'	Tomato	13	NA	S, 0
Farm 'D'	Tomato	14	R, 100	S, 0
Farm 'D'	Eggplant	15	S, 0	S, 0
Farm 'A'	Tomato	16	S, 0	S, 0
Farm 'B'	Tomato	17	S/R, 55/45	S, 0
Farm 'H'	Tomato	18	R, 75	S, 0
Farm 'I'	Tomato	19	S/R, 55/45	S, 0
Lab	Tomato	FLA	S, 0	S, 0
Lab, field	Tomato	FLB	S, 0	S, 0
Lab, field	Tomato	FLC	S, 0	S, 0
Lab, field	Tomato	FLD	S, 0	S, 0
Lab, field	Tomato	FLE	S, 0	S, 0
Lab, field	Tomato	FACCE	S, 0	S, 0
Lab	Bean	G	S, 0	S, 0
Lab	Bean	S	S, 0	S, 0
Lab	Tomato	TA	S, 0	S, 0

3.4 Abamectin and bifenthrin resistant populations display multiresistance

The mortality data from abamectin and bifenthrin toxicity bioassays were used for establishing multiresistance status of populations. Three populations (12, 16, 18) were found to be resistant to both acaricides ($R^2=0.3177$), while the majority of populations were susceptible to both acaricides (Figure 3.9). In conclusion, Ontario GH-collected populations display cases of multiresistance between acaricides.

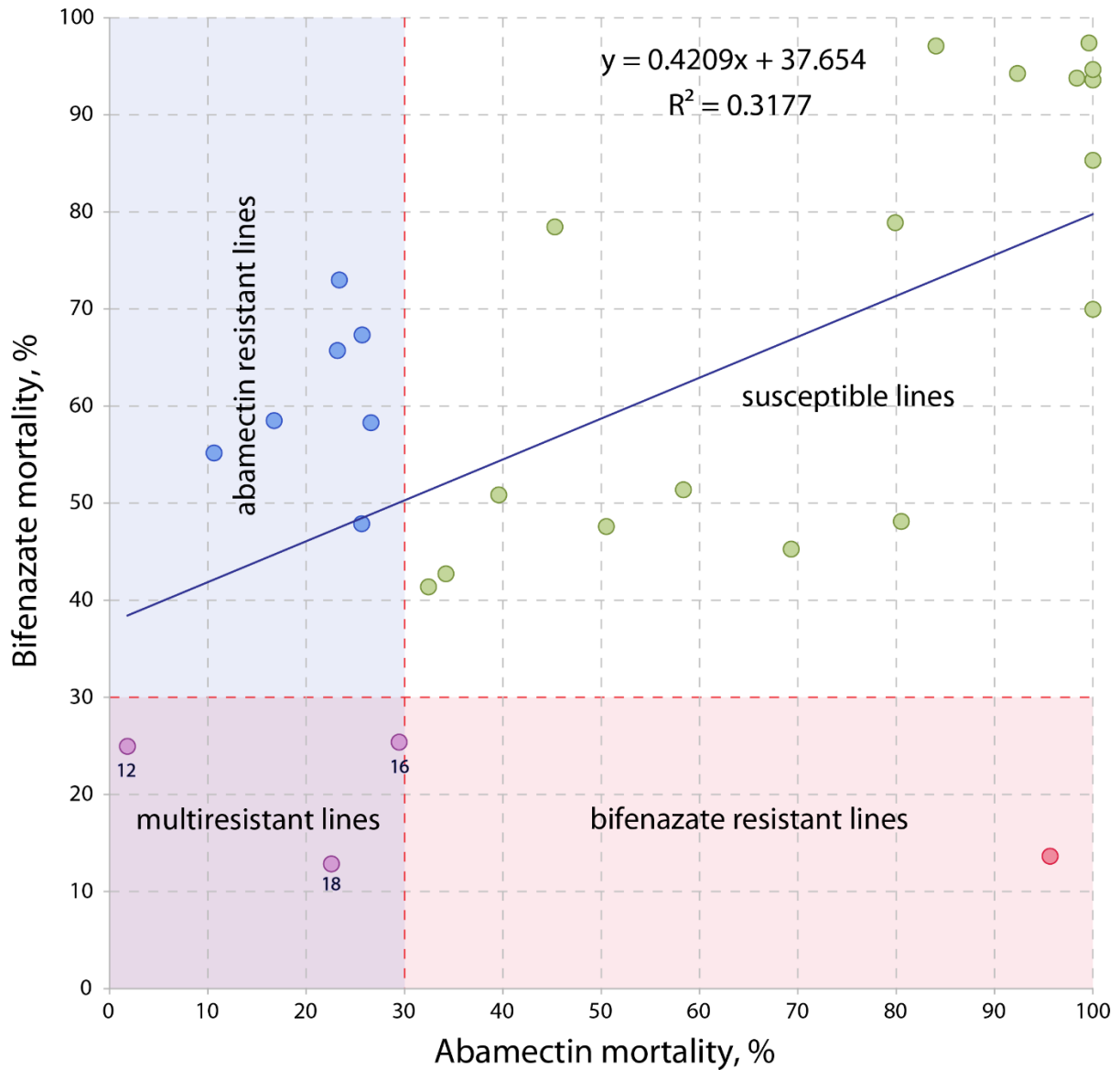


Figure 3.7 Analysis of multiresistance between abamectin and bifenazate in Ontario greenhouse-collected and laboratory TSSM populations. Green dots represent susceptible GH and laboratory populations. Blue dots represent lines that are resistant only to abamectin. Red dot represents bifenazate only resistant line. Purple dots represent lines resistant to both abamectin and bifenazate. A linear regression line is shown with the blue line.

