Impact Assessment of Neonicotinoid Insecticides on the Reproductive Biology of the Two-Spotted Spider Mite *Tetranychus urticae* Koch (Acari: Tetranychidae)

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des akademischen Grades eines
Doktors der Gartenbauwissenschaften
-Dr. rer. hort.-
genehmigte
Dissertation
von

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geboren am 07. November 1975 in Yade, Togo

2006

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Tag der Promotion	29.11.06

Abstract

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Mouhoube Ako

The use of neonicotinoid insecticides to control agricultural insect pests such as aphids and whiteflies in orchards and hops has been reported to trigger field population outbreaks of two-spotted spider mite (TSSM) *Tetranychus urticae* Koch. This phenomenon has become a topic of concern for the use of these insecticides in integrated pest management (IPM) programs because of increasing risk for TSSM damages. The here presented laboratory and greenhouse trials aimed at elucidating the possible mechanisms behind these observations by assessing the population growth potential of two-spotted spider in relation with the use of neonicotinoid insecticide, especially imidacloprid under controlled laboratory and greenhouse conditions.

A series of experiments has been carried out using French bean as host plant. In the first trial, the effect of a field relevant dose rate of imidacloprid (100ppm) on the reproduction of four different TSSM strains with different acaricide resistance backgrounds has been studied. The results showed no influence of imidacloprid on fecundity as well as on the proportion of F1 females in acaricide-resistant TSSM strains while significant decreases in fecundity and in the proportion of F1 females were observed in the susceptible strain. Egg fertility was not affected by imidacloprid.

In order to obtain detailed estimates of driving factors for population growth of TSSM under the influence of imidacloprid treatments, a second greenhouse experiment was set up, in which important life table parameters of these TSSM strains were determined using the Jackknife method. By comparing the 4 TSSM strains without insecticide treatment, important variations were observed in the net reproductive rate R_0 among the strains. Results revealed

that in all tested TSSM strains, intrinsic rate of increase (Rm), finite rate of increase (λ), and (R_0) were higher in the control compared with drench application of imidacloprid, while in foliar application, Rm and λ did not show important variations compared with the control treatment. In addition, the doubling time (Dt) for TSSM population was shorter in control treatments compared with drench but not with foliar applications. The mean generation time (T) did not vary among the treatments and TSSM strains with or without imidacloprid treatment.

In an attempt to understand more the reasons for the depression of TSSM fecundity observed with drench application of imidacloprid, the influence of imidacloprid treatments on the activity of relevant stress enzymes namely carboxylesterase (CarE) and superoxide dismutase (SOD) were investigated in the third experiment. Results showed higher CarE activity with foliar treatment and moderate activity with soil application compared with the control on the first day and third day after treatment. After six days however, no differences were observed in CarE activity among the treatments. For SOD, higher activity was observed in foliar treatment compared with the control only on the third day after treatment. It was concluded that uptake and metabolism of imidacloprid by the mites causes a certain unspecific stress and might interfere with physiological processes involved, resulting in a fitness cost (e.g. detoxification). Such fitness cost on a sublethal level can be expressed by a decrease in the fecundity of TSSM.

It remains however unclear, whether imidacloprid or its metabolites are involved in this process, insofar as some metabolites of imidacloprid are highly active compared with imidacloprid itself. Therefore, the effects of an important metabilite, 6-chloronicotinic acid (6-CNA) on the reproduction of TSSM was investigated. The results again gave no indication towards negative effects on TSSM fecundity. However, the sex ratio was more male-biased with 6-CNA treatments than observed in the normal TSSM population.

After looking into the influence of imidacloprid and its metabolites on the reproductive biology and physiology of TSSM in controlled experiments in the laboratory, a field-simulated greenhouse experiment was set up in order to

study the influence of this insecticide under more realistic conditions without restriction of TSSM distribution on the plant. Results showed overall higher densities of different TSSM developmental stages on the upper canopy, when leaves were sprayed with insecticide. This observation may results from a mass migration of mobile TSSM stages towards the upper parts of the plant, probably because of the stress effect of imidacloprid, as shown in a previous experiment. However, the overall number of eggs, immature stages and adults (lower and upper canopies combined) did not vary among the treatments.

Since all our laboratory and greenhouse investigations have failed to find any indication towards a population growth of TSSM due to imidacloprid treatments, an additional experiment was set up, which took into consideration the fertilization status with nitrogen as an important factor of variability between laboratory and field environments. The influence of inorganic nitrogen in combination with imidacloprid on the reproduction of TSSM was investigated. Results showed positive effects of nitrogen on the fecundity and the number of immature stages, while the combination of imidacloprid and nitrogen did not affect significantly the fecundity and the number of immature stages compared with the nitrogen treatment only.

Key words: Neonicotinoid insecticides, *Tetranychus urticae*, life table parameters, nitrogen fertilization

Zusammenfassung

Einfluß von Insektiziden aus der Gruppe der Neo-nicotinoide auf die Reproduktionsbiologie der Spinnmilbe *Tetranychus urticae* Koch (Acari: Tetranychidae)

Mouhoube Ako

In den letzten Jahren wurde verschiedentlich darüber berichtet, dass die Anwendung von Insektiziden aus der Gruppe der Neo-nicotinoide zur Kontrolle von Blattläusen und Weißen Fliegen im Obst- und Hopfenbau zu Massenvermehrungen der Gemeinen Spinnmilbe (TSSM) *Tetranychus urticae* führen kann. Dieses Phänomen hat wegen der Gefahr zunehmender Schäden durch TSSM Besorgnis aufkommen lassen, die entsprechenden Wirkstoffe weiterhin in Rahmen von Integrierten Pflanzenschutzkonzepten (IPM) einzusetzen. Die hier vorgestellten Labor- und Gewächshausversuche haben zum Ziel, die möglichen Grundlagen dieser Beobachtungen aufzuklären. Dazu wurde das Entwicklungspotential der Gemeinen Spinnmilbe bei Applikation von neonicotinoiden Wirkstoffen, insbesondere Imidacloprid, unter kontrollierten Labor- und Gewächshausbedingungen untersucht.

Es wurde eine Serie von Experimenten mit der Gartenbohne *Phaseolus vulgaris* als Wirtspflanze für die Spinnmilben durchgeführt. In der ersten Versuchsreihe wurde der Einfluß einer praxisrelevanten Aufwandmenge von Imidacloprid (100 ppm) auf das Reproduktionspotential von vier unterschiedlichen TSSM Stämmen untersucht. Die Stämme unterschieden sich insbesondere hinsichtlich ihres Resistenzniveaus gegenüber verschiedenen Akariziden. Anwendungen von Imidacloprid zeigten bei Akarizid-resistenten Stämmen weder einen Einfluß auf die Fekundität noch auf den Anteil von Weibchen in der F1 Generation, wohingegen eine signifikante Abnahme der Fekundität und des Anteiles der F1-Weibchen bei empfindlichen Stämmen beobachtet werden konnte. Die Schlupfrate aus den Eiern war in keinem Fall beeinträchtigt.

Um detaillierte Daten über Schlüsselfaktoren der Populationsdynamik von TSSM unter dem Einfluß von Imidacloprid zu gewinnen, wurde ein weiteres

Gewächshausexperiment angelegt, in dem Lebenstafelparameter der TSSM – Stämme mittels der “Jackknife Methode“ bestimmt wurden. Der Vergleich der vier TSSM – Stämme ohne Insektizideinfluß zeigte zunächst deutliche Unterschiede in der Reproduktionsrate (R_0) zwischen den Stämmen. Die Ergebnisse der Imidacloprid-Behandlungen ergaben für alle geprüften Stämme höhere Werte für die „intrinsic rate of increase (Rm)”, “finite rate of increase (λ)” und R_0 in den unbehandelten Kontrollen im Vergleich zu einer Substratapplikation (Gießbehandlung) von Imidacloprid, während bei Blattbehandlungen Rm and λ keine Veränderungen im Vergleich zu Kontrollen zeigten. Zusätzlich war die „doubling time (Dt)” der TSSM Population kürzer in den Kontrollbehandlungen im Vergleich zu Gießbehandlungen, während keine Unterschiede zu Blattbehandlungen zu erkennen waren. Die „mean generation time (T)” variierte nicht bei den verschiedenen Behandlungen und TSSM Stämmen beim Vergleich von Kontroll- und Imidaclopridbehandlungen.

Um mehr Informationen über die Gründe für den Rückgang der Fekundität von TSSM bei Gießbehandlungen zu gewinnen, wurde der Einfluß von Imidaclopridbehandlungen auf die Aktivität relevanter Stressenzyme wie der Carboxylesterase (CarE) and der Superoxide Dismutase (SOD) bestimmt. Die Ergebnisse zeigten eine höhere CarE-Aktivität bei Blattbehandlungen sowie eine moderate Aktivitätserhöhung bei Substratbehandlungen im Vergleich zur Kontrolle einen und drei Tage nach Behandlung. Nach sechs Tagen konnten keine Unterschiede in der CarE-Aktivität mehr festgestellt werden. Für SOD wurden höhere Aktivitäten bei Blattbehandlungen im Vergleich zur Kontrolle aber nur am dritten Tag nach der Applikation beobachtet. Die Daten lassen den Schluss zu, dass die Aufnahmen und Metabolisierung von Imidacloprid für die Milben einen unspezifischen Stress darstellt und daraus gewisse Fittness-Kosten resultieren (e.g. durch die Entgiftungsreaktionen). Derartige Effekte können durchaus eine reduzierte Fekundität nach sich ziehen.

Es war jedoch weiterhin unklar, ob Imidacloprid selbst oder Metabolite dieser Substanz für diese Effekte verantwortlich waren. Es ist bekannt, dass einige Metabolite von Imidacloprid selbst hohe insektizide Aktivität haben können. Um dieses Problem genauer zu hinterfragen wurde der Einfluß eines Hauptmetaboliten, 6-Chloronicotin Säure (6-CNA) auf die Reproduktion von

TSSM untersucht. Es ergaben sich erneut keine Hinweise auf eine Beeinflussung der TSSM Fekundität. Nur das Geschlechterverhältnis war nach 6-CNA Behandlungen zu den Männchen hin verschoben.
Nach Analyse möglicher Effekte von Imidacloprid und seiner Metaboliten auf die Reproduktionsbiologie und Physiologie von TSSM in kontrollierten Laborexperimenten wurde eine Studie mit simulierten Gewächshausbedingungen durchgeführt, um die Imidacloprid-Anwendung zusätzlich unter mehr praxisrelevanten Bedingungen zu überprüfen, insbesondere bei freien Verteilungsmöglichkeiten der Milben auf der Pflanze. Die Ergebnisse zeigten nach Blattapplikationen von Imidacloprid durchgehend höhere Dichten aller TSSM Entwicklungsstadien auf den oberen Blättern. Diese Beobachtung ist vermutlich das Ergebnis einer gezielten Wanderung der mobilen TSSM Stadien, möglicherweise hervorgerufen durch den vorher beschriebenen Stress-Effekt von Imidacloprid. Wurden jedoch die Anzahlen der Milben auf oberen und unteren Blättern summiert, ergaben sich in der Gesamtzahl von Eiern, Nymphen und Adulten keine Unterschiede zwischen den Behandlungen.
Da alle Labor- und Gewächshausexperimente keine Hinweise auf eine direkte Imidacloprid bedingte Erhöhung der Wachstumsrate von TSSM Populationen ergaben, wurde ein zusätzliches Experiment durchgeführt, in dem als zusätzlicher Faktor ein unterschiedlicher Düngungsstatus mit Stickstoff integriert wurde. Es lag die Hypothese zugrunde, dass möglicherweise durch den Wirkstoff bedingte Effekte erst bei einem suboptimalen N-Niveau relevant werden. Erwartungsgemäß steigerten hohe N- Dosierungen zwar die Fekundität und damit die Anzahl der Nymphenstadien von TSSM auf den Pflanzen aber erneut konnte die Kombination verschiedener N-Niveaus mit Imidacloprid keinen Hinweis auf wirkstoffbedingte Einflüsse auf das Populationswachstum der Milben erbringen.

Stichworte: Neonicotinoide Insektizide, *Tetranychus urticae*, Lebenstafel, Stickstoffdüngung

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Abbreviations

ANOVA	Analysis of Variance
λ	Finite rate of increase (Fertility life table parameter)
6-CNA	6-Chloronicotinic Acid
CarE	Carboxylesterase
df	Degree of freedom
Dt	Doubling time (Fertility life table parameter)
EDTA	Ethylene Diamine Tetraacetic Acid
EEPS	Endogenous Enzymes of Protective System
GLM	General Linear Procedure
GSS	German Susceptible Strain (a strain of Two-Spotted Spider Mite)
HPLC	High Performance Liquid Chromatography
IPM	Integrated Pest Management
IPP	Institute of Plant Diseases and Plant Protection
METI	Mitochondrial Electron Transport Inhibitor
NADH	Nicotinamide Adenine Dinucleotide
NBT	Nitroblue Tetrazolium
P	*P*-value Statistical significance level
PMS	Phenazine Methosulfate
PPM	Parts Per Million
R_0	Net reproductive rate (Fertility life table parameter)
RH	Relative Humidity
Rm	Intrinsic rate of increase (Fertility life table parameter)
ROS	Reactive Oxygen Species
SAS	Statistical Analyses System
SE	Standard error of the mean
SL	Soluble Concentrate
SOD	Superoxide Dismutase
t	Statistical *t*-value
T	Mean generation time (Fertility life table parameter)
TSSM	Two-Spotted Spider Mite
USA	Strain USA of Two-Spotted Spider Mite
Vmax	Maximum Velosity
WI	Wiesmor (a strain of Two-Spotted Spider Mite)

CHAPTER I

1. General Introduction

Two-spotted spider mite: biology, reproduction and control strategies

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch, equally known as glasshouse red spider mite, red spider mite or simply red mite, is the major spider mite pest of ornamental plants and vegetable crops grown in greenhouses in temperate zones. Furthermore, this ubiquitous spider mite is a serious pest of numerous ornamental plants in home landscapes, and is of considerable importance as a pest of food and fibre crops throughout the world (Osdone et al., 1985). Originally from Eurasia, TSSM has become a cosmopolitan pest with a host range of more than 900 plant species (Navajas, 1998). TSSM belongs to the Phylum Arthropoda, Order Arachnida and is a member of the family Tetranychidae, which contains many harmful species of plant-feeding mites (Osborne et al., 1985). Cagle (1949) provided an account of the characteristics of males and females. The body of the female is oval-shaped and rounded posteriorly. The female adult is about 0.5 mm long and has eight short legs. Its colour varies from light yellow or green to dark green, straw colour, brown, black and various shades of orange (figure 1.1). The male is much smaller and is considerably more active. The body is narrow and distinctly pointed posteriorly. The colour of the male varies from pale to dark green, brownish, or at times, orange.