3.5 Etoxazole

3.5.1 Majority of Ontario greenhouse-collected *Tetranychus urticae* populations carry I1017F SNP associated with etoxazole resistance

To examine the pattern and presence and the frequencies of this reported SNP in Ontarian populations, GH and laboratory non-selected strains were genotyped and analyzed. As seen from Table 3.5, the genotyping revealed that all but four Ontario GH-collected populations carry I1017F mutation in *CHSI* gene at very high frequencies, while the frequency of this mutation in laboratory populations and four GH-collected populations were undetectable. To conclude, despite the late introduction and overall limited use of etoxazole, Ontario GH-collected populations possess I1017F mutation at high frequency in line with other reported cases of etoxazole resistance globally.

Table 3.5 Etoazole resistance-associated SNP of greenhouse-collected populations genotyped four months after the collection from greenhouses and laboratory reared populations. Substitution mutation genotyped was I1017F found in *CHS1*. The presence of SNP is indicated by ‘R’ (resistant), while the absence is indicated by ‘S’ (susceptible).

			2019
			S/R, frequency %
Producer	Crop	ID	I1017F
Farm 'A'	Tomato	01	R, 100
Farm 'B'	Tomato	02	R, 100
Farm 'A'	Pepper	03	S
Farm 'D'	Tomato	04	R, 100
Farm 'E'	Tomato	05	R, 100
Farm 'I'	Tomato	06	R, 100
Farm 'I'	Tomato	07	S
Farm 'I'	Tomato	08	R, 100
Farm 'F'	Tomato	09	R, 100
Farm 'F'	Tomato	10	R, 100
Farm 'C'	Tomato	11	R, 100
Farm 'C'	Cucumber	12	S
Farm 'G'	Tomato	13	R, 100
Farm 'D'	Tomato	14	R, 100
Farm 'D'	Eggplant	15	S
Farm 'A'	Tomato	16	R, 100
Farm 'B'	Tomato	17	R, 100
Farm 'H'	Tomato	18	R, 100
Farm 'I'	Tomato	19	R, 100
Lab	Tomato	FLA	S
Lab, field	Tomato	FLB	S
Lab, field	Tomato	FLC	S
Lab, field	Tomato	FLD	S
Lab, field	Tomato	FLE	S
Lab, field	Tomato	FACCE	S
Lab	Bean	G	S
Lab	Tomato	TA	S

3.5.2 Ontario greenhouse-collected *Tetranychus urticae* populations display phenotypic sensitivity to etoxazole

To establish reference data, a reference susceptible TU-LND strain was used for testing the range of etoxazole concentrations, 10x dilution from 5 ppm to 0.00005 ppm; the field dose of etoxazole is listed as a range between 30 and 60 ppm.

At low etoxazole concentrations the hatchability of TU-LND eggs was not affected, however, at high doses, egg mortality reached 100% (Figure 3.10a). This indicates that etoxazole concentrations tested are suitable to identify the LC₉₀, the concentration that renders 90% of eggs inviable. The negative control consisting of water treated TU-LND resulted in close to 100% egg hatchability; this indicates that the protocol execution did not negatively affect egg hatchability (not shown). Using the Probit Analysis of dose response data, the TU-LND LC₉₀ to etoxazole was determined to be 0.0196 ppm (with field dose ranging 30-60 ppm). This concentration was subsequently used as a discriminatory dose to determine the etoxazole resistance status of Ontario GH and laboratory populations. The resistance to etoxazole was inferred if <30% of eggs did not hatch in response to the LC₉₀ discriminating etoxazole concentration. The hatchability of water-treated TU-LND eggs was around 100%, as expected (not shown). Consistent with the dose responses shown in Figure 3.10a, the mortality of eggs of TU-LND reference populations (G) treated with LC₉₀ etoxazole concentration was close to 100%. Only two GH-collected and three laboratory populations displayed intermediary resistance to etoxazole, between 30 and 70% mortality (Figure 3.10b). The rest of GH-collected and laboratory populations were highly susceptible with mortalities above 70% (Figure 3.10b). To conclude, all Ontario GH-collected populations display phenotypic sensitivity to etoxazole.

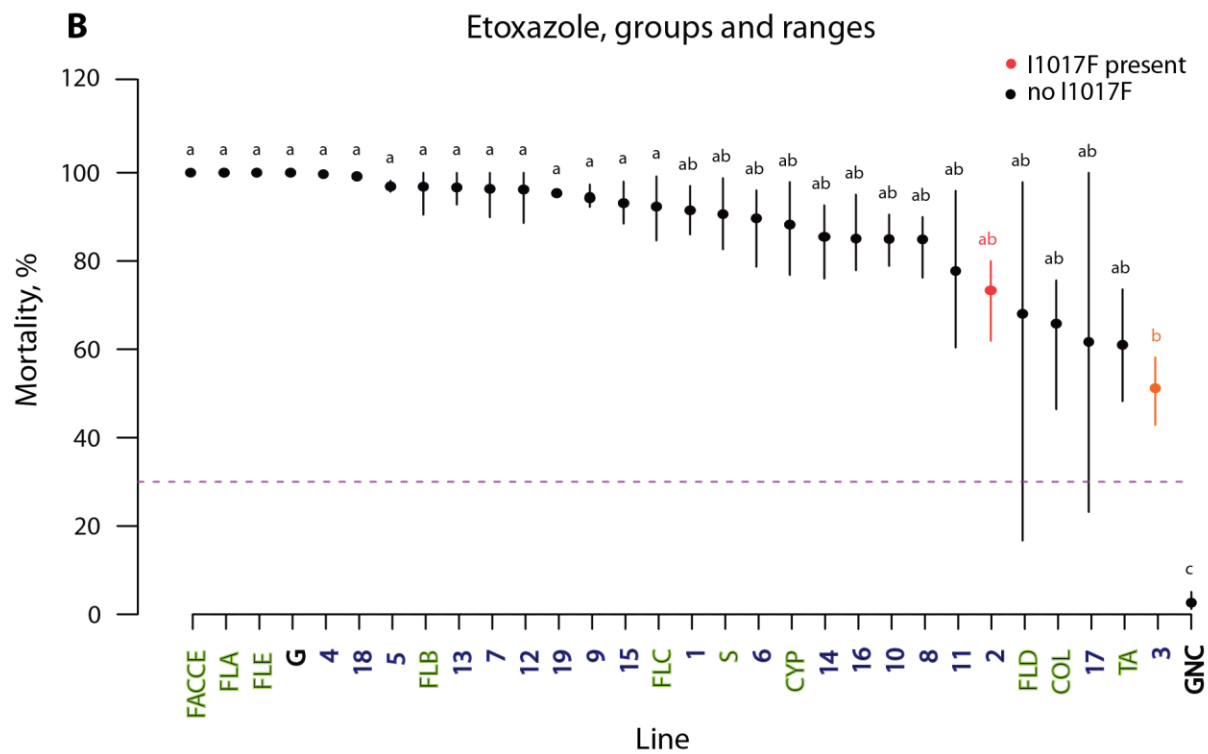
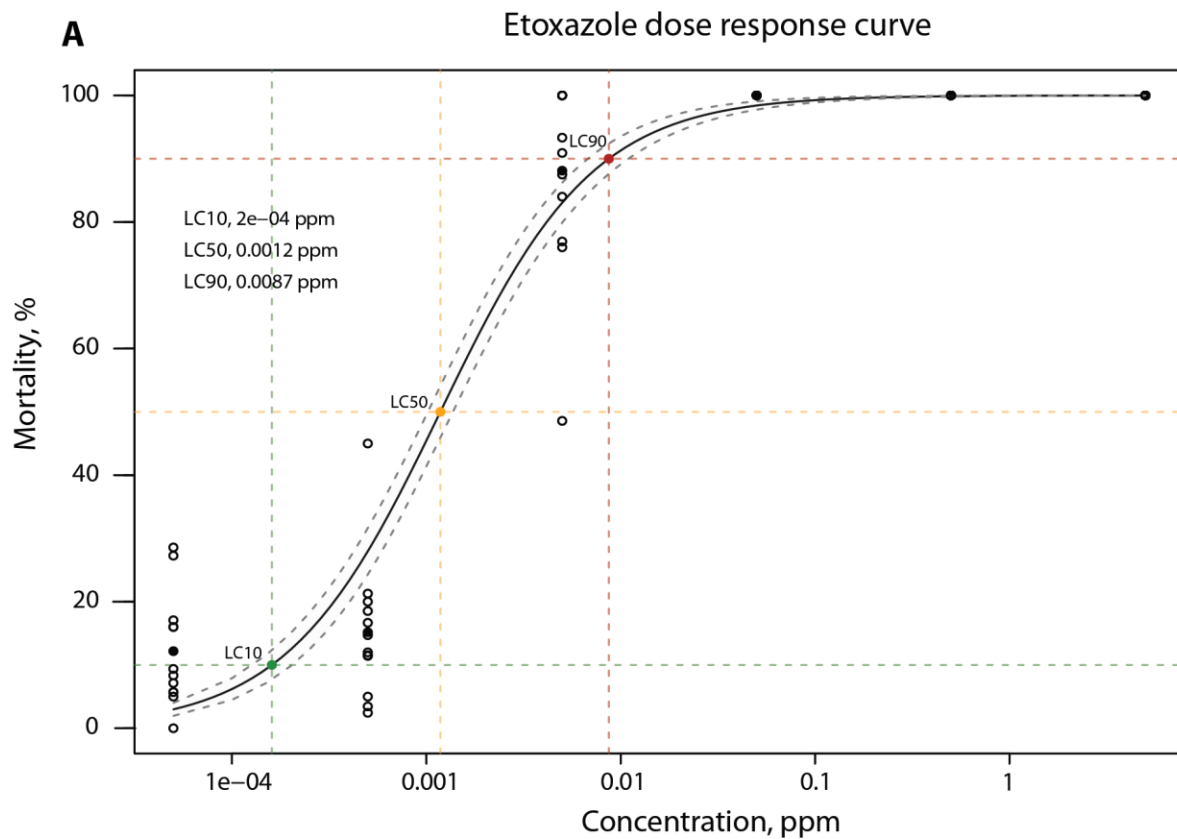


Figure 3.8 The analysis of mite susceptibility to etoxazole. (A) Dose-response curve showing the mortality of sensitive reference population (TU-LND) treated with serial dilution 0.00005, 0.0005, 0.005, 0.05, 0.5, and 5 ppm of etoxazole. Shown are proportions of eggs that did not hatch at tested concentrations (n = 60 adult females/trial, performed in three independent trials). (B) Ovicidal toxicity bioassay of mite populations that were reared unchallenged on beans for 14 months after their collection from greenhouses. Greenhouse mite populations (01 through 19) are shown in blue, and laboratory populations are shown in green. Populations were treated with discriminating concentration of etoxazole ($LC_{90} = 0.0196$ ppm) in three independent trials (n=60/trial). TU-LND (in black) was used as positive (G) control. Shown are proportions of eggs that did not hatch (as a %) \pm 95% confidence intervals (CI). The red dots with CI represent egg mortality of populations carrying I1017F mutation in *CHS1* based on genotyping '2020' (Table 3.6). The black dots with CI represent egg mortality of populations that lack I1017F mutation in *CHS1* based on genotyping '2020' (Table 3.6). Letters represent significant differences between means using Tukey's HSD test at $\alpha = 0.05$.

3.5.3 Repeated genotyping points to the loss of etoxazole resistance associated I1017F SNP in Ontario greenhouse-collected *Tetranychus urticae* populations

As seen from bioassays done on Ontario GH populations, the presence of SNP in 15 Ontario GH-collected populations did not coincide with these populations being phenotypically resistant to etoxazole. However, 14 months passed between sample collection for genotyping and the bioassay, so there was a possibility that the frequency of the TSSM populations changed meanwhile and that the informative SNP was being lost. Thus, the populations were re-genotyped about a year after the initial genotyping, and about three months after the bioassays were performed. During the year between genotyping analyses, the populations were maintained on beans without selection pressure.