Damages of TSSM to host plants

Spider mites have tiny mouthparts, modified for piercing individual plant cells and removing the contents. This results in tiny yellow or white speckles. When many of these feeding spots occur near each other, the foliage takes on a

yellow or bronzed cast. Once the foliage of a plant becomes bronzed, it often drops prematurely (Shetlar, 1992)

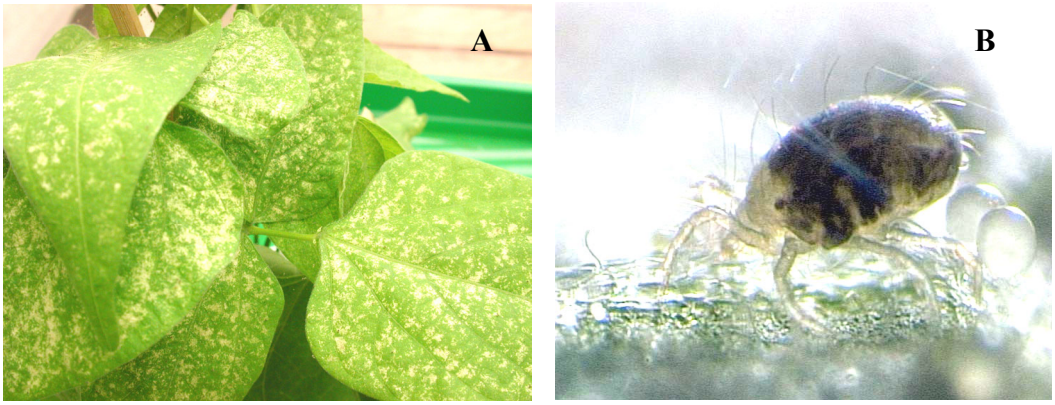

Figure 1.1. Damages due to TSSM on French beans (A) and a female adult TSSM (B)

Damage to plants is effectuated in several ways: First, feeding causes the destruction and disappearance of chloroplasts, which then leads to basic physiological changes in the plant like a reduced photosynthetic capacity and stomata closure. Stomatal closure can be a primary host plant response, and in such cases, uptake of CO_2 decreases, resulting in a marked reduction in transpiration and photosynthesis (Sances et al., 1979a, 1979b). Secondly, the mites release phytotoxic substances into the plant when feeding (Liesering, 1960; Avery and Briggs, 1968; Jeppson et al., 1975). Finally, the stippling or speckling of the upper leaf surface, plus the webbing produced by protonymphs, deutonymphs, and adults, leads to aesthetic injury, particularly in the case of ornamental plants.

Developmental and reproductive biology of TSSM

In both male and female TSSM, development proceeds through the following stages: egg, larva, protonymph, deutonymph, and adult. The larval, protonymphal, and deutonymphal stages are further divided into feeding

(active) and quiescent (resting) stages. The quiescent stages are referred to as nymphochrysalis (protochrysalis), deutochrysalis, and teleiochrysalis for larval, and protonymphal, and deutonymphal for nymphal stages, respectively. (Laing, 1969; van de Vrie et al., 1972).

Females normally lay eggs on the underside of leaves. According to Cagle (1949), the spherical egg is about 0.14 mm in diameter. The newly deposited egg is clear, but turns opaque and glassy as incubation progresses. Just before hatching, the egg is straw-coloured and the carmine "eyespots" of the embryo becomes visible (Cagle, 1949). The larva has three pairs of legs (hexapod). The first non-feeding resting stage between the larval and protonymphal stages is called protochrysalis. The protonymph has four pairs of legs (octapod) and is somewhat larger than the larva (Cagle, 1949). At the end of the feeding stage, the protonymph attaches to the substrate, enters the quiescent stage (deutochrysalis), and is later transformed into a deutonymph.

The octapod deutonymph is generally larger than the protonymph, although similar in its colour pattern. At this stage, the males can usually be distinguished from the females because of their smaller size and the wedge-shaped posterior of the females (Cagle, 1949; Laing, 1969). The octapod adult eventually emerges from teliochrysalis.

Developmental time of TSSM is generally variable with conditions such as temperature, humidity, host plant, leaf age, etc. However, temperature is the most important factor that influences the rate at which mites develop. The lower threshold for development is about 12°C, whereas the maximum upper limit for the development is about 40°C (Jeppson et al., 1975) and relative humidity (RH) can fluctuate from 55% to 98%. Under these conditions, mites developed from egg to adult on average in 16.5 days. Shih et al. (1976) reared TSSM on lima beans at 27°C and 90±5% RH. In this case, mites developed from egg to adult on average in 7.6 days. Sabelis (1981) determined the developmental time required for an egg to develop into a female capable of

laying eggs. The regimens studied were 25-35°C and 10-20°C for which he determined the developmental times to be 8.3 and 28.2 days, respectively.

In a given colony of TSSM, both adult males and females can usually be found; however, females are normally about three times more abundant than males. Generally, adult males can be found in close association with quiescent female deutonymphs. Quiescent female deutonymphs release a sex pheromone, which attracts males and keep them in close proximity until adult emergence leading to immediate fertilization (copulation) of young females with eventually several males (Cone et al., 1971a, 1971b; Penman and Cone, 1972, 1974). However, only the first mating is effective for female (Satoh et al., 2001)

The lifespan of the adult female is divided into the pre-ovipositional period and the ovipositional period, the former being the time between emergence from the teleiochrysalis to the deposition of the first egg. Apparently, the pre-ovipositional period (9% of the time required to develop from egg to egg) can last less than 0.5 day and as long as 3 days, depending on the temperature. The ovipositional period can last from 10 days at 35°C to 40 days at 15°C (Sabelis, 1981). An individual female can deposit over 100 eggs in her lifetime (Shih et al., 1976; Carey and Bradley, 1982). The total number of eggs laid per female and the eggs laid daily per female can, however, vary with age, temperature, species of host plant, relative humidity, nutrition of host plant, exposure to pesticides, etc. (van de Vrie et al., 1972; Karban and Carey, 1984). Temperature and age of the female are especially important determinants for egg production (fecundity). However, Sabelis (1981) determined that fecundity was little affected at temperatures between 20-35°C. In his study, peak oviposition (i.e., 161 eggs/female) occurred at 25°C, with the maximum rate (i.e., 12 eggs/female/day) occurring two days after the first eggs had been laid. The effect of temperature is particularly evident in greenhouses, where spider mite populations often develop rapidly soon after the onset of summer temperatures.

Sex determination in TSSM (as in many other spider mites) is arrhenotokous, i.e., females develop from fertilized eggs and have the normal two sets of chromosomes (diploid), whereas males develop from unfertilised eggs and have only one set of chromosomes (haploid). Unmated females give rise to males only whereas mated females can produce either female or male.

Control strategies for TSSM

Early detection of spider mites, before damage is noticed, is important. For detection of spider mites, a 10X to 15X magnifying glass is a necessity. Examine the undersides of the leaves closely for mites, cast skins and webbing. A more efficient technique is to place a sheet of white typing paper beneath the leaves and strike the foliage sharply. The mites will fall onto the paper and can be more easily observed and identified than on the green foliage. Several control strategies i.e. cultural, chemical and biological controls are available and combined in an integrated spider mite control (Blindeman and Van Labeke, 2003) in which the compatibility between some acaricides (chlorfenapyr spiromesifen and bifenazate) and the predatory mites (*Neoseiulus californicus*) permitted a simultaneous use of both control methods for an integrated management of spider mite (Cloyd et al., 2006)

Cultural control

Syringing and water management: Since rainy weather seems to knock off spider mites, using a forceful jet of water from a hose can perform the same task. Regular water spraying can keep spider mites under control on most ornamental plants in the field. This technique also helps conserve natural enemies like predators. Proper irrigation to prevent drought stress is the key cultural practice for avoiding mite outbreaks. However, once mite populations have established, irrigation cannot reduce mite densities. Nitrogen fertilization tends to promote mite infestations, but reducing N application rates to manage spider mites is often economically not feasible (Peairs, 1998).

Quarantine and inspection: TSSM is often introduced through infested bedding and plants. When purchasing new plants, the lower leaf surface should be carefully inspected for any signs of mite activity. New imported plants should be quarantined from other plants until it is sure that no mites are present (Shetlar, 1992).

Chemical control

"Soft" pesticides: Most spider mites can be controlled with insecticidal oils and soaps. Horticultural oils can be used on perennial and woody ornamentals during the summer at a rate of 1 to 2% Higher rates of horticultural oil (3 to 4 %) or dormant oil are useful for killing mite eggs and dormant adults in the fall and spring (Shetlar, 1992).

Acaricides: Pesticides claiming to be efficient "for mite suppression" are usually weak acaricides and often do not perform well. There are few products available to homeowners. Dicofol is registered for over-the-counter use in the USA but is difficult to find there. Acephate, dimethoate, chlorpyrifos, diazinon, disulfoton, and malathion have over-the-counter product labels but are considered to be weak acaricides (Shetlar, 1992).

Acaricides such as avermectin, bifenthrin, dienochlor, fenbutatin-oxide, fluvalinate, oxamyl, oxydemeton-methyl, oxythioquinox, and propargite are restricted-use pesticides, available only to licensed applicators in the US (Shetlar, 1992).

Biological control

Many natural enemies are associated with spider mites under field conditions. These enemies are either predators or pathogens. One group of small, dark-coloured lady beetles known as the "spider mite destroyers" (*Stethorus* species) are specialized predators of spider mites. Minute pirate bugs, big-eyed bugs (*Geocoris* species) and predatory thrips can be important natural enemies. The predacious phytoseiid mite *Phytoseiulus persimilis, Amblyseius*

fallicus, Neoseiulus californicus, Amblyseius cucumeris, Galandromus occidentalis and Metaseiulus occidentalis are the major species used to control TSSM in greenhouses.

Pathogens occur naturally under field conditions and appear to be an important regulator of spider mite populations. The fungus *Hirsutella thompsonii* Fisher (Hyphomycetes) has been proposed as a potential entomopathogen for control of TSSM in greenhouses, but has been proven effective only under laboratory conditions (Gardner et al., 1982).

Neonicotinoid insecticides: generalities and mode of action

The chloronicotinyl insecticides (Figure 1.2) represent a new class of insecticides acting on insect nicotinic acetylcholine receptors in the central nervous system (Elbert, 1990; Bai et al., 1991). As with nicotine, binding of imidacloprid to the nicotinic acetylcholine receptors (nAChR) results in excitation, followed by paralysis and death. Because of their systemic and translaminar properties, chloronicotinyl insecticides are particularly effective in controlling sucking pests such as aphids, leafhoppers and whiteflies. Imidacloprid was the first commercially available representative of chloronicotinyl insecticides. It was synthesized in 1985 and the first registration was obtained in France (1991) for sugar beet (Sur et al., 2003). Imidacloprid has a broad insecticidal spectrum, excellent systemic and translaminar properties, and high residual activity (Elbert et al., 1990; Takahashi et al., 1992). According to Elbert et al. (2001), imidacloprid is the most successful active ingredient, which has been marketed in the last decade. Thiacloprid, acetamiprid and thiamethoxam belong to the same class of insecticides and are efficient in controlling sucking insect pests and show more or less the same activity as imidacloprid (Figure 1.2). These insecticides are commercialised under different names, depending on the formulations and regions where they are sold. For example, imidacloprid is the active ingredient in Admire, Gaucho, Impower, Merit and Advantage, in Canada. Worldwide it is used in 100 countries on 70 crops (Heather, 2002).

Imidacloprid has a mixed reputation regarding its safety to natural enemies of pests (James and Price, 2002). It has low toxicity to spiders, some predatory beetles (carabids, staphylinids) (Kunkel et al., 1999, James and Vogele 2001), and some predatory bugs (anthocorids, lygaeids, pentatomids, reduviids) (Hough-Goldstein and Whalen 1993, Elzen 2001, James and Vogele 2001). However, Mizell and Sconyers (1992), Delbeke et al. (1997), Stark et al. (1995), Sclar et al. (1998) and James and Vogele (2001), showed that imidacloprid was highly toxic to other species from most of these families. Similarly, some predatory mite (Phytoseiidae) species were tolerant of imidacloprid (Mizell and Sconyers 1992, James, 1997, James and Vogele 2001) while others were susceptible (James and Coyle 2001). Sublethal effects of imidacloprid on natural enemies reported to date, include reduction in prey consumption (Elzen 2001) and alteration in locomotory. behavior (Smith and Krischik 1999, Vincent et al., 2000).

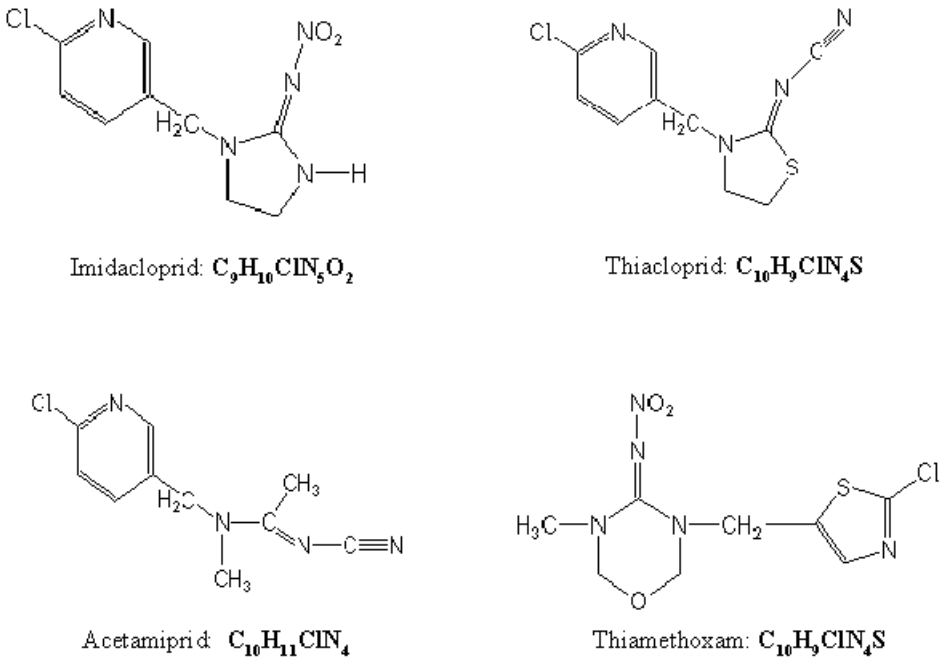

Figure 1.2. Names and chemical structures of four neonicotinoid insecticides

Uptake, translocation and metabolism of imidacloprid in plants

Experiments have been carried out to determine uptake, translocation and metabolic pathways of imidacloprid after different types of application (foliar, soil and seed treatment) on various plant species including apples (Vogeler et al., 1992), maize (Vogeler and Dräger, 1989), cotton (Vogeler and Brauner, 1993), eggplant (Yoshida, 1992), rice (Kurogochi and Araki, 1989; Kurogochi et al., 1989; Sakamoto, 1991). After soil application or seed dressing, imidacloprid is taken up through roots, translocated acropetally within the xylem and degraded into secondary products or metabolites.