As seen from the Table 3.6, I1017F SNP was lost in nearly all but two populations that used to carry it previously. The frequency of the SNP was undetectable in all GH-collected populations, except for populations 02 and 03. In conclusion, it is evident that Ontario GH-collected populations have lost the SNP associated with etoxazole resistance after a year of being unchallenged with the acaricide. These genotyping data are consistent with the results of the bioassays, so the phenotypic etoxazole susceptibility status coincides with the loss of the SNP.

Table 3.6 Side-by-side comparison of the presence of etoxazole associated SNP in greenhouse-collected mite populations genotyped four months ('2019') and 17 months ('2020') after their collection from greenhouses and laboratory reared populations. Substitution mutation genotyped was I1017F found in *CHS1*. The presence of SNP is indicated by 'R' (resistant), while the absence is indicated by 'S' (susceptible). '*' denotes heterozygosity. SNP frequency is shown in percentages.

Producer	Crop	ID	2019	2020
			S/R, frequency %	
			I1017F	I1017F
Farm 'A'	Tomato	01	R, 100	S
Farm 'B'	Tomato	02	R, 100	R*, 50
Farm 'A'	Pepper	03	S	R*, 30
Farm 'D'	Tomato	04	R, 100	S
Farm 'E'	Tomato	05	R, 100	S
Farm 'I'	Tomato	06	R, 100	S
Farm 'I'	Tomato	07	S	S
Farm 'I'	Tomato	08	R, 100	S
Farm 'F'	Tomato	09	R, 100	S
Farm 'F'	Tomato	10	R, 100	S
Farm 'C'	Tomato	11	R, 100	S
Farm 'C'	Cucumber	12	S	S
Farm 'G'	Tomato	13	R, 100	S
Farm 'D'	Tomato	14	R, 100	S
Farm 'D'	Eggplant	15	S	S
Farm 'A'	Tomato	16	R, 100	S
Farm 'B'	Tomato	17	R, 100	S
Farm 'H'	Tomato	18	R, 100	S
Farm 'I'	Tomato	19	R, 100	S
Lab	Tomato	FLA	S	S
Lab, field	Tomato	FLB	S	S
Lab, field	Tomato	FLC	S	S
Lab, field	Tomato	FLD	S	S
Lab, field	Tomato	FLE	S	S
Lab, field	Tomato	FACCE	S	S
Lab	Bean	G	S	S
Lab	Tomato	TA	S	S

4.0 Discussion

Safe and sustainable agricultural production is hindered not only by the fast growth of human population but also ongoing global warming that will change the occurrence as well as the geographical distribution of agricultural pests. It is projected that the two-spotted spider mite (TSSM), *Tetranychus urticae* (Koch) will migrate further north with the increasing temperatures, becoming an invasive species in places where it has not been before. Even though there is the potential for successful biocontrol of TSSM using natural predators such as *Amblyseius californicus* (McGregor), *Stethorus punctillum* (Weise), *Conwentzia psociformis* (Curtis), and *Phytoseiulus macropilis* (Garcia-Mari and Gonzalez-Zamora 1999; Oliveira *et al.* 2007), chemical pesticides are the “go-to” option against TSSM. However, frequent use of acaricides contributes to the development of acaricide resistance, and multi-resistance. There are two main mechanisms of pesticide resistance and multiresistance under investigation in this study: target site mutations (SNPs) and metabolic resistance. While the mechanism of cross-resistance development is beyond the scope of this research, I was interested in the implications of multiresistance with regards to the development of a pesticide resistance screening tool. There are multiple documented cases of multiresistance based on SNP genotyping in the literature (Demaeght *et al.* 2014; Ferreira *et al.* 2014; Sato *et al.* 2005; Van Leeuwen *et al.* 2006; Van Nieuwenhuysse *et al.* 2009; Yorulmaz and Ay 2009) that were used as sources of information to this end.

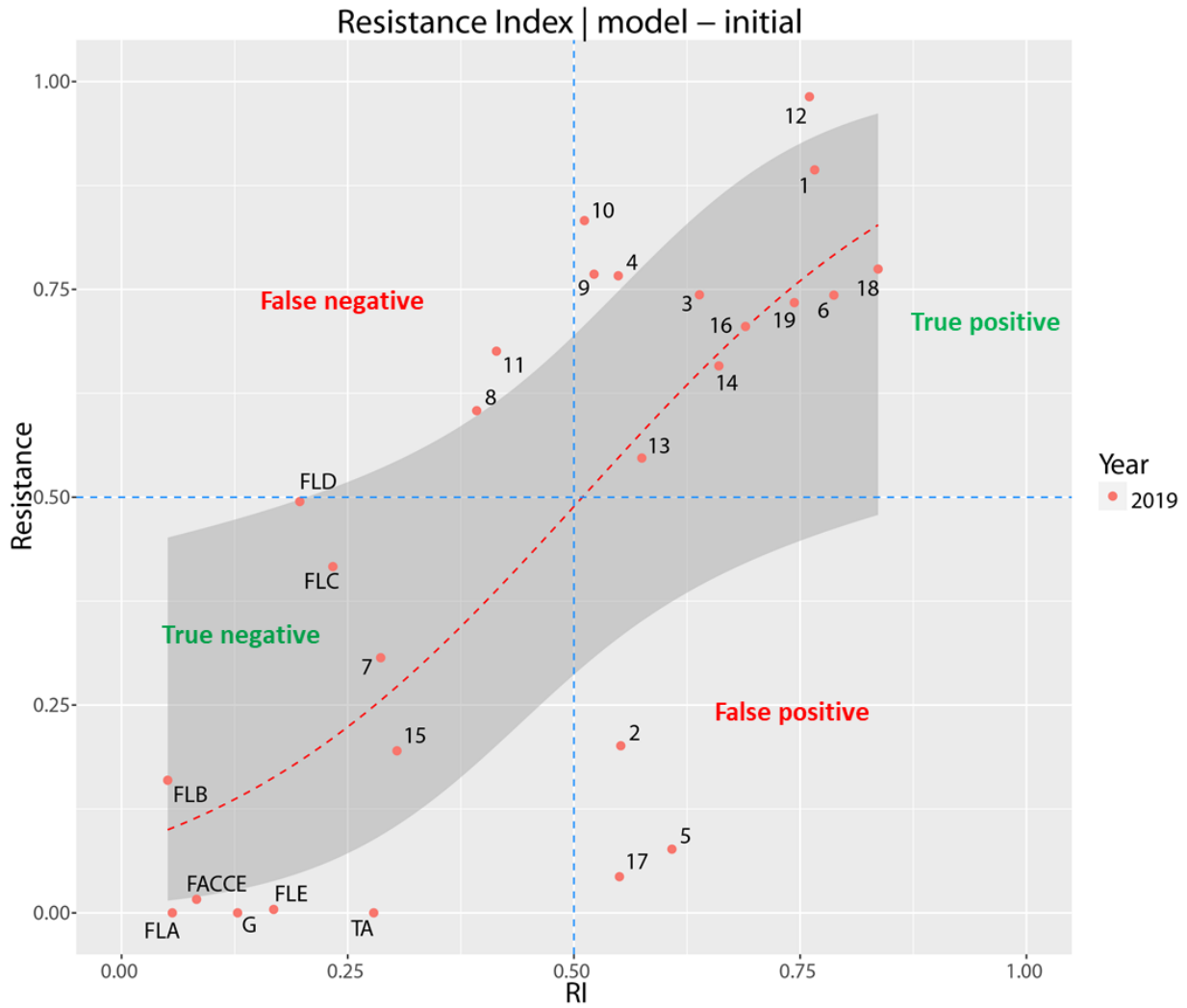
Based on results of SNPs genotyping in previous studies, resistance mechanisms are dependent on two main factors: geographical location and the past selection history including host plants and acaricide exposure of a TSSM population. Because there are no resistance-associated genotyping data for Canadian TSSM populations, I examined 19 populations from Ontario collected from various vegetable-producing greenhouses and compared the SNPs presence and frequencies to the populations collected from different locations and with different selection histories.

From a geographical point of view, TSSM population collected from different geographical locations display different patterns of abamectin resistance. To illustrate, TSSM from a rose greenhouse in Greece (Dermauw *et al.* 2012) and field-collected (watermelon, melon, eggplant, and tomato) TSSM populations from China (Xu *et al.* 2018) displayed high abamectin resistance and carried two of the SNPs associated with abamectin resistance: G323D and G326E found in

GluCl1 and *GluCl3* respectively. In contrast, Turkish vegetable (bean and cucumber) greenhouse-collected populations (Cagatay *et al.* 2018), Pacific North-West USA populations (Adesanya *et al.* 2018; Piraneo *et al.* 2015; Wu *et al.* 2019) and a Greek gypsum greenhouse-collected population (Papapostolou *et al.* 2021) did not carry any of the aforementioned SNPs despite showing a highly resistant abamectin phenotype. Furthermore, Papapostolou *et al.* (2021) identified another novel SNP, I321T in *GluCl3*, that has been linked to abamectin resistance. Xue *et al.* (2020) examined European field populations and showed that G323D and G326E were found in abamectin resistant populations collected from fields in UK, Italy and Spain but not from Belgium, and the novel I321T marker was found in one of the resistant populations from Spain. Based on the survey of target site mutations for genotypic resistance to abamectin and toxicity bioassays for phenotypic resistance to abamectin, it was found that Ontarian populations did not show segregation of G323D and G326E SNPs, yet they displayed high frequency of I321T target site mutation that aligned with phenotypic resistance of these populations well (Figure 3.3; Table 3.1). That is, the presence of high frequency of I321T mutation coincided with abamectin resistance and vice versa. However, there were some discrepancies in the correlation. Populations 03 and 12 that did not have the SNP were very resistant, 17, 05 and 02 had the SNP but were very susceptible (Figure 3.3). This suggests that there must be other factor(s) contributing to abamectin resistance. In fact, abamectin resistance is polygenic in nature. For example, not only is target site insensitivity (SNP) involved in abamectin resistance but also increase in detoxification through metabolism. Note that metabolic resistance could be triggered by mite responses to the plant host and may not reflect the selection against the pesticide itself (Van Leeuwen *et al.* 2013). Riga *et al.* (2014) investigated Greek TSSM populations and found overexpression of three cytochrome P450 genes *CYP392A16*, *CYP392D8*, and *CYP392D10* in an abamectin resistant population. The *in vitro* study concluded that *CYP392A16* can metabolize abamectin to a less toxic form and is involved in abamectin resistance. Piraneo *et al.* (2015) showed that abamectin resistant Pacific North-West USA populations overexpressed *CYP392D8*, suggesting a potential role in abamectin resistance as well. Xue *et al.* (2020) have also reported a possibility of *CYP392D8* contributing to metabolic resistance of European field populations. Xu *et al.* (2020), using RNAi silencing of cytochrome P450 genes in Chinese abamectin resistant populations, showed that *CYP392D8*, *CYP389C10*, *CYP392A11*, and *CYP392A12*, play a role in abamectin metabolic resistance. I looked at *CYP392A16*, *CYP392D8*, and *CYP392D10* that were reported as genes potentially associated with the abamectin resistance

at the time of the experimental work for this study. Although Riga *et al.* (2014) reported *CYP392A16* as the promising metabolic marker, this P450 did not show any correlation with the resistance status of Ontarian populations (Table 3.3). The expression levels of *CYP392D8* and *CYP392D10* were more informative, displaying correlation with the susceptibility of Ontarian populations.