Translocation experiments have shown that imidacloprid is highly mobile within the xylem. It is transported to shoots and leaves on one hand. On the other hand, imidacloprid showed a poor basipetal translocation to sinks, i.e. storage organs, roots and fruits because of its negligible phloem mobility (Sur and Stork, 2003). Consequently, higher residues are expected to occur in the older leaf parts of the plants. Xylem and phloem have different pH values of about 5 and 8, respectively. Therefore, especially weak acids, e.g. 6-chloronicotinic acid, an important metabolite of imidacloprid, tend to accumulate in the phloem sieve tubes (Sur and Stork, 2003).

Despite the wide variety of crops and application types having been investigated, a rather uniform picture of the metabolic behaviour of imidacloprid in plants was found, consisting of three principal biotransformation pathways (Figures 1.3, 1.4, and 1.5). Especially after soil application or seed treatment a quick degradation of the a.i. was observed after root uptake of the a.i. (Sur and Stork, 2003). When sprayed, only a part of

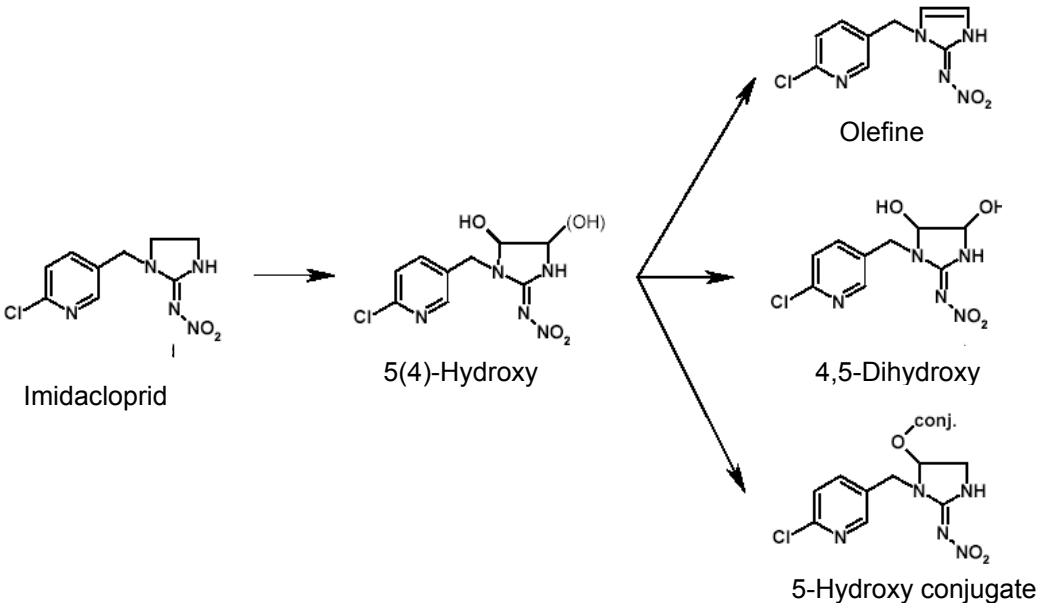

Figure 1.3. Metabolism of imidacloprid (I): ethylene-bridge hydroxylation of the imidazoline ring and elimination of water.

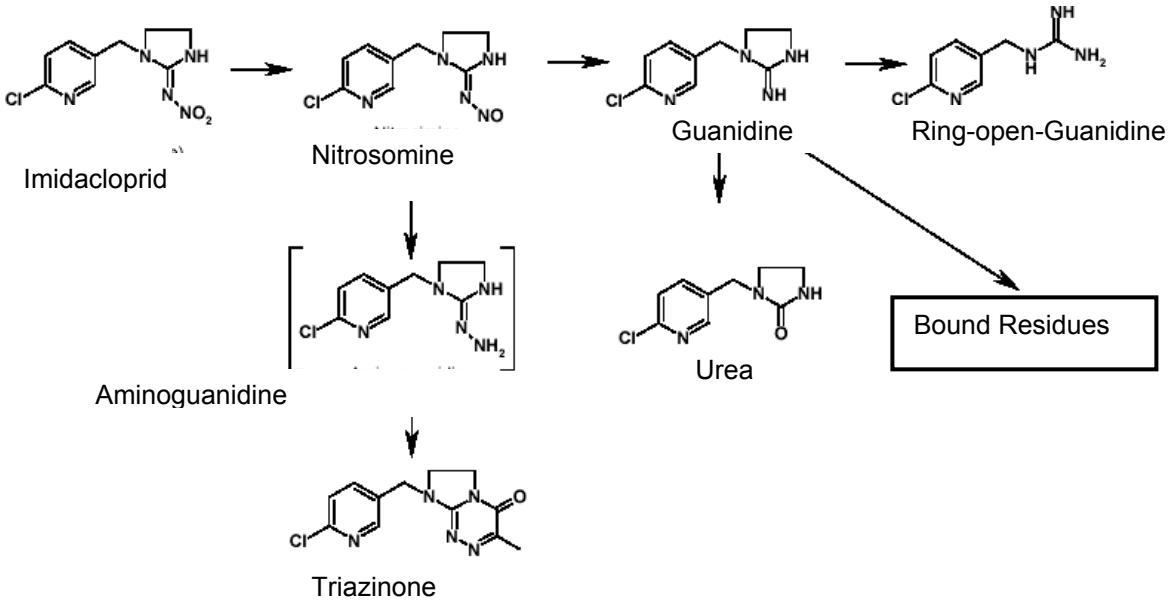

Figure 1.4. Metabolism of imidacloprid (II): nitro-group reduction to nitrosimine and further loss of NO to form guanidine.

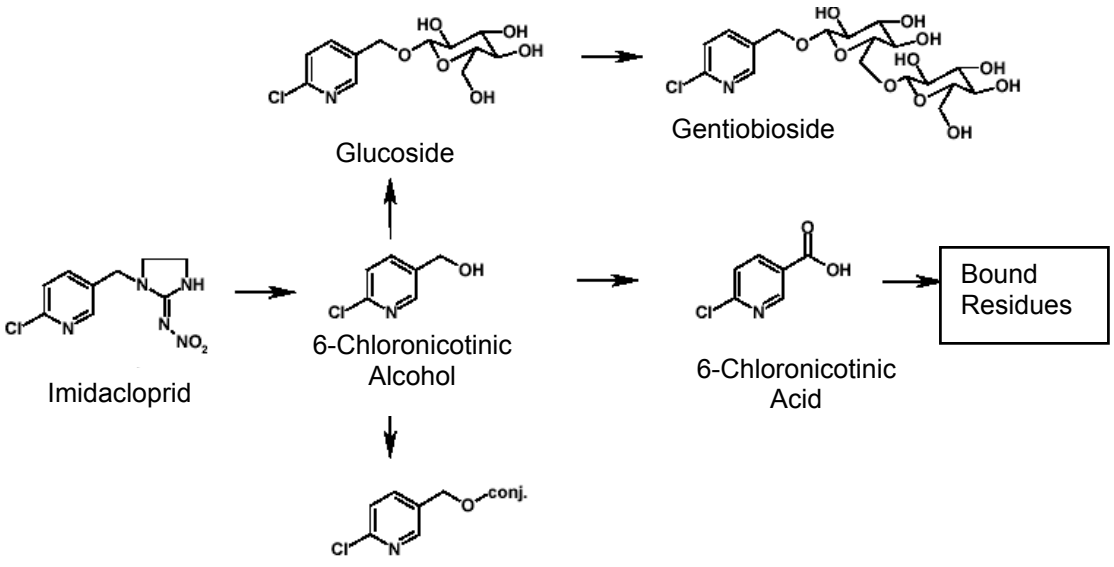

Figure 1.5. Metabolism of imidacloprid (III): oxidative cleavage of the methylene bridge to form 6-chloropicolyl alcohol and subsequent oxidation to 6-chloronicotinic acid.

the a.i. is translocated into the plant and metabolised there, so the degree of metabolism tends to be lower here (Sur and Stork, 2003).

Neonicotinoid insecticides and the reproduction of TSSM

Recent field and greenhouse studies focussing on the side effect of neonicotinoid insecticides on the reproduction of TSSM have yielded contradictory results. One of the first observations of abnormal field increases of TSSM populations following the use of neonicotinoids was done by Sclar et al. (1998). In a series of field experiments, the authors found that marigolds, (*Tagetes erecta* L.), treated with either a soil drench or soil granular formulation of imidacloprid was observed to sustain significantly greater damage from TSSM, than untreated plants. However, in a similar experiment conducted in the greenhouse, the same authors observed no such effects of imidacloprid on spider mite populations or injury on marigolds like observed in the field. In laboratory experiments in which leaf discs were placed on water-saturated cotton wool were used, James and Price (2002) showed that spray

and systemic applications of imidacloprid at rates used in Washington hop yards have led to significant increases fecundity in TSSM. However, the leaf cut technique used by James and Prices (2002) was for many technical reasons not reproducible in one experiment we undertook to study the effect of 4 main neonicotinoids' active ingredient i.e. imidacloprid, thiacloprid acetamiprid and thiamethoxam, on the bionomics of TSSM (Ako et al., 2004). Hence the sticky ring technique (Nauen et al., 2001; Stumpf and Nauen, 2001), which has the advantage of allowing the use of living plants, which are more appropriate for testing systemic insecticides, was used. The results showed no evidences of a potential increase of TSSM in greenhouse as has already shown Sclar et al. (1998). Beers et al. (2005), examining the effect of neonicotinoids on integrated mite control in Washington apple reported important field increases in mite density with 4 or more applications of acetamiprid at field relevant dose rates. However the same authors reported lower mite densities with the same insecticide, when applied only two times. Moreover, the occurrence of elevated mite density was not constant but varied significantly from trial to trial with the same treatments, showing certain instability in the occurrence of increased mite population in the field. Beers and Himmel (2002) found that the use of imidacloprid can enhance the reproduction of both TSSM and predatory mites such as *Galendromus occidentalis* Nesbitt and *Neoseiulus fallacis* Garman (both Acari: Phytoseiidae) at sublethal doses. The same phenomenon of egg production enhancement was reported for TSSM, when exposed to carbaryl or DDT (Dittrich et al., 1974). Similarly, an increased egg production and population development was reported for the Australian predatory mite *Amblyseius victoriensis* (Womersley) (Acari: Phytoseiidae) at sublethal doses of imidacloprid (James, 1997). This phenomenon of reproductive stimulation of pests or beneficials was described by Luckey (1968) as hormoligosis and is equally observed with green peach aphids when exposed to residues of azinphosmethyl (Lowery and Sears, 1986), and with citrus thrips on leaves containing dicofol or malathion residues (Morse and Zareh, 1991).

The concept of chemical hormoligosis or hormesis has a long history, originating over a century ago from the research of H. Schulz, (Schultz, 1888, cited by Edward et al., 1988) who recorded that many chemicals were able to stimulate growth and respiration of yeast at low doses but were inhibitory at higher levels. This concept of a generalized low-dose stimulation-high-dose inhibition was gradually supported by similar observations with other chemicals and eventually became known as the Arndt-Schulz law. Although Schulz (Edward et al., 1988) ushered in the so-called modern concept of hormesis, Paracelsus (Stebbing, 1982), writing in the 16th century, likewise noted that various toxic substances may be beneficial in small quantities.

Statement of research problem and research objectives

Two-spotted spider mites (TSSM), *Tetranychus urticae* Koch, have been a key pest for field and greenhouse crops for many years, and continue to be of concern for growers although both successful chemical and biological control methods exist. Recently, population outbreaks of TSSM following the use of neonicotinoid insecticides in the field have been reported (Sclar et al., 1998; Beers and Himmel, 2002; Beers et al., 2005). For both producers and scientists, this phenomenon of TSSM population outbreaks constitutes a real treat in terms of Integrated Pest Management (IPM) in an already existing complex context of increasing agricultural pests' resistance to pesticides, which required the employment of new and environmental friendly methods of pest management, for which neonicotinoid insecticides were seen acceptable because of their selectivity for insects and relatively low toxicity to non target organisms (Elbert et al.,1990; 1991; Hough-Goldstein and Whalen, 1993; James, 1997; Elzen, 2001). It would be, for these reasons and for avoiding such effects while developing new insecticides in the near future to look into the biological, physiological and biochemical implications of neonicotinoid insecticides in observed TSSM population outbreaks in the field and the different factors contributing to this phenomenon. To this end, different

laboratory and greenhouse experiments were conducted as described in this document.

The main objective of the present research work was to evaluate, in detailed laboratory and greenhouse experiments the influence of imidacloprid and its metabolite, the 6-chloronicotinic acid on the reproductive biology as well as on the physiology of TSSM. In addition the influence of an important field factor, i.e. inorganic nitrogen fertilization, on the evolution of TSSM populations was evaluated in combination with imidacloprid.

CHAPTER II

2. The reproduction of acaricides-resistant and -susceptible strains of *Tetranychus urticae* Koch (Acari: Tetranychidae) as sffected by imidacloprid [1]

2.1. Abstrsact

Occasional reports linking neonicotinoid insecticide applications to field population outbreaks of two-spotted spider mite (TSSM) *Tetranychus urticae* Koch have been a topic of concern in agricultural production systems, particularly in apples and hops. In order to identify the factors, which may contribute to the occasional field population increase of TSSM, following the application of neonicotinoid insecticides, greenhouse experiments have been set up. Four different TSSM strains, namely GSS (acaricide susceptible), WI (organophosphate-selected), USA (largely uncharacterized strain) and Akita (METI (mitochondrial electron transport inhibitor) acaricide resistant and cross-resistant to dicofol) were compared for their fecundity without insecticide treatment and for their ovipositional response to foliar and drench application of field relevant dose rates of imidacloprid (100ppm). Without insecticide treatment, strain GSS laid significantly more eggs (162.50 ± 5.43) when compared with the multiple resistant strain Akita (139.90± 5.54) in a 16-day oviposition period. With imidacloprid treatment, the highest effect was observed with GSS, with a significantly reduced number of eggs in drench (143.40±4.22) and foliar (144.60±5.85) applications. For the strains Akita and

[1] Partly published as Ako, Mouhoube, Hans-Michael Poehling, Christian Borgemeister and Ralf Nauen, 2006. The reproduction of acaricide-resistant and -susceptible strains of Tetranychus urticae Koch (Acari: Tetranychidae) as affected by imidacloprid. *Pest Management Science* 62: 419-424.

USA, no significant differences were observed in oviposition between imidacloprid treatments and control. The proportion of F_1 female offspring decreased significantly with drench application for GSS and WI, while no differences were observed among the strains for the survival of F_1 immature stages, except strain USA. The viability of eggs was relatively high (from 82.9 ± 4.5% for USA to 95.2 ± 1.2% for GSS) and not affected by imidacloprid treatments.