The collected data contributed to a model that would be able to support growers' decision on what pesticide to apply in each season because growers lack a reliable decision support tool so far. Based on data that I have generated, Dr. Vladimir Zhurov and Prof. Dan Lizotte generated a model that integrated collected data. It is referred to as the Resistance Index (RI). RI is a value that is able to predict whether a population is resistant or susceptible to a given pesticide. For abamectin resistance, it is based on following data: a) genotype at I321T - *GluC13* SNP; b) the gene expression for *CYP392A16*, *CYP392D10*, and *CYP392D8*, and c) TSSM mortality in abamectin bioassays. Based on the data obtained from 2019 populations, a generalized linear model was created (Figure 4.1). The data obtained from 2020 populations were tested using this model (Figure 4.2). An RI response scale ranges from 0 to 1. Populations with RI over 0.5 are considered to be resistant while populations with RI below 0.5 are considered to be susceptible. The biggest concern of such a model are false negative outcomes, when populations are predicted to be susceptible, yet in the reality they are resistant. As seen from both graphs (Figures 4.1 and 4.2, this model is quite robust as very few populations out of 31 tested falls under false negative category. Sensitivity, specificity, and accuracy are very high.



Sensitivity

True positive / Condition positive = 12 / 14 = 0.86

Specificity

True negative / Condition negative = 10 / 13 = 0.77

Accuracy

True outcomes / All outcomes = 22 / 27 = 0.82

Figure 4.1 Resistance Index (RI) of greenhouse and laboratory populations based on 2019 analysis. RI was calculated based on mortality data obtained from toxicity bioassays, the expression of *CYP392A16*, *CYP392D8*, *CYP392D10* and the presence of SNP in *GluCl3* (I321T). The analysis of these parameters was performed in 2019 (red dots). The data are shown on a response scale. True negative populations are predicted to be susceptible. True positive populations are predicted to be resistant. False negative populations are predicted to be susceptible, even though they are resistant. False positive populations are predicted to be resistant, even though they are susceptible.



Sensitivity

True positive / Condition positive = 14 / 17 = 0.82

Specificity

True negative / Condition negative = 28 / 35 = 0.8

Accuracy

True outcomes / All outcomes = 42 / 52 = 0.807

Figure 4.2 Resistance Index (RI) of greenhouse and laboratory populations based on 2019 and 2020 data analyses. RI was calculated based on mortality data obtained from toxicity bioassays, the expression of *CYP392A16*, *CYP392D8*, *CYP392D10*, and the presence of SNP in *GluCl3* (I321T). The analysis of these parameters was performed in 2019 (red dots) and 2020 (blue dots). The data are shown on a response scale. True negative populations are predicted to be susceptible. True positive populations are predicted to be resistant. False negative populations are predicted to be susceptible even though they are resistant. False positive populations are predicted to be resistant even though they are susceptible.

Further, the predictability of P450 genes reported in Xu *et al.* (2020) were subsequently investigated in greenhouse-collected TSSM populations by other members of Grbic group. Their expression appears as a good predictor of TSSM resistance to abamectin in conjunction to the target site I321T SNP. Thus, the results presented here demonstrate the need for continuous optimization and testing of known and newly discovered genetic and metabolic markers in developing a screening protocol for the purposes of determining TSSM resistance status to pesticides. As more populations from Ontario are tested and more markers become available, the screening platform should become both more accurate and more precise in its predictions and recommendations to growers, especially given the close geographic proximity of many of the greenhouses where the mite samples are coming from and common vegetable crops serving as plant hosts. It is also clear that abamectin resistance genetic make-up is variable and one should identify markers that are informative for the local TSSM populations. At present, it is not clear if TSSM populations from other production systems will share the resistance patterns with one characterised here based on 19 greenhouse populations. The survey of mite pesticide resistance patterns that would span agricultural production systems but remained focused on Southern Ontario will help establish if it is the influence of crop or relatedness among TSSM populations that shape the pesticide resistance pattern in TSSM populations.

Furthermore, I observed that resistance associated SNP frequency decreased in the absence of selection by abamectin, to the point of being undetectable. This is likely explained by the fitness cost, i.e., pleiotropic effect, of the SNPs (Bajda *et al.* 2018; Fotoukkaiaii *et al.* 2020; Sato *et al.* 2005; Stocco *et al.* 2016). In particular, Ontarian populations were re-genotyped after being reared under non-selected conditions for about a year and toxicity bioassays were performed again around the same time (Figure 3.4; Table 3.2). Genotyping confirmed the loss of I321T amino acid substitution, and bioassays showed increased susceptibility of populations to abamectin. This suggests that the genetic marker is not stable in the populations, and it is probably explained by fitness cost of bearing the SNP when it is not needed for the survival. Congruent with this finding were the studies of Brazilian populations (Nicastro *et al.* 2010; Sato *et al.* 2005) that also showed a complete loss of resistance to abamectin after being non-challenged for six months. In contrast, Stumpf & Nauen (2002) and Xue *et al.* (2020) observed that the resistance to abamectin was stable for a year under laboratory conditions. The discrepancy between measures of abamectin resistance stability may arise from differences in mechanism of field- and laboratory-based selection as well

as the previous acaricide use, host and geographical history of populations. RT-qPCR performed around three months after the initial RT-qPCR did not show any correlation between detoxification gene expression and the abamectin resistance status of TSSM populations (Table 3.3). This suggests that there is a need to search for further metabolic markers, assuming this mechanism of resistance is consistently associated with abamectin resistance in all populations.