2.2. Introduction

Two-spotted spider mites (TSSM) *Tetranychus urticae* Koch (Acari: Tetranychidae) are pests of greenhouse and field crops, especially under dry and warm conditions (van de Vrie et al., 1972 Ebeling, 1975). TSSM is an extremely polyphagous herbivore, feeding on a wide range of host plant species throughout the world (Navajas, 1998). Damage due to TSSM include a reduction in crop yield as well as aesthetic injuries, because of the webbings produced by proto- and deutonymphs and the adults, particularly in ornamentals. In the field, TSSM are mainly controlled by synthetic acaricides, whereas in greenhouses inundative releases of predatory mites such as *Phytoseiulus persimilis* Athias-Henriot and *Amblyseius andersoni* Chant (Acari: Phytoseiidae) have become the common practice for combating TSSM outbreaks (Amano et al., 1978a, 1978b; Hamlen, and Lindquist. 1981; Field and Hoy, 1984).

One of the most important insecticides used for insect pest control is imidacloprid, belonging to the chemical class of neonicotinoids (Elbert et al., 1990; Nauen et al., 2001, Nauen and Bretschneider 2002). Since its introduction in 1990, imidacloprid has been successfully used for control of sucking insect pests such as aphids and whiteflies, and also several beetle, fly and moth species; however, imidacloprid is not toxic to spider mites (Elbert et al., 1990, 1991). Other neonicotinoids commercialised are acetamiprid, thiamethoxam, thiacloprid, dinotefuran and clothianidin.

Recently field outbreaks of TSSM following acetamiprid (Assail) treatments have been reported (Beers and Himmel, 2002). Moreover, in laboratory experiments, James and Price (2002) recorded a significant increase in egg production in TSSM after spray and systemic applications of imidacloprid at field-relevant dose rates for hop yards (James and Price, 2002). In contrast a significantly reduced oviposition in TSSM following drench or foliar applications of imidacloprid and acetamiprid at field-relevant rates has been observed in another study (Ako et al., 2004). Since the latter authors used a different TSSM strain as James and Price (2002), we hypothesise that the effects of neonicotinoids on the reproduction of TSSM are most likely strain-dependent. Thus the aim of this study was to compare the oviposition in four different TSSM strains after foliar and drench application of imidacloprid at field-relevant dose rates.

2.3. Materials and methods

Plant material

The trials were carried out in a greenhouse at the Institute of Plant Protection and Plant Diseases (IPP), University of Hanover, Germany. Two-week-old French beans (*Phaseolus vulgaris* L., var. Saxa) were used in the experiments. The choice of French beans as host plants is justified by the polyphagious caracter of *T. urticae*, which reproduce and establish very well on a variety of plant species, including French beans. In previous experiments, in which hops (*Humulus lupulus* L.) plants were used as host plant compared with French beans, we observed no substantial variations in terms fecundity or duration egg-adult in *T. urticae* (data not shown). The commercially available substrate “Fruhstorfer Erde Type P” (Archut GmbH, Lauterbach-Wallenrod, Germany) was used as plant growing medium. The substrate is composed of humus, clay, and peat in the proportion of 15:35:50, and due to its high content of peat it has a high water holding capacity. Plants were produced in plastic pots (5 cm diameter, 7cm hight) under greenhouse conditions (25±1°C and 65±5% RH).

Mite strains

Four different TSSM strains were compared, i.e. 'WI' (Wiesmoor), 'GSS' (German Susceptible Strain), 'Akita' and 'USA'. The WI strain is an organophosphate-selected strain maintained in the laboratories of BayerCropscience (Monheim, Germany) since 1954 under a biannual selection with the organophosphate oxydemeton-methyl (0.3g/l) (Nauen et al., 2001). GSS, an acaricide susceptible strain has been maintained in culture since 1965, without exposure to any acaricides (Nauen et al., 2001). Strain USA was originally collected from a back yard in 1999 in Washington State (USA) and was kindly provided by Dr. D. James, Washington State University, and is the same strain as used by James and Price (2002) in their study on effects of imidacloprid on the fecundity of TSSM. Strain Akita is characterised by a stable and inherited resistance to METI (Mitochondrial Electron Transport Inhibitor) acaricides and exhibits a cross-resistance to dicofol (Nauen et al., 2000). All stock cultures are maintained on French beans.

Sticky ring technique

The 'sticky ring' technique was used to keep the mites in a restricted area of the leaf, and hence to facilitate a precise monitoring of their activities (Ako et al., 2004; Nauen et al., 2001). An arena of 3 cm in diameter was created on the upper side of a bean leaf, using a plastic cylinder of 9 cm height and 3 cm diameter coated with a sticky substance made out of a mixture of two different insect trap adhesives (Raupenleim Brunonia, F. Schacht GmbH & Co, Braunschweig, Germany). Individual female mites were transferred into the sticky ring arena, where they could feed and lay eggs.

Synchronized culture of *T. urticae*

In order to obtain homogeneous individuals in terms of age for the experiments, a synchronized mite culture was established on French beans in greenhouse for all strains tested. Twenty mated females collected from the

original cultures of each strain were placed in a sticky ring for six hours. Afterwards, the females were carefully removed with a soft brush while the eggs remained inside the rings. These infested plants were maintained in the greenhouse under the previously described conditions until the progeny had developed into pre-ovipositional females, which were then used in the experiments. In order to avoid the arrhenotokous parthenogenesis in *T. urticae* where unmated females give rise to males only. In order to increase the number of male mites and thereby the mating chance of young pre-ovipositional females, 10 additional males collected from the stock culture were transferred into each sticky ring, shortly before the deuteronymphal stage of the synchronized population (9 days after the eggs were laid).

Bioassays

The commercial imidacloprid formulation Confidor® 200SL (soluble concentrate, 200g a.i./l) (BayerCropscience AG., Monheim, Germany) was used to prepare a solution of 100 ppm imidacloprid to be used for either foliar or drench applications. Prior to the insecticide treatment, leaves of 2-week old bean plants were provided with sticky rings. Young pre-ovipositional females from the synchronized strain cultures were then transferred into the sticky ring with a soft brush. Only one female was added to each sticky ring. The experiment consisted of three treatments (see below), replicated three times. Per replicate and per treatment, 10 individual females were monitored. In total, 30 individual females were tested per treatment. The treated plants were then kept in the greenhouse under the previously described conditions (Ako et al., 2004).

Control or water-only treatment. Control plants were irrigated daily with tap water until the end of the experiments.

Drench application. In order to assure the uptake and translocation of the active ingredient into and within the plant tissues, the drench application was performed 48 hours prior to introducing the mites into the sticky rings. For the same reasons plants were not watered for two days following the insecticide

application; thereafter plants were watered daily with tap water. The drench application consisted of applying 100 ml of insecticide solution (100ppm) directly to the soil in the pots with a pipette (Combitips 50ml, Eppendorf Ltd., Hamburg, Germany).

Foliar application. Plants were sprayed using a manual pressure sprayer (Pico 3235, MESTO®, Freiberg, Germany) with an internal pressure of 3 bars. Before treatment, females were introduced into the sticky rings arena and both sides of the leaves were evenly sprayed with the Confidor® 200SL solution until run-off. To avoid mites being washed off while spraying, the sprayer nozzle was constantly kept at approximately 50 cm distance to the treated leaves.

Fecundity, hatch rate of eggs, larval survival and sex ratio

For the determination of fecundity all eggs were counted every two days for 16 days in total, starting from day 2 after the treatments. Eggs were counted under a stereomicroscope (MZ6, Leica Microsystems, Bensheim, Germany), using a manual counter. After recording the number of eggs, the female mite was carefully transferred from the previous to a new sticky ring on the same leaf with a soft brush. Eggs laid were left untouched in the original sticky ring for subsequent determination of the hatch rate of the eggs, pre-imaginal survivorship and sex ratio of the F_1. The hatch rate of the eggs was determined in the third and sixth ring, 8 days after the eggs were laid. It was calculated by subtracting the number of eggs that did not hatch from the original number of eggs in the ring. The pre-imaginal survivorship was determined by recording the number of offspring, which survived until adulthood in each ring. Larvae that had died in the sticky ring substance were excluded from the analysis. Usually, very low numbers of larvae (less than 10%) are trapped in the sticky ring substance. The sex ratio was calculated as the proportion of female offspring out of the total number of offspring in the third ring (i.e., eight days after the insecticide treatment), two days after the emergence of the adults.

Statistical analyses

Data was analysed by means of analysis of variance (ANOVA), using the general linear model procedure (Proc GLM) in SAS. Initially data were checked for variance homogeneity and transformation were applied whenever necessary. Data on fecundity, recorded as number of eggs, were log-transformed, while data on sex ratio, recorded as percentage of female offspring, were arcsine-square root transformed. In case the ANOVA yielded a significant F-value, means were separated using the Bonferroni (Dunn) *t*-tests at a significance level of α = 0.05.

2.4. Results

Fecundity of TSSM without the impact of imidacloprid

Without imidacloprid treatment, the four TSSM strains differed significantly in their fecundity, expressed as the cumulative number of eggs produced during 16 days (table 2.1).

Table 2.1. Mean (± SE) total fecundity (16 days) of four TSSM strains without imidacloprid treatment.

Mean (± SE) fecundity (16 days) of 4 TSSM strains				Statistics		
GSS	WI	Akita	USA	N	F	p>F
162.5± 5.4a	153.8± 4.8ab	139.9± 5.5b	155.4 ± 6.8ab	120	3.23	0.02

Means within a row followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests.

The highest fecundity was recorded in the GSS strain, with significantly higher number of eggs than in Akita. Approximately similar levels of fecundity were observed in strains USA and WI, which did not differ significantly from GSS or Akita (Table 2.1). In all strains the same ovipositional pattern was observed, with a rapid increase in oviposition from day 2 until day 6, where always almost

the highest numbers of eggs were recorded, and subsequently a constant decrease from day 6 onwards. Since the four strains naturally differed concerning fecundity, the comparisons were done only between treatments within each TSSM strain. The between strains comparison was not statistically feasible, as the strains were basically different from each other in their oviposition.

Effects of imidacloprid on the fecundity of four TSSM strains

In strain WI a foliar, and in GSS also a drench application of imidacloprid significantly reduced the number of eggs laid compared to the untreated control (Tables 2.2 and 2.3) while no such effects were observed in strains Akita and USA (Tables 2.4 and 2.5).

Table 2.2. Mean (± SE) hatch rate of eggs, preimaginal survivorship and sex ratio (± SE) of strain GSS of *T. urticae* treated with imidacloprid at 100ppm

Treatments	Sex ratio (%F)	Survival (% I)	Hatch rate (%)	Fecundity
Control	77.1 ± 3.3a	86.23 ± 3.6a	95.2 ± 1.2a	162.5 ± 5.4a
Drench	60.4 ± 2.4b	82.17 ± 5.1a	91.10 ± 2.2a	143.4 ± 4.2b
Foliar	72.62 ± 3.8a	83.47 ± 3.8a	92.35 ± 2.6a	144.6 ± 5.8b
N	90	89	89	90
df	2, 87	2, 86	2, 86	2, 87
F-Value	6.34	0.78	0.59	6.87
P>f	0.0027	0.4614	0.5567	0.0015

Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests. %F, percentage females; %I, percentage immature stages.

Table 2.3. Mean (± SE) hatch rate of eggs, preimaginal survivorship and sex ratio (± SE) of strain WI of *T. urticae* treated with imidacloprid at 100ppm

Treatments	Sex ratio (%F)	Survival (%I)	Hatch rate (%)	Fecundity
Control	74.5 ± 3.2a	76.4 ± 5.2a	93.2 ± 1.8a	153.8 ± 4.8a
Drench	60.9 ± 3.0b	72.1 ± 5.9a	92.2 ± 2.3a	138.5 ± 4.1ab
Foliar	65.3 ± 3.3ab	69.6 ± 5.9a	88.0 ± 2.3a	136.3 ± 4.9b
N	90	88	87	90
df	2, 87	2, 85	2, 84	2, 87
F Value	4.62	2.30	0.97	3.40
p>F	0.0238	0.1065	0.3831	0.0378

Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests. %F, percentage females; %I, percentage immature stages

Table 2.4. Mean (± SE) hatch rate of eggs, preimaginal survivorship and sex ratio (± SE) of strain Akita of *T. urticae* treated with imidacloprid at 100ppm

Treatments	Sex ratio (%F)	Survival (%I)	Hatch rate (%)	Fecundity
Control	66.8 ±3.7 a	85.0 ± 5.2a	88.3 ±4.2 a	139.9 ± 5.5a
Drench	63.5 ± 3.8a	78.6 ±5.6 a	86.9 ±3.1 a	138.3 ± 5.3a
Foliar	67.3 ± 3.7 a	80.2 ± 5.7 a	84.8 ± 4.8 a	136.1 ± 6.1a
N	90	88	87	90
df	2, 87	2, 85	2, 84	2, 87
F Value	0.90	1.01	0.48	0.19
p>F	0.4104	0.3684	0.6202	0.8272

Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests. %F, percentage females; %I, percentage immature stages.

Table 2.5. Mean (± SE) hatch rate of eggs, preimaginal survivorship and sex ratio (± SE) of strain USA of *T. urticae* treated with imidacloprid at 100ppm

Treatments	Sex ratio (%F)	Survival (%I)	Hatch rate (%)	Fecundity
Control	69.3 ± 3.9a	73.5 ± 5.6a	84.0 ± 3.4a	155.4 ± 6.8a
Drench	64.6 ± 3.2a	63.8 ± 4.6b	85.2 ± 2.5a	148.4 ± 6.5a
Foliar	67.2 ± 3.7a	71.5 ± 6.8ab	82.9 ± 4.5a	146.3 ± 6.8a
N	90	88	88	90
df	2, 87	2, 85	2, 85	2, 87
F Values	0.87	4.98	0.22	1.89
p>F	0.4224	0.0091	0.8027	0.1571

Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests. %F, percentage females; %I, percentage immature stages.

Effects of imidacloprid on the sex ratio, pre-imaginal survivorship, and hatch rate of eggs in four TSSM strains

In strains GSS and WI a drench application of imidacloprid led to a significant decrease in the sex ratio of the F1, i.e. in the proportion of female offspring while no such effects were recorded following a foliar treatment with the insecticide (Tables 2.2 and 2.3). In GSS but not in WI a significantly lower sex ratio was recorded in the drench compared to the foliar application of imidacloprid. In contrast, in both Akita and USA the imidacloprid treatments, i.e. either foliar or drench had no effects on the sex ratio of the F_1 (Tables 2.4 and 2.5). Except for USA, the pre-imaginal survivorship was not significantly affected by either foliar or drench applications of imidacloprid (Tables 2.2, 2.3 and 2.4). In USA, a significantly lower proportion of immature survived in the drench treatment compared to the control; however, no differences were observed in the proportion of surviving immature between the foliar application and the control treatments (Table 2.5). In all four strains the hatch rate of eggs was not influenced by the imidacloprid treatments (Tables 2.2-2.5). In general the highest hatch rate was observed in GSS, followed by WI, Akita and USA.