In terms of TSSM resistance to bifenazate, there is only one mechanism of resistance thus far observed – target site insensitivity. Reports from others have characterized a few SNPs that potentially correlate with population level resistance to bifenazate in different countries. G126S, I136T, S141F, D161G and P262T were identified in TSSM *cytochrome b* by Van Leeuwen *et al.* (2008). The G126S and S141F, G126S and I136T, and P262T, individually or in combinations, have been reported as mutations contributing to strong bifenazate resistance in populations from greenhouses in the Netherlands (Van Leeuwen *et al.* 2008; Van Nieuwenhuysse *et al.* 2009). Several Belgium populations also had a high frequency of the P262T mutation (Van Leeuwen *et al.* 2010). Netherlands rose greenhouse populations had either fixed G126S, fixed combination of G126 and I136T, or no fixed P262T in populations with low or moderate resistance while the rest of the mutations were not found at all (Khajelhali *et al.* 2011). Some moderately resistant Korean rose greenhouse populations revealed G126S and P262T as representative resistance markers, but I136T, S141F, and D161G were not observed (Kwon *et al.* 2015). However, a few resistant populations from the same study revealed themselves to have no known resistant markers present at all. Piraneo *et al.* (2015) found that several of the tested Pacific Northwest US hops field TSSM populations carried only the G126S substitution and displayed low to moderate resistance. The investigation of Greek ornamental greenhouse populations (Papapostolou *et al.* 2020) and a study of several Chinese populations (Xu *et al.* 2018) report very low resistance levels and the absence of any reported target site mutations. Such a genetic variability associated with TSSM resistance to bifenazate suggests that the resistance state is not genetically constrained, but can be derived via multiple independent alterations in the *cytochrome b*. How these SNPs translate to the function of the mitochondrial complex III and the bifenazate binding is not yet clear but is expected to result in the various degrees of resistance and fitness costs.

Ontario populations were analyzed for the presence of G126S, I136T, S141F, D161G and P262T mutations in *cytochrome b*. Only G126S was detected in a few populations conferring

moderate resistance (Table 3.4). This is in line with other studies described above that suggest very recent start of the bifentazate use as well as low resistance levels associated with the SNP. Strains 16 and 17 display highly resistant phenotype, however they do not carry any of the SNPs (Figure 3.8; Table 3.4). This suggests that more informative markers and other mechanisms contributing to bifentazate resistance should be investigated. Namely, Sugimoto and Osakabe (2019) suggest the role of detoxification mechanism. Fotoukkaiaii *et al.* (2020) identified two novel mutations in European field collected TSSM strains in the same Q₀ site where previous bifentazate resistance mutations have been revealed: G132A and G126S+A133T that can be included in future analysis of Ontario populations.

Canadian populations have also been predicted to have multiresistance. Based on abamectin and bifentazate bioassays and genotyping data analysis, a few Canadian populations proved to have developed cross-resistance to abamectin and bifentazate (Figure 3.9). According to other studies, it is very likely that these populations would also confer resistance to other acaricides such as milbemectin (Nicastro *et al.* 2010; Sato *et al.* 2005), acequinocyl (Khajehali *et al.* 2011), and other pesticides that have similar modes of actions that were not tested in this work. Multiresistance needs to be further tested and is beyond the scope of this work.

Etoxazole resistance is monogenic in nature; that is, it is known to be associated only with target site insensitivity as the mechanism of resistance. Previous studies showed a high correlation of TSSM etoxazole resistance with I1017F mutation in *chitin synthase 1* (Van Leeuwen *et al.* 2012). Ilias *et al.* (2014) did a global survey of this mutation in populations from various greenhouses, mostly ornamental crops, and the presence of this mutation was observed in Greek, Italian, Netherlandish, Belgian, Kenyan, Japanese, Australian, Cypriot, and Turkish populations. Osakabe *et al.* (2017) and Herron *et al.* (2018) also report the presence of I1017F mutation in Japanese and Australian field populations, respectively. Given the high correlation between the mutation and the phenotypic resistance status of TSSM populations across the world, it was predicted that Ontarian strains would also have the high correlation between this SNP and the etoxazole resistance. However, the presence of the I1017F SNP at high frequencies was not correlated with the phenotypic resistance status of Ontario populations at all (Figure 3.10; Table 3.5). The reason for this discrepancy likely arises due to the large time gap between the first genotyping of the populations and bioassays performed to examine the mortality status. It is likely

that populations have lost their resistance due to fitness cost caused by this mutation (Bajda *et al.* 2018), before there was a chance to assess the presence of such a correlation. Thus, the Ontario populations were re-genotyped two months after the toxicity bioassays, and it was found that strains had lost the I1017F SNP that was initially present at high frequency in the populations (Table 3.6). This might explain the loss of phenotypic resistance. As for the future directions, it would be interesting to see if etoxazole resistance can be restored by the selection with the acaricide as described in Liu *et al.* (2020). Once the resistance is restored, the Ontario populations should be re-genotyped to investigate whether the I1017F mutation was back and, indeed, correlated with the resistance to etoxazole.

5.0 Summary & Conclusions

Based on the three pesticides studied, it is evident that TSSM pesticide resistance and multi-resistance is a growing problem in Canada. Thus, IPM decision needs to be supported with a tool that would be able to provide information on what pesticide to use once a crop is infested with TSSM. Not only will it save time, money and help preserve environment, but it will also eliminate the need to use multiple pesticides in hopes that one will be effective. Effective use of pesticides will contribute to the prevention of the rapid development of resistance and cross-resistance. Even though bioassays are often used as means for resistance monitoring (R4P Network 2016), they are challenging in many ways such as being labor intensive, or because of a need of pest maintenance in a laboratory facility etc. With an increasing knowledge of molecular markers associated with resistance, high throughput, reliable and fast monitoring tool based on the molecular and metabolic markers can be developed. This idea has been recently put forward for vectors of malaria (Donnelly *et al.* 2016; Vontas *et al.* 2020). Thus, there is potential of creating such a pesticide resistance monitoring tool that would be able to monitor and assess resistance of various TSSM populations and provide growers with decision support. Osakabe *et al.* (2017) have developed a diagnostic method based on the frequency of I1017F mutation that is strongly associated with TSSM resistance to etoxazole. RED-ddCt ($\Delta\Delta$ Ct method that uses real-time PCR for genomic DNA) method includes digestion of the susceptible allele by restriction endonucleases that is followed by quantitative real-time PCR amplification to quantify the frequency/presence of the etoxazole resistant allele in a pooled sample. Three years later, Maeoka *et al.* (2020) optimized this method even further, making it less time consuming and more efficient. The new optimized

method uses quantitative real-time PCR with primer set specific for I1017F mutated allele in *CHS1* (Maeoka *et al.* 2020). Even though it is beyond the scope of my work, the monitoring tool is being developed for Ontario populations based on abamectin resistance mutation (I321T in *GluCl3*), toxicity bioassays and RT-qPCR data representing metabolic resistance markers (Figures 4.1 and 4.2). So far, the sensitivity, specificity, and accuracy of the tool are high; however, more informative genetic and metabolic markers would make it even more precise and usable. Overall, it can be expected that the resistance prediction tool will be able to contribute to acaricide resistance management strategies and applied to a wider range of acaricides.