2.5. Discussion

In this study we compared the oviposition potential of four different strains of TSSM on French bean plants without and with exposure to field relevant dose rates of imidacloprid. In the none-insecticide experiment the four strains significantly differed in their egg laying capacity, which might be due to variations in local environmental factors, which could induce adaptative responses in mite strains in relation to the host plant and the geographic origin of the strain (Egas et al., 2003). Another factor is acaricide resistance in the different strains, which has been characterized recently (Stumpf et al., 2001; Stumpf and Nauen, 2001). Detoxification enzyme activities differ considerably among strains and these higher levels of enzyme activity may result in fitness costs, i.e. reduced fecundity as observed in strain Aktita. The acquired adaptative response, like genotypic modifications, could lead to phenotypic variability in different strains of the same mite species collected in different geographical areas (Navarjas, 1988; Tsagkarakou et al., 1999). The observed differences in oviposition capacity could also suggest differences in the pest potential among the four tested mite strains in their capability of damaging host plants, though no such data were gathered in this study. This is particularly supportable if it is assumed that the immature stage mortality as well as the proportion of female offspring does not vary significantly among the TSSM strains in the natural environment. Our study did not provide any data on the possible variation in oviposition in different temperature scales, which is equally known to be a relevant factor affecting the fecundity in Tetranychidae family (Sabelis, 1981). However, it is known from other investigations that the temperature requirements for optimal spider mite reproduction are ranging between 25°C and 30°C, which fit with our experimental conditions chosen (Sabelis, 1981; Bounfour and Tanigoshi, 2001).

When testing their sensitivity to imidacloprid, all strains of mites exhibited different fecundity levels, when treated by both foliar and drench application using a field relevant dose of imidacloprid. One reason for this observation is

that they were collected from different agro-ecological regions as stated above. Having evolved under different acaricide treatment regimes, the TSSM strains also have developed a resistance or insensitivity to certain acaricides, e.g. strong resistance against METI-acaricides in strain Aktita, but there is no significant influence of imidacloprid on their reproduction (Stumpf and Nauen, 2001). Strain USA has been collected from a back yard in Washington and may have developed a resistance to some commonly used acaricides in this region. Preliminary trials using strain USA with diagnostic doses for a range of acaricides revealed a lower susceptibility to abamectin, but not pyridaben, spirodiclofen and hexythiazox (Nauen, data not shown). Strain Akita was also collected from the field several years ago and has been shown to exhibit a stable and partially dominant inherited resistance to METI acaricides and a cross-resistance to dicofol (Stumpf and Nauen, 2001). Strain GSS in contrast, showed the highest decrease in oviposition following imidacloprid treatments. GSS is a laboratory reference strain, susceptible to numerous commercially available acaricides (Nauen et al., 2001). The absence of any resistance to acaricides in strain GSS and its high sensitivity to imidacloprid support a possible correlation between the insecticide resistance background of a given mite strain and its susceptibility to imidacloprid. This phenomenon has been also observed by Yang et al., (2002) in a trial, in which dimethoate (organophosphate)-exposed *T. urticae* was shown to have developed a 15.9-fold resistant to bifenthrin (pyrethroids) compared with unselected control mites (Yang et al., 2002). In our experiments the relationship between field resistance to acaricides in mites and their susceptibility to imidacloprid has been shown. However, the mechanism, which may lead to this kind of resistance or greater insensitivity to imidacloprid in mites, remains unclear. The insect selective nature of neonicotinoids and the fundamental differences between their mode of action and that of other classes of neuroactive insecticides and acaricides, excludes the chance for a physiologically acquired resistance to imidacloprid (Yamamoto et al., 1998, Tomizawa and Casida, 2003). Neonicotinoids act as nicotinic acetylcholine receptor agonists, at the

post-synaptic membrane, where the active ingredient (e.g. imidacloprid) binds to nicotinic acethylcholine receptors (Nauen et al., 2001; Tomizawa and Casida, 2003; Jeschke and Nauen, 2005). Mechanisms of neonicotinoid resistance are best described in *Bemisia tabaci*, and a major detoxification pathway is the hydroxylation of the 5-membered imidazoline ring system in either 4- or 5-position, less detailed studies were also performed in other target pests (Rauch and Nauen, 2003; Nauen and Denholm, 2005). However, elevated levels of monooxygenases were at least also described in strain Akita and these may serve to explain the different effects on fecundity when compared with strain GSS (Stumpf and Nauen, 2001).

Following the results of the present greenhouse trials, it could be concluded that the ovipositional response of TSSM to field recommended dose rates of imidacloprid is likely strain-dependent. However, none of the strains tested (and particularly not the strain USA) exhibited an increased fecundity in our trial. Therefore it could be concluded simply that the treatment of TSSM with imidacloprid alone is not responsible for the observed phenomenon. However mites may behave differently under the much more complex field situations, where several factors (e.g. climate, wind, agronomic practices, pest resistance, etc.) could interact and modify the mite reaction potential, thus yielding modified and less predictable results as those compared to a greenhouse study under controlled conditions. In summary it is, from this point, still not clear whether imidacloprid and most likely neonicotinoids in general, taken as active ingredients used at their field recommended rates, are the sole factors contributing to propagating mite pests in the field by stimulating their oviposition. For this, the following experiment will provide us with detailed information on the influence of imidacloprid on the fertility life table parameters, which are important indicators of the population dynamics of a given species.

CHAPTER III

3. Determination of Jackknife fertility life table parameters of four *Tetranychus urticae* Koch (Acari: Tetranychidae) strains exposed to imidacloprid [2]

3.1. Abstract

Occasional field increases of two-spotted spider mite (TSSM) (*Tetranychus urticae* Koch), populations consecutive to applications of neonicotinoid insecticides, have gained a special attention because of increasing field damages due to TSSM. In order to elucidate basic mechanisms behind such observations, the influence of soil and foliar applications of imidacloprid on the population growth potential in TSSM greenhouse experiments were carried out, in which the effect of field recommended dose rate of imidacloprid on fertility life table parameters of four different TSSM strains with different acaricide resistance backgrounds, was determined following Jackknife method. The sticky ring technique was used to keep mites in a restricted area on the leaf for a precise monitoring of their activities. The parameters estimated were intrinsic rate of increase (Rm), finite rate of increase (λ), the net reproductive rate (R_0), the doubling time (Dt) and the mean generation time (T). Results showed overall higher influence of imidacloprid on fertility life table parameters, when used as drench application compared to foliar treatment and control. However, the pesticide resistance backgrounds in TSSM strains did not greatly affect the fertility life table parameters as suspected. In all tested

[1] To be published as Ako, Mouhoube, Hans-Michael Poehling, Christian Borgemeister and Ralf Nauen. Determination of Jackknife fertility life table parameters of four *Tetranychus urticae* Koch (Acari: Tetranychidae) strains exposed to imidacloprid in *Pest management Science.*

TSSM strains, Rm, λ, and R_0 were higher in control treatments compared with drench application, while foliar application did not differ significantly from the control treatment, only for Rm and λ. In addition, Dt for TSSM population was shorter in control compared with drench but not with foliar application. No significant variations were observed in mean generation time (T) among the treatments and TSSM strains. Hence, the laboratory data could not support the field observations. The possible role of other field specific factors such as pest control strategies, inorganic soil fertility management and their possible combination with neonicotinoids are discussed.

3.2. Introduction

Imidacloprid is a worldwide used neuroactive insecticide, which belongs to the neonicotinoid or chloronicotinyl class of insecticides, exhibiting systemic and contact activity against a wide range of phytophagous insect pests. Neonicotinoids are nicotinic acethylcholine receptor agonists at the postsynaptic membrane (Nauen et al., 2001; Nauen and Bretschneider 2002) and are described as selective for insects (Matsuda et al., 2005; Tomizawa et al., 2000; Tomizawa and Casida, 1999, 2003; Nauen et al., 1998; Yamamoto et al., 1998) and as harmless to phytophagous mites (Elbert et al., 1990, 1991). Imidacloprid, the most important representative of the group is commonly used in diverse cropping systems to control sucking insect pests such as aphids and whiteflies, but is also applied against some important coleopteran and moth pests (Elbert et al., 1998).

A decade after their introduction to the market, neonicotinoids such as acetamiprid and imidacloprid have been reported to occasionally boost spider mite populations, when used against insect pests. Several reports have described abnormal increase of TSSM populations, consecutive to neonicotinoid insecticide applications to control aphids and/or whiteflies (Raupp et al., 2004; James and Prices, 2002; Beer et al., 2005; Sclar et al., 1998).

The reasons for neonicotinoid-stimulated population outbreaks in TSSM remain unknown, although hormoligosis and/or adverse effects on natural enemies, with a possible combination with agricultural practices such as soil fertility management could be possible reasons (Altieri and Nicholls, 2003, Chaboussous, 1970). Moreover, Beer et al., 2005 reported that the number of annual applications of acetamiprid was positively correlated with the increase in TSSM population in the field, which could indicate some kind of additive effect. They concluded that reducing the number of applications might limit the population increase of TSSM.

Since the onset of field TSSM outbreaks following the use of neonicotinoids, several greenhouse trials have been undertaken in order to find out possible factors and mechanisms, which could be the reason for such observations. Greenhouse experiments designed to determine the effects of four different neonicotinoid insecticides on the bionomics of a standard TSSM strain yielded no conclusive results in terms of population increase of TSSM (Ako et al., 2004). In another experiment (see chapter 2), the reproductive responses to imidacloprid of different TSSM strains with different acaricide backgrounds was evaluated and no evidences of potential TSSM population increases were observed (Ako et al., 2006). Sclar et al. (1998) have found no significative increase in TSSM population while studying the effect of soil application of imidacloprid on TSSM under greenhouse conditions. However, the same authors observed a significant increase in TSSM population when setting up the experiments in the field.

The conclusion out of these observations is that the enhancement of field TSSM populations following neonicotinoid applications may be a phenomenon, which is due to complex interactions most probably involving several environmental factors and probably climatic conditions (Altieri and Nicholls, 2003, Chaboussous, 1970).

An attempt to shade light on the phenomenon may require stepwise studies of the whole system, first with details of pest-pesticide interaction, later focusing on environmental factors. Therefore, first basic knowledge such as a detailed

study of the effects of neonicotinoids on fertility life table parameters of TSSM in a controlled environment is required as starting point. Hence, the present trial aimed at providing a complete estimation of the Jackknife fertility life table parameters of four different TSSM strains with different acaricide resistance backgrounds, when exposed to imidacloprid.

3.3. Materials and methods

Plant materials

The trials were conducted in a greenhouse at the Institute of Plant Protection and Plant Diseases (IPP), University of Hannover, Germany. Two-week-old French bean plants (*Phaseolus vulgaris* L., var. Saxa) were used in the experiments as hosts for *T. urticae.* The substrate "Fruhstorfer Erde Type P" (Archut GmbH, Lauterbach-Wallenrod, Germany) was used as plant growing media. The substrate is composed of humus, clay, and peat in the proportion of 15:35:50, and due to its high content of peat it has a high water holding capacity. Plants were produced in plastic pots (5 cm in diameter, 7cm in height) under greenhouse conditions (25±1°C and 65±5% RH).

Mite materials

Four different TSSM strains i.e. 'WI' (Wiesmoor), 'GSS' (German Susceptible Strain), 'Akita' and 'USA', were compared. The WI strain is an organophosphate-selected strain maintained in the laboratories of Bayer CropScience (Monheim, Germany) since 1954 under a biannual selection with the organophosphate oxydemeton-methyl (Nauen et al., 2001). GSS, a standard acaricide susceptible strain has been maintained in culture since 1965, but without exposure to any acaricides (Nauen et al., 2001). The USA strain was originally collected from a back yard in Washington State (USA) and kindly provided by Dr. D. James, Washington State University, and is the same strain as used by James and Price (2002) in their study on the effects of imidacloprid on the fecundity of TSSM. Akita is characterised by a stable

resistance to METI (Mitochondrial Electron Transport Inhibitor) acaricides and exhibits a cross-resistance to dicofol (Stumpf and Nauen, 2001).

Bioassays

The commercial imidacloprid formulation Confidor® 200SL (Bayer CropScience Ltd., Monheim, Germany) was used to prepare a solution of 100 ppm imidacloprid (active ingredient) to be used as either foliar or drench application. Prior to the insecticide treatment, leaves of 2-week old bean plants were provided with sticky rings.

The sticky rings (Stumpf and Nauen, 2001; Ako et al., 2004; 2006) were used to keep the mites in a restricted area of the leaf, and hence to facilitate a precise monitoring of their activities. A sticky ring, (3 cm in diameter) was created on the upper side of a bean leaf, using a plastic cylinder (9 cm in height and 3 cm in diameter) coated with a sticky substance made out of a mixture of two different insect trap adhesives (Raupenleim Brunonia, F. Schacht GmbH & Co Kg, Braunschweig, Germany). Individual female mites were transferred into the sticky ring arena.

A synchronised culture of each TSSM strain was established in order to obtain a homogeneous population in terms of age. To increase the mating chance of females, because of the arrhenotokous parthenogenesis in *T. urticae* (Helle and Overmeer 1973), males collected from the stock culture were added to the synchronised culture at deuteronymplal stage. Young pre-ovipositional females from the synchronized strain cultures were transferred individually into the sticky ring with a soft brush, where they could feed and lay eggs. Each experiment consisted of three treatments (see below), repeated three times with 10 females per replicate, in a completely randomised design. The treated plants were then kept in the greenhouse under the previously described conditions.

Control or water-only treatment. Control plants were irrigated daily with tap water until the end of the experiments.

Drench application. In order to assure sufficient uptake and translocation of the active ingredient into and within the plant tissues respectively, the drench application was performed 48 hours prior to introducing the mites into the sticky rings. For the same reason plants were not watered for two days following the insecticide application; thereafter plants were watered daily with tap water. The drench application consisted of applying 100 ml of a Confidor® 200SL solution directly to the soil in the pots with a pipette (Combitips 50ml, Eppendorf Ltd., Hamburg, Germany).

Foliar application. Plants were sprayed using a manual pressure sprayer (Pico 3235, MESTO®, Freiberg, Germany) with an internal pressure of 3 bar. First, females were introduced into the sticky rings. Thereafter, both sides of the leaves were evenly sprayed with the Confidor® 200SL solution until the formation of suspended droplets at the edge of the leaves. To avoid mites being washed off, while spraying the nozzle was constantly kept at approximately 50 cm distance to the treated leaves.

Fertility life table parameters

The determination of fertility life table parameters, which are used to estimate the population growth, was done after the treatments. The following parameters were estimated: (1) The net reproductive rate (Ro), (2) the intrinsic rate of increase (r_m) (3) the mean generation time (T), (4) the doubling time (Dt) and (5) the finite rate of increase (λ).

The information needed for construction of fertility life table parameters are as followed (Maia et al., 2000):

- *Number of females per group or cohort*: It is the number of mated females in each group or replicate i.e. cohort.
- *Initiation of adult stage*: It is the time interval between the day of oviposition that yielded a female i of a group, and the day when this female becomes an adult. The obtention of young pre-ovipositional females mites required 12 days of successive developmental stages for the experimental conditions chosen.

- *Longevity*: It is a time interval between the day of oviposition that yielded a female of a group and the day of its death.
- *Number of eggs*: The number of eggs laid by a female i of a group in each pivotal age x. The pivotal age corresponds to the age of female + 0.5, the day on which eggs were counted. The addition of 0.5 is a requirement of the program. It represents the midpoint of the interval between two pivotal ages.
- *Total number of eggs*: The total number of eggs laid by all females of a group G at a pivotal age.
- *Ratio of females:* The ratio of females is calculated as the number of females divided by total number of offspring in the same sticky ring.
- *Immature stage survivorship*: The pre-imaginal survivorship is the number of offspring females that survived until adulthood.

Three groups of ten female spider mites, taken from the synchronised culture were placed individually into sticky rings and observed every two days for oviposition. After the number of eggs was recorded, the experimental mite was carefully transferred into a new ring in such a way to keep eggs untouched in the former ring for subsequent observations. The eggs were counted for the whole lifetime of the experimental mites. Eggs were kept on leaves until hatching and the pre-imaginal stages were carefully monitored until the adult stage. The pre-imaginal survivorship was then determined as the number of individuals, which reached adulthood, out of the total number of emerged larva. The ratio of females was calculated as the proportion of females out of the total number of individuals (males and females) that reached adulthood. The experiment was replicated three times overtime with a group of 10 females per replicate and per treatment.

Statistical analyses

The algorithms developed in SAS/STAT by Maia et al. (2000) were used to compute the fertility life table parameters. The advantages of this method over the MS DOS-based program developed by Hulting et al. (1990) is the possibility to obtain all parameters with their respective confidence intervals.

Moreover a multiple comparison approach (done using the t-test for pairwise comparison) with more than two treatments, like in the present case, was made possible by this method and was automatically computed along with other parameters. For details on data sheet organization refer to Maia et al. (2000).

3.4. Results

Doubling time

The doubling time (Dt) of a TSSM population was significantly lower in the control when compared with drench application for all TSSM strains tested (Tables 3.1-3.4). Wheras no differences were observed in Dt between control and foliar application in GSS and Akita, Dt was shorter in foliar application compared with drench application in both strains (Tables 3.1 & 3.3). In strain WI, Dt was lower in control compared with foliar application (Table 3.2) while no significant differences were observed in strain USA for both treatments (Table 3.4). Without insecticide treatment, no significant differences were observed in Dt among the strains, except between GSS and USA (Table 3.5)

Intrinsic rate of increase

The intrinsic rate of increase of TSSM populations (Rm) was significantly higher in the control compared with drench application in all tested TSSM strains (Tables 3.1-3.4). Contrary to strain WI, the control treatment did not differ significantly from foliar application in Rm for both GSS and Akita (Tables 3.1, 3.2 & 3.3). For strain USA, neither did the control nor drench application vary significantly in comparison with foliar application (Table 3.4). For both GSS and Akita, Rm values were higher in foliar application compared with drench treatment (Tables 3.1&3.3). Without insecticide treatment, Rm did not differ significantly among the strains except between GSS and USA (Table 3.5).

Table 3.1. Population dynamics parameters of strain GSS as affected by imidacloprid: t-test for pairwise (group) comparison of treatments (C: Control, D: drench; f: foliar application) of Jackknife estimates of doubling time (D_T), finite rate of increase (λ), intrinsic rate of increase (R_m), net reproductive rate (R_0) and mean generation time (T)

Para.	GA	GB	Mean A	SE A	Mean B	SE B	P_T
D_T	C	D	2.8600	0.0508	3.0167	0.0206	0.0332*
D_T	C	F	2.8600	0.0508	2.8935	0.0137	0.5505
D_T	D	F	3.0170	0.0206	2.8935	0.0137	0.0016*
λ	C	D	1.2740	0.0055	1.2583	0.0019	0.0420*
λ	C	F	1.2740	0.0055	1.2707	0.0014	0.5682
λ	D	F	1.2580	0.0019	1.2707	0.0014	0.0013*
R_m	C	D	0.2420	0.0043	0.2298	0.0015	0.0413*
R_m	C	F	0.2420	0.0043	0.2395	0.0011	0.5662
R_m	D	F	0.2300	0.0015	0.2395	0.0011	0.0013*
R_0	C	D	132.8040	4.4610	85.6146	3.6470	0.0000*
R_0	C	F	132.8040	4.4610	96.3072	4.1271	0.0003*
R_0	D	F	85.6150	3.6470	96.3072	4.1271	0.0887
T	C	D	20.1690	0.4953	19.3698	0.2976	0.2118
T	C	F	20.1690	0.4953	19.0705	0.2360	0.0944
T	D	F	19.3700	0.2976	19.0705	0.2360	0.4546

* Significant two-tailed *P* values for pairwise group comparison.

Para.: parameters; GA: group A; GB: group B; Mean A, mean group A; Mean B mean group B, SE A; standard error of Mean A; SE B, standard error Mean B; P_T, two-tailed probability.

Table 3.2. Population dynamics parameters of strain WI as affected by imidacloprid: t-test for pairwise (group) comparison of treatments (C: Control, D: drench; f: foliar application) of Jackknife estimates of doubling time (D_T), finite rate of increase (λ), intrinsic rate of increase (R_m), net reproductive rate (R_0) and mean generation time (T)

Para.	GA	GB	Mean A	SE A	Mean B	SE B	P_T
D_T	C	D	2.9120	0.02150	3.0093	0.02546	0.02008*
D_T	C	F	2.9120	0.02150	3.0217	0.03198	0.02495*
D_T	D	F	3.0090	0.02546	3.0217	0.03198	0.76880
λ	C	D	1.2690	0.00223	1.2590	0.00246	0.01937*
λ	C	F	1.2690	0.00223	1.2578	0.00307	0.02254*
λ	D	F	1.2590	0.00246	1.2578	0.00307	0.76688
R_m	C	D	0.2380	0.00176	0.2303	0.00195	0.01942*
R_m	C	F	0.2380	0.00176	0.2294	0.00244	0.02277*
R_m	D	F	0.2300	0.00195	0.2294	0.00244	0.76707
R_0	C	D	111.1500	3.57760	85.8504	2.85172	0.00066*
R_0	C	F	111.1500	3.57760	78.7290	3.64305	0.00022*
R_0	D	F	85.8500	2.85172	78.7290	3.64305	0.16446
T	C	D	19.7940	0.27208	19.3326	0.26392	0.25867
T	C	F	19.7940	0.27208	19.0361	0.38541	0.15128
T	D	F	19.3330	0.26392	19.0361	0.38541	0.54559

* Significant two-tailed *P* values for pairwise group comparison.

Para., parameters; GA, group A; GB, group B; Mean A, mean group A; Mean B mean group B, SE A; standard error of Mean A; SE B, standard error Mean B; P_T, two-tailed probability.

Table 3.3. Population dynamics parameters of strain Akita as affected by imidacloprid: t-test for pairwise (group) comparison of treatments (C: Control, D: drench, F: foliar application) of Jackknife estimates of doubling time (D_T), finite rate of increase (λ), intrinsic rate of increase (R_m), net reproductive rate (R_0) and mean generation time (T)

Para.	GA	GB	Mean A	SE A	Mean B	SE B	P_T
D_T	C	D	2.9432	0.01821	3.0616	0.01660	0.00138*
D_T	C	F	2.9487	0.01821	2.9432	0.02215	0.85202
D_T	D	F	3.0616	0.01660	2.9487	0.02215	0.00417*
λ	C	D	1.2655	0.00184	1.2541	0.00154	0.00152*
λ	C	F	1.2655	0.00184	1.2650	0.00224	0.85057
λ	D	F	1.2541	0.00154	1.2650	0.00224	0.00501*
R_m	C	D	0.2355	0.00145	0.2264	0.00123	0.00150*
R_m	C	F	0.2355	0.00145	0.2351	0.00177	0.85072
R_m	D	F	0.2264	0.00123	0.2351	0.00177	0.00491*
R_0	C	D	90.3638	3.21297	77.5190	2.98800	0.01918*
R_0	C	F	90.3638	3.21297	83.8860	2.98887	0.17833
R_0	D	F	77.5190	2.98800	83.8860	2.98887	0.17036
T	C	D	19.1257	0.26146	19.2186	0.25884	0.80716
T	C	F	19.1257	0.26146	18.8456	0.24574	0.45758
T	D	F	19.2186	0.25884	18.8456	0.24574	0.32669

* Significant two-tailed *P* values for pairwise group comparison.

Para., parameters; GA, group A; GB, group B; Mean A, mean group A; Mean B mean group B, SE A; standard error of Mean A; SE B, standard error Mean B; P_T, two-tailed probability.

Table 3.4. Population dynamics parameters of strain USA as affected by imidacloprid: t-test for pairwise (group) comparison of treatments (C: Control, D: drench; f: foliar application) of Jackknife estimates of doubling time (D_T), finite rate of increase (λ), intrinsic rate of increase (R_m), net reproductive rate (R_0) and mean generation time (T)

Para.	GA	GB	Mean A	SE A	Mean B	SE B	P_T
D_T	C	D	3.0184	0.04045	3.1498	0.02852	0.03193*
D_T	C	F	3.0184	0.04045	3.0652	0.02305	0.35153
D_T	D	F	3.1498	0.02852	3.0652	0.02305	0.05133
λ	C	D	1.2581	0.00386	1.2461	0.00249	0.03605*
λ	C	F	1.2581	0.00386	1.2537	0.00214	0.35976
λ	D	F	1.2461	0.00249	1.2537	0.00214	0.05000
R_m	C	D	0.2296	0.00307	0.2200	0.00200	0.03558*
R_m	C	F	0.2296	0.00307	0.2261	0.00170	0.35890
R_m	D	F	0.2200	0.00200	0.2261	0.00170	0.05010
R_0	C	D	89.4146	2.53367	76.0500	2.98155	0.00951*
R_0	C	F	89.4146	2.53367	78.7842	3.06508	0.02911*
R_0	D	F	76.0500	2.98155	78.7842	3.06508	0.54043
T	C	D	19.5694	0.20512	19.6852	0.31735	0.76844
T	C	F	19.5694	0.20512	19.3125	0.30498	0.50714
T	D	F	19.6852	0.31735	19.3125	0.30498	0.42184

* Significant two-tailed *P* values for pairwise group comparison.
Para., parameters; GA, group A; GB, group B; Mean A, mean group A; Mean B mean group B, SE A; standard error of Mean A; SE B, standard error Mean B; P_T, two-tailed probability; P_L, lowertailed probability; P_U, uppertailed probability.

Table 3.5. Population dynamics parameters of strain GSS, WI, Akita and USA without insecticide treatment: t-test for pairwise (group) comparison of Jackknife estimates of D_T, λ, R_m and R_0

Para.	GA	GB	Mean A	SE A	Mean B	SE B	P_T
D_T	GSS	WI	2.860	0.05089	2.912	0.02150	0.38195
D_T	GSS	AKI	2.860	0.05089	2.943	0.01821	0.18255
D_T	GSS	USA	2.860	0.05089	3.018	0.04045	0.04187*
D_T	WI	AKI	2.912	0.02150	2.943	0.01821	0.30382
D_T	WI	USA	2.912	0.02150	3.018	0.04045	0.05888
D_T	AKI	USA	2.943	0.01821	3.018	0.04045	0.14483
λ	GSS	WI	1.274	0.00553	1.269	0.00223	0.39876
λ	GSS	AKI	1.274	0.00553	1.266	0.00184	0.19957
λ	GSS	USA	1.274	0.00553	1.258	0.00386	0.04772*
λ	WI	AKI	1.269	0.00223	1.266	0.00184	0.30529
λ	WI	USA	1.269	0.00223	1.258	0.00386	0.05208
λ	AKI	USA	1.266	0.00184	1.258	0.00386	0.13471
R_m	GSS	WI	0.242	0.00434	0.238	0.00176	0.39691
R_m	GSS	AKI	0.242	0.00434	0.235	0,00145	0.19768
R_m	GSS	USA	0.242	0.00434	0.230	0,00307	0.04698*
R_m	WI	AKI	0.238	0.00176	0.235	0.00145	0.30512
R_m	WI	USA	0.238	0.00176	0.230	0.00307	0.05274
R_m	AKI	USA	0.235	0.00145	0.230	0.00307	0.13572
R_0	GSS	WI	132.804	4.46101	111.150	3.57760	0.00581
R_0	GSS	AKI	132.804	4.46101	90.364	3.21297	0.00009*
R_0	GSS	USA	132.804	4.46101	89.415	2.53367	0.00011*
R_0	WI	AKI	111.150	3.57760	90.364	3.21297	0.00261*
R_0	WI	USA	111.150	3.57760	89.415	2.53367	0.00151*
R_0	AKI	USA	90.364	3.21297	89.415	2.53367	0.82267

* Significant two-tailed *P* values for pairwise group comparison.

Para., parameters; GA, group A; GB, group B; Mean A, mean group A; Mean B mean group B, SE A; standard error of Mean A; SE B, standard error Mean B; AKI, strain Akita; P_T, two-tailed probability.

Finite rate of increase

For all tested TSSM strains the finite rate of population increase (λ) values were higher in the control compared with drench application (Tables 3.1-3.4). While λ did not vary between control and foliar application for GSS and Akita (Tables 3.1&3.3), it was significantly higher in control treatment compared with foliar application for strain WI (Table 3.2). A decrease was observed in λ with drench application compared with foliar application for both GSS and Akita strains (Tables 3.1&3.3), whereas no variations were observed in λ for WI and USA strains with the same treatments (Table 2&4). For strain USA, λ didn't vary between control and foliar application (Table 3.4). Without insecticide treatment, significant differences were observed in λ only between GSS and USA (Table 3.5)

Net reproductive rate

The net reproductive rate (R_0) or the number of female offspring per female was higher in the control compared with either drench or foliar application in all the tested TSSM strains (Tables 3.1, 3.2 & 3.4), except Akita, for which the control treatment did not differ from foliar application (Table 3.3). The proportions were 35.5, 22.7, 14.9 and 13.8% more female offspring in control compared with drench application, respectively for GSS, WI, USA and Akita respectively. The net reproductive rate did not vary in foliar application compared with drench application in all tested strains. Without insecticide treatment R_0 was significantly higher in GSS compared with Akita and USA but not with WI. Moreover, WI differed significantly from Akita and USA in R_0 values (Table 3.5).

The mean generation time

The mean generation time (T) was not affected by the treatments for all TSSM strains (Tables 3.1-3.4).

3.5. Discussion

The life table parameters obtained from the present field-simulated greenhouse trials are overall not supporting the general hypothetical concept linking the population increase of TSSM to application of imidacloprid, particularly in a greenhouse with above described conditions, using field recommended dose rates. The estimate of population growth of TSSM on the basis of fertility life table parameters obtained from the present greenhouse experiments consistently showed a decrease in TSSM population with drench application of imidacloprid, particularly obvious from the intrinsic and finite rates of population increase in different strains. Moreover, no important variations were observed in fertility life table parameters with different TSSM strains, contrary to previous greenhouse trials, in which bionomics of TSSM were studied and where acaricide resistant strain Akita showed a relatively lover fecundity, compared with the reference strain (GSS) (Ako et al., 2006). The life table parameters of TSSM and other mite species in relation with insecticides in general are very poor documented and for neonicotinoids the literarature on these parameters does not exist probably because of the recent nature of the phenomenon neonicotinoids/TSSM. However azadirachtin was the only insecticide in the literature, in relation with which the life table parameters of TSSM were studied. The results were similar as those obtained with imidacloprid treatments, with lower λ, R_m and R_0. with azadirachtin-treated TSSM compared with untreated individuals (Martínez-Villar et al., 2005).