The data obtained in this work shows that TSSM tend to lose their resistance to a pesticide over time (Figure 3.5); thus, pesticide and crop rotation may be an effective strategy for growers to implement to extend the use of current pesticides. This observation aligns with the previous works of Sato *et al.* (2005), Nicastro *et al.* (2010).

Furthermore, the modes of actions of currently used pesticides are very restricted because TSSM has evolved the resistance to most of them. Yet, the development of new pesticides with new modes of actions becomes more and more challenging. Thus, a need for a novel approach is needed. Further investigation of biocontrol using TSSM predator mites or insects may be of an interest. Biological control offers no plant and human exposure to toxic chemicals, very low risk of environmental pollution, and no resistance development (Lenteren 2000). Opit *et al.* (2009) demonstrated that it is possible to effectively control TSSM with *Phytoseiulus persimilis* in an economically efficient way, despite the complex interaction between TSSM and predators in different crop and pest management systems. While *P. persimilis* is routinely used for TSSM biocontrol in vegetable-producing greenhouses, its efficiency is very limited on tomato crop. Thus, growers have to rely on pesticides to control TSSM populations in tomato-producing greenhouses. A novel appealing pest control product that combines advantageous ideas of both chemical and biological controls is RNAi-based control method. This genetic tool is environmentally friendly (Baum *et al.* 2007; Whyard *et al.* 2009) with high specificity for gene families contributing to TSSM's ability of overcoming plant defenses. RNAi's mode of action differs drastically compared to the existing pesticides (Bensoussan *et al.* 2020); thus, the lack of resistance to RNAi-based pesticide is expected. Furthermore, potential novel RNAi protocols would enable high throughput genetic screens and development of RNAi-based control of TSSM (Bensoussan *et al.* 2020).

6.0 Future directions

Since the completion of this work additional genetic markers associated with abamectin resistance have been reported (Xu *et al.* 2020). These markers should be tested in Ontario greenhouse collected mite populations to see if they can increase the precision of the RI model. In addition, Adesanya *et al.* (2019) suggested the presence of mite metabolic resistance to etoxazole. Identification of genes encoding detoxification enzymes that may contribute to mite etoxazole resistance should be characterized and tested for their ability to predict mite resistance to etoxazole. Additional mite populations from different agricultural settings should be collected and tested for their pesticide resistance to abamectin, etoxazole and bifenazate in order to test the predictability of currently known and future genetic markers of mite pesticide resistance. Moreover, more TSSM populations should be collected and tested for the new and existing markers to improve the acaricide resistance monitoring tool's accuracy, specificity and sensitivity to support IPM decisions.

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Appendices

R studio information:

```
R version 4.0.5 (2021-03-31)
Platform: x86_64-w64-mingw32 (64 bits)
Running under: Windows 10 x64 (build 18362)
Matrix products: default
```

locale:

```
[1] LC_COLLATE=English_United States.1252;LC_CTYPE=English_United
States.1252;LC_MONETARY=English_United
States.1252;LC_NUMERIC=C;LC_TIME=English_United States.1252
```

R packages used for dose response analysis:

```
[1] drc ggplot2 tictoc
```

R packages used for toxicity bioassay analysis:

```
[1] reshape dplyr tidyr amap gplots ggplot2 RColorBrewer pheatmap agricolae
```

Curriculum Vitae

ACADEMIC TRAINING

Master of Science (Biology) **May 2019 – Oct 2021**
University of Western Ontario, London, Ontario, Canada
MSc thesis: Establishment of a pesticide resistance monitoring tool for the two-spotted spider mite, *Tetranychus urticae*

Bachelor of Science Honors **Sep 2014 – Apr 2019**
University of Western Ontario, London, Ontario, Canada
Honors Specialization in Biology, Minor in Genetics

RESEARCH EXPERIENCE

Work Study Student **2018 – 2019**
Department of Biology
Principal investigator: Dr. Vojislava Grbić
University of Western Ontario, London, Ontario, Canada

TEACHING EXPERIENCE

Teaching Assistant **2019 – 2021**
Department of Biology
University of Western Ontario, London, Ontario, Canada

CONFERENCES AND PRESENTATIONS

Poster Presentation: Establishment of a pesticide resistance monitoring tool for the two-spotted spider mite, Tetranychus urticae
Ontario Fruit and Vegetable Convention **Feb 2020**
Niagara Falls, Ontario

Poster Presentation: Establishment of a pesticide resistance monitoring tool for the two-spotted spider mite, Tetranychus urticae
Ontario Pest Management Conference **Nov 2019**
Guelph, Ontario

Conference Presentation: Establishment of a pesticide resistance monitoring tool for the two-spotted spider mite, Tetranychus urticae
Biology Graduate Research Forum **Oct 2019**
UWO, London, Ontario

Thesis Day Presentation: Characterization of detoxification capability of five tomato-adapted spider mite strains – Award winning presentation
UWO, London, Ontario **Apr 2018**

Conference Presentation: Characterization of detoxification capability of five tomato-adapted spider mite strains
Ontario Biology Day Conference **Mar 2018**
University of Waterloo, Ontario

SCHOLARSHIPS AND AWARDS

Dean's Honor List (Undergraduate) **2018**