Contradictory results have been already reported between greenhouse and field trials while studying the population dynamics of TSSM under the influence of neonicotinoids. In greenhouse trials, Sclar et al. (1998) observed no effects of soil application of imidacloprid on TSSM populations or damage caused by mites, although they reported significantly greater damage by TSSM in the field after a soil application of the same insecticide. The authors rejected the hypothesis of hormoligosis and phytotoxicity and concluded that the adverse effects on predators may be important reason for the increase in spider mite injury and abundance after soil application of imidacloprid in the field.

These contradictory results in the effects of neonicotinoid insecticides on population outbreaks of TSSM between field trials (James and Price, 2002, Beers and Himmel, 2002) and greenhouse experiments (Sclar et al., 1988, Ako et al., 2004) suggest the existence of determinant field factors, which may act in combination with insecticides to yield the TSSM outbreak.

The control of insect pests using efficient and selective insecticides like neonicotinoids, may lead to an important decrease in insect population, thus eliminating potential competitors of spider mites in terms of food and space on plant leaves, hence reducing inter-specific competition. The reduced inter-specific competition between insects and spider mites sharing the same biotope affects evidently the mite population outbreak after neonicotinoids application. Moreover, an efficient elimination of insects using insecticides, which are known to be less toxic to herbivorous mites (Elbert et al., 1991, 1998), may act synergistically with a reduced inter-specific competition to yield an increase in spider mite population, wherever neonicotinoids or any insecticide exhibiting the same characteristics are used to control insects. Another synergistic factor for TSSM population increases in the field could be inorganic soil amendment, especially with nitrogen fertilisation. The relationship between intensive use of inorganic fertilizers and susceptibility of plants to pests and diseases is fully documented (Henneberry, 1962; Barker, 1975; Luna, 1988; Altieri et al., 1990, 1998; Letourneau et al., 1996; Brodbeck et al., 2001). This topic will be discussed more in details in the following chapters.

The life table parameter of TSSM as obtained in the present greenhouse experiments in relation with imidacloprid treatments are not helping for the interpretation of the phenomenon of field populations increase of TSSM consecutive to the use of imidacloprid in the field. Results obtained from trials in controlled environments could once again support the hypothesis that the factors contributing to abnormal increases in TSSM population after neonicotinoid insecticides' application may be tied to some field factors and/or their synergistic interactions with neonicotinoids. Of these factors are intrinsic

characteristics of different TSSM strains like pesticide resistance/susceptibility, as the observed field increases of TSSM were consistently reported only in certain regions (in the USA) and that the previous experiments (See chapter 2) showed important differences in fecundity in different TSSM strains collected from different regions

Looking into the biochemical implications of imidacloprid on TSSM like stress enzyme activities would provide additional information, which could be used as starting point for subsequent physiological and biochemical investigations. For this, the following experiment will focus on the variation in stress enzymes activities in TSSM as affected by imidacloprid.

CHAPTER IV

4. Effect of imidacloprid on carboxylesterase and superoxide dismutase activities in two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) [3]

4.1. Abstract

The effects of neonicotinoid insecticides on the reproduction of two-spotted spider (TSSM) mite *Tetranychus urticae* Koch are a problem of concern especially in orchards and hops. In order to help elucidate the effects of imidacloprid on the physiology of TSSM, greenhouse experiments were carried out to assess general carboxylesterase (CarE) and superoxide dismutase (SOD) activities in TSSM on first (day1), third (day3) and sixth (day6) after treatment (AT). Results showed higher CarE activity in foliar application (F) compared with control (C) and drench application (D) on day1 AT, with 66.87±2.63mOD/min/mite (mOD) (mOD = milli-optical density), while no differences were observed between C (51.45±2.33mOD) and D (53.66±2.07mOD). On day3 AT, CarE activity varied significantly in all treatments, with increased activity to 73.59±1.97mOD in F, whereas D and C showed respectively 66.45±2.26mOD and 57.61±1.72mOD. No differences were observed in CarE activity among the treatments, on day6 AT. SOD activity didn't vary among the treatments on day1 and day6 AT. On day 3 AT, higher SOD activity was observed in F (154.01±2.48mOD) compared with both D (162.40±2.80mOD) and C (168.92±2.59mOD), which didn't differ from each other.

[3] To be published as Ako, Mouhoube, Hans-Michael Poehling and Ralf Nauen. Effect of imidacloprid on carboxylesterase and superoxide dismutase activities in two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) in *Pesticide Biochemistry and Physiology*

In addition, the total protein concentration was assessed in individual mites. Results showed no significant variations in total protein concentration among the treatments, with 2.08±0.09; 2.05±0.08 and 2.03±0.10 μgProtein/ml for C, D and F respectively.

4.2. Introduction

Carboxylesterase (CarE) and superoxide dismutase (SOD) belong to a wide range of detoxifying and protective enzymes present in living cells (Gregory et al., 1974, Fridovich, 1977). Their involvements in biochemical detoxification processes and protection of living cells against harmful effects of carboxylesters and reactive oxygen species respectively are well established (Perrin et al., 1990; Sogorb and Vilanova, 2002, Wheelock et al., 2004). Carboxylesterases are a class of enzymes that hydrolyze compounds, which contain ester bonds to the corresponding alcohol and carboxylic acid (hydrolysis products) (Satoh and Hosokawa, 1998; Wheelock et al., 2005a; 2005b). These enzymes play an important role in the metabolism and subsequent detoxification of many xenobiotic and endogenous compounds, including pyrethroids and OPs (Wheelock et al., 2005b; Abernathy and Casida, 1973). Besides CarE, living cells produce a number of protective enzymes of which SOD, which is an important antioxidant agent present in all oxygen-metabolizing cells (Gregory et al., 1974). SOD protect cells from damages due to reactive oxygen species i.e. hydroxyl radicals, superoxide, hydrogen peroxide, and peroxynitrite (Fiers et al., 1999; Nicholls and Budd, 2000).

In many insect species i.e. peach-potato aphid, *Myzus persicae* Sulzer and the tobacco aphid, *Myzus nicotianae* Blackman, metabolic resistance to organophosphate, carbamates and pyrethroids was shown to be associated to elevated levels/activities of carboxylesterases (Nauen and Elbert, 1997; Field and Devonshire, 1992; Abdel-Aal et al., 1992; Devonshire and Field, 1991; Devonshire, 1989). However, other abiotic factors like toxic metals, i.e. zink and cadnium were shown to enhance CarE activity in carabid beetle *Poecilus cupreus*, (Wilczek et al., 2003), suggesting that some non-carboxylester

compounds can lead to an increased CarE activity. The elevated antioxidant enzymes i.e. SOD activity has been shown to be closely related to the resistance of organisms to unfavorable environments either physical (exposure to cold, heat) or chemical (exposure to chemicals) conditions (Packer, 1984, Grubor-Lajsic et al., 1997). Recently, Zhang et al. (2004) reported increased SOD activity in carmine spider mite *Tetranychus cinnabarinus* (Boisduvals) exposed to simulated acid rain prepared at different pH levels.

In previous *in planta* experiments, the bionomics of two-spotted spider mite (TSSM) (*Tetranychus urticae* Koch) exposed to field relevant dose rates of imidacloprid were studied (Ako et al., 2004, 2006). Results showed significant variations in oviposition and sex ratio in TSSM in two imidacloprid-treated mites strains with no or little acaricide resistance background; and because higher detoxification enzymes activities have been linked to resistance in insects and mites to pesticides and/or unfavorable environmental conditions, we hypothesized that looking into the effects of imidacloprid on the activities of important stress enzymes such as CarE and SOD of TSSM would provide us with additional information of physiological implications of imidacloprid on TSSM. Therefore, the present experiment aimed at assessing general carboxylesterase and superoxide dismutase activities as affected by imidacloprid applications.

4.3. Materials and methods

French bean plants and spider mites

French bean (*Phaseolus vulgaris* L.) plants (Saxa) were used in all experiments. Plants were produced under greenhouse conditions at 25±1°C, 65±5%RH and a photoperiod of 16:8 (L:D). Seeds were sown in pot (7 cm in height and 5 cm in diameter) filled with the commercially available substrate “Fruhstorfer Erde Type P” (Archut GmbH, Lauterbach-Wallenrod, Germany) and used for the experiments two weeks later at 2 leaves stage.

The strain Wiesmor (WI) of two spotted spider mites (TSSM) was used in the trials. The strain is an organophosphate-selected strain maintained in the

laboratories of BayerCropscience (Monheim, Germany) since 1954 under a biannual selection with the organophosphate oxydemeton-methyl (Nauen et al., 2001).

Bioassay

The commercial imidacloprid formulation Confidor® 200SL (Soluble Concentrate) (BayerCropscience Ltd., Monheim, Germany) was used to prepare a solution of 100 ppm imidacloprid to be used as either foliar or drench applications.

In order to obtain a homogeneous population to reduce systematic errors due to age of experimental mites, a synchronised culture of TSSM was established. For this, 20 young mated female mites were placed in a sticky ring (Stumpf and Nauen, 2001, Ako et al., 2004, 2006) on a bean leaf for 6 h. Thereafter, the adults were removed, and the offspring were kept under greenhouse conditions at 25±°C and 65±5% RH. The F 1 offspring were maintained on the leaves until the first adults emerged, which under the above-mentioned conditions took 12 days. To increase the mating chance of females, because of the arrhenotokous parthenogenesis in *T. urticae* (Helle and Overmeer 1973), males collected from the mother culture were added to the synchronised culture at deteuronymphal stage. Young pre-ovipositional females from the synchronized strain cultures were transferred onto French bean leaves using a soft brush at a rate of 10 mites per leaf. Thereafter, plants were subjected to different treatments as described below.

Control or water-only treatment. Control plants were irrigated daily with tap water until the end of the experiments.

Drench application. In order to assure the uptake and translocation of the active ingredient into and within the plant tissues, the drench application was performed 48 hours prior to introducing the mites into the sticky rings. For the same reasons plants were not watered for two days following the insecticide application; thereafter plants were watered daily with tap water. The drench application consisted of applying 100 ml of imidacloprid solution directly to the

humus earth in the pots with a pipette (Combitips 50ml, Eppendorf Ltd., Hamburg, Germany).

*Foliar application***.** The foliar application was carried out in an automated spray chamber (dimensions 120cm x 100m x 80cm; Hürner/M Kunststoff Anlagen, Germany) equipped with four multidirectional nozzles. One hundred ml of insecticide solution were sprayed at a pressure of $1.5x10^5$ Pa. The treated plants were kept in the greenhouse under the same conditions as described above.

Enzyme preparation

The enzyme preparation was performed on first (day1), third (day3) and sixth day (day6) after treatment (AT). For that, mites from treated plants were collected at appointed dates for laboratory essays for assessing CarE and SOD activities. The chemical products used in CarE and SOD essays i.e. triton-X-100, α-naphthylacatate, Fast Blue RR salt, Phenazine Methosulfate (PMS), Nitroblue Tetrazolium (NBT), Ethylene Diamine Tetraacetic Acid (EDTA) and Nicotinamide Adenine Dinucleotide (NADH) were purchased from Sigma (Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany).

Carboxylesterase

The total CarE activity was essayed according to the method described by Stumpf and Nauen (2002) and Rauch and Nauen (2003). Mites were homogenized individually with a plastic pestle in an Eppendorf tube containing 30 µl ice-cold sodium phosphate buffer (0.1 mol/l, pH 7.6) mixed with 0.1% triton-X-100. The crude homogenate was incubated for 10 min and centrifuged at 10.000 g for 10 min at 4°C in a freezing centrifuge (Microfuge® R, Beckman, Krefeld, Germany). The resulting supernatant was used in essay for enzyme activity as source of enzyme.

Superoxide dismutase

SOD essay was done following the method described by Ewing and Janero (1995). Mites were collected and homogenized individually in ice-cold sodium phosphate buffer (0.05 mol/l, pH 7.4) containing EDTA at 1 mmol/l. The resultant extract was clarified by centrifugation at 10.000g for 10min at 4°C and used as enzyme source for SOD essay.

Essay for enzyme activity

The enzyme activity of CarE and SOD was monitored with spectrophotometric essay using microplate reader. 96-well microplates were used in all tests.

Carboxylesterase

The total CarE activity was monitored with α-naphtylacetat as substrate (Stumpf and Nauen, 2002; Rauch and Nauen, 2003; Zhang et al., 2004). The substrate was prepared at 0.1 mol/l in aceton and stored at –20°C. It was mixed with a freshly prepared Fast Blue RR solution at 10^{-2} V/V (substrate / Fast Blue RR) and use as substrate solution. Per microwell, the reaction system consisted of 25μl enzyme source, 25 μl sodium phosphate buffer (0.2 mol/l, pH 6.0) and 200 μl freshly prepared Fast Blue RR solution containing the substrate at 1 mmol/l. In control wells, the enzyme source was replaced with 25 μl sodium phosphate buffer (0.1 mol/l, pH 7.6) previously used for mites' homogenization. After the preparation, the microplate was immediately read under kinetic mode at 24°C for 20 min using ThermoMax microplate reader (ThermoMax, Molecular Devices, USA). The absorbance was determined at 450 nm and expressed in mOD/min/mite. Each essay was replicated 4 times overtime. and each replicat consisted of 10 individual mites.

Superoxide dismutase

SOD activity was essayed by monitoring the reduction of NBT by aerobic mixture of NADH and phenazine methosulfate (Ewing and Janero, 1995). Each microwell was filled with a complete reaction solution of 250 μl consisting of 25

µl enzyme source, 200 µl freshly prepared 0.1mmol/l EDTA, 62µmol/l NBT, 98µmol/l NADH in 50 mmol/l phosphate buffer, pH 7.4 and 25 µl of a freshly prepared mixture of 33µmol/l PMS, 0.1mmol/l EDTA in 50 mmol/l phosphate buffer, pH 7.4 to initiate the reaction. The prepared microplate was incubated for 5 min and read in endpoint measurement mode with "automix" function activated using spectrophotometer (microplate reader) (SpectraFluor, TECAN, Grödig, Austria). As already shown by Ewing and Janero (1995), the rate of NBT reduction though NADH-PMS has a decreasing relationship with the absorbance. Consequently, the highest SOD activity will be expressed on the graphs by the lowest absorbance (Figures 4-6). The absorbance was determined at 550 mn and expressed in mOD/min/mite. Four replicates were made for each essay.

Total protein determination

The purpose of the total protein determination (TPD) was to quantify the total amount of proteins in relation to imidacloprid treatments and the variation in CarE and SOD activities on day 3 AT. The TPD is important for verifying the general level of protein in tested orgnanism. Any important variation in protein level among the treatments would bias also the enzymatic activity levelBradford method was used for TPD. The protein extraction was done by homogenizing individual mites in tube containing 30 µl ice-cold sodium phosphate buffer (0.1 mol/l, pH 7.6) mixed with 0.1% triton-X-100. The crude homogenate was incubated for 5 min at 25°C and thereafter centrifuged at 5.000 g for 10 min at 4°C in a freezing centrifuge. The resulting supernatant was used in TPD essay. The reaction solution consisted of 20µl homogenate, 480µl distilled water and 500µl Bradford Reagent in Acryl tubes for spectrophotometer. The control consisted of 500 µl distilled water and 500 µl Bradford Reagent. The absorbance was determined at 595nm after 5min incubation at 25°C using an UV visible sprectrophotometer (Ultrospec 200, Pharmacia Biotech, Cambridge, England).

Statistical analysis

CarE and SOD activities' data were analysed using GLM procedure in SAS/STAT. The absorbance values expressed in mOD/min/mite or OD/min/mite were checked for Gaussian distribution through box plot. Thereafter, log transformation was applied in order to adjust the absorbance variable to normal (Gaussian) distribution. Means separation was performed using Bonferroni (Dunn) t-test for pairwise means comparision at significance level α = 0.05. The frequency distributions of individual TSSM according to different absorbance classes for CarE and SOD were plotted using GraphPad Prism for Windows.

4.4. Results

Carboxylesterase

The total CarE activity in TSSM varied according to imidacloprid treatments and time after treatment (Table 4.1). Moreover in Figure 4.1 the frequency distribution of individual mites according to different absorbance (Vmax = maximal velocity, a kinetic constant expressing the rate of enzymatic activity) classes on day1, day3 and day6 AT, with a bin center adjusted to the control treatment. Figure 4.1 gives a general overview of enzyme activity on different dates and according to treatments. On day1 AT, CarE activity was higher in mites collected from plants treated with foliar application compared with those collected from drench application and control plants, while no differences were observed between control treatment and foliar application (Table 4.1). The highest effect of imidacloprid on total CarE activity in mites was observed on day3 AT, where CarE activity in mites collected from foliar application increased significantly compared with both drench application and control (Table 4.1). The lowest CarE activity on day3 AT was obtained with control treatment, which varied significantly compared with drench application (table 4.1).

Table 4.1. Effect of imidacloprid on CarE activity (mean ±SE) in TSSM

	CarE activity (mOD/min/mite)		
Treatments	Day1	Day3	Day6
Control	51.45 ± 2.33b	57.61 ± 1.72c	55.72 ± 1.81a
Drench	53.65 ± 2.07b	66.45 ± 2.25b	56.51 ± 2.10a
Foliar	66.86 ± 2.63a	73.58 ± 1.97a	60.88 ± 1.85a
N	119	120	120
df	2, 116	2, 117	2, 117
F value	12.44	16.05	2.07
Pr>F	< 0.001	< 0.0001	0.1303

Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests.

On day6 AT the effect of imidacloprid on CarE activity in mites was no more variable among the treatments with no significant differences observed in CarE activity (Table 4.1).

Superoxide dismutase

The frequency distributions shown in Figure 4.2 gives an overview of the repartition of individual mites according to different treatments and absorbance (Vmax) classes. SOD activity in TSSM did not vary significantly among the treatments on day1 AT (Table 4.2). The effect of imidacloprid on SOD activity was observed only on day3 AT, with higher SOD activity in foliar application compared with control treatment (Table 4.2). However, neither did SOD activity vary significantly between foliar and drench applications nor between drench application and control treatment (Table 4.2). On day6 AT, SOD activity did not vary significantly among the treatments (Table 4.2).

Total protein determination

The total protein content was around 2 μg protein/ml and did not vary significantly among the treatments, suggesting that the variations in CarE and SOD activities were not due to a general variation in protein content in TSSM (Table 4.3).

Table 4.2. Effect of imidacloprid on SOD activity (mean ± SE) in TSSM

	SOD activity (OD/min/mite)		
Treatments	Day1	Day3	Day6
Control	0.171 ± 0.002a	0.169 ± 0.003b	0.173 ± 0.003a
Drench	0.165 ± 0.002a	0.162 ± 0.003ab	0.169 ± 0.002a
Foliar	0.163 ± 0.002a	0.154 ± 0.002a	0.168 ± 0.002a
N	119	120	120
df	2, 116	2, 117	2, 117
F value	1.43	8.11	0.87
Pr>F	0.2435	0.0005	0.4213

Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests.

Table 4.3. Effect of imidacloprid on total protein content in TSSM

Treatments	Control	Drench	Foliar
Protein content (μg/ml)	2.08 ± 0.09a	2.05 ± 0.08a	2.03 ± 0.10a

Means within a row followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests.

4.5. Discussion

In this study, we assayed the activity of CarE and SOD in TSSM reared on French beans plants, which were treated with either foliar or drench applications of imidacloprid at field relevant dose rates. However, it would be

important to remind that imidacloprid is an insecticide and have no lethal effect on TSSM at field recommended doe rates, which are used in this experiment. The insensitivity of TSSM to imidacloprid is partly due to the structural differences between the nicotinic acethylcholine receptors at postsynaptic membrane of insects and mites´ nervous systems (Tomizawa et al., 2000). Due to theses structural differences, imidacloprid is not recognize by nicotinic acethylcholine receptors of mammals and many other organisms, which explains also the selectivity of imidacloprid for insects (Zhang et al., 2002).

The study of physiological responses of TSSM to neonicotinoids is a part of a series of field and greenhouse experiments undertaken in attempt to shade light on the factors contributing to reported field outbreaks of TSSM consecutive to the use of neonicotinoids to control insect pests. (James and Price, 2002; Beer et al., 2005; Sclar et al., 1998; Ako et al., 2004; 2006). The monitoring of enzyme activity in TSSM exposed to imidacloprid aimed at obtaining additional information about the physiological changes in TSSM exposed to this insecticide, which may contribute to clarifying the relationships between the observed field population build up of TSSM and the application neonicotinoids. The effects of conventional insecticides on the physiology and especially the enzyme activity in TSSM is poor documented, probably because there have been so far no scientific interests in such a topic prior to the announcement of field outbreaks of TSSM following the application of neonicotinoid insecticides. However, several investigations have been carried out, which have cleary established a strong relationship between elevated CarE and SOD activity and insecticide resistance in insect pests especially aphids and whiteflies (Nauen et al., 1996; 2001; Loxdale et al., 1998; Sun et al., 2005),

In this experiment, total CarE and SOD activity could be significantly linked to the applications of imidacloprid. CarE activity in TSSM increased quickly especially with foliar application, starting from the first DAT with a peack on third DAT, and disappeared on the sixth DAT,

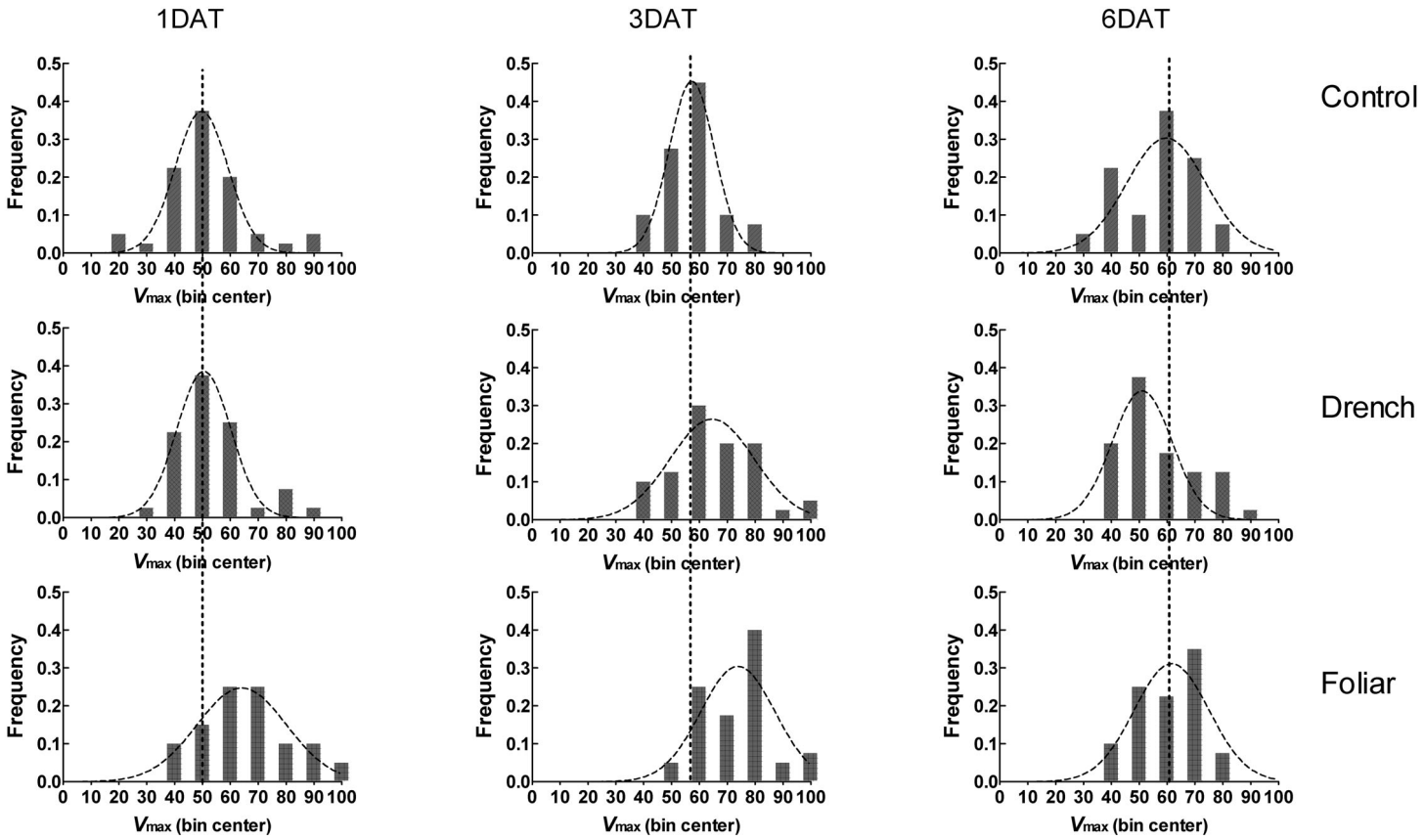

Figure 4.1. Frequency distributions of individual TSSM following the absorbance (*Vmax*) classes for CarE activity

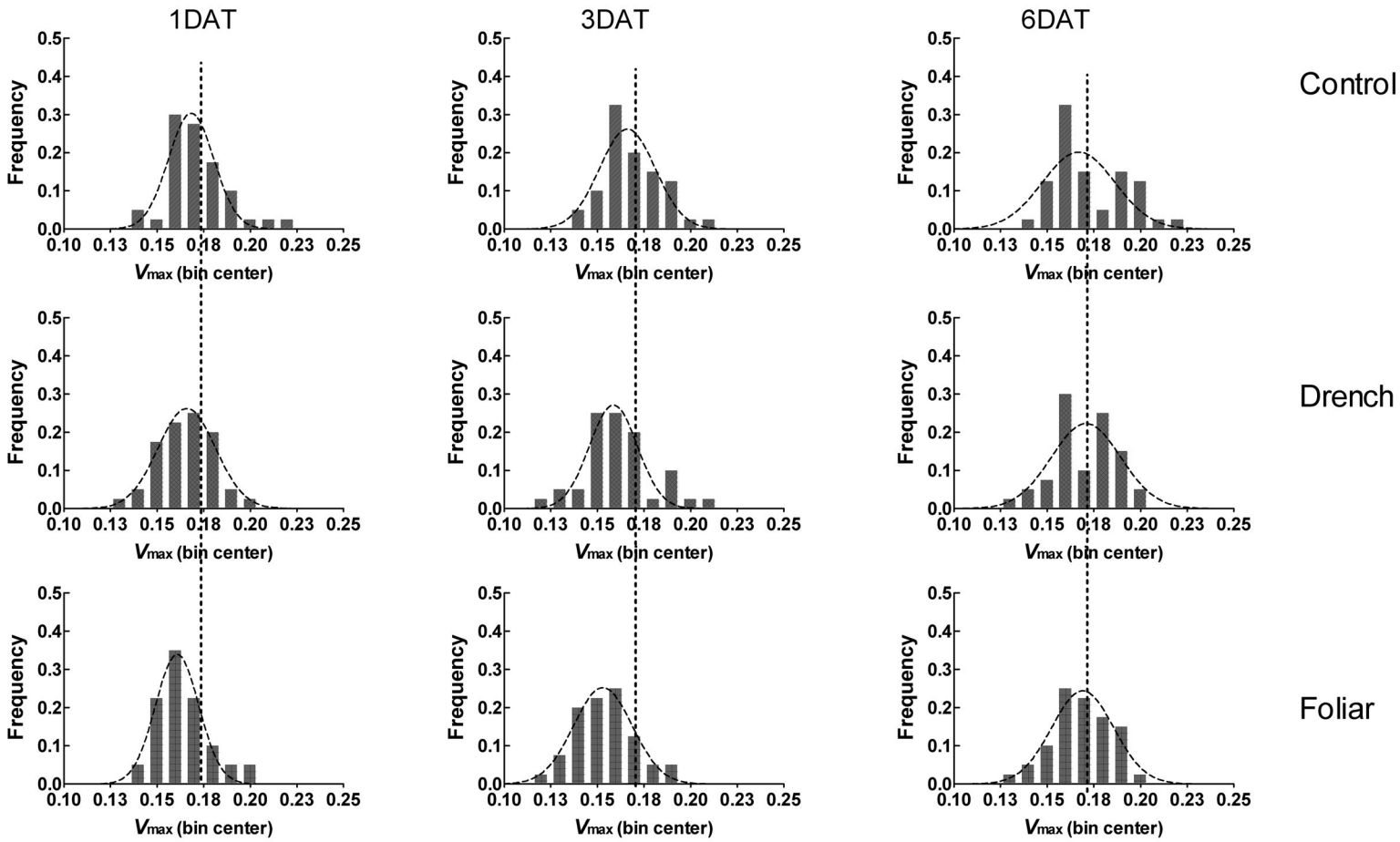

Figure 4.2. Frequency distributions of individual TSSM following the absorbance (*Vmax*) classes for SOD activity

suggesting the quick ability of mites to recover from the effects, either physical or chemical of imidacloprid treatments. The enhancement of CarE activity is a proof of the occurrence of a detoxification process in TSSM exposed to imidacloprid. Van Leeuwen et al. (2005) equally observed an increase in esterase activity in acaricides multiple-resistant and cross-resistant field TSSM strain compared with a susceptible strain. Moreover, these results support partially the non-lethal character of imidacloprid on TSSM as previously reported by Elbert et al. (1990, 1991). However, the chemical process leading to increased CarE activity in mites remains not elicited, since in its basic chemical structure imidacloprid does not contain any carboxyl ester link, which could be targeted by CarE like in the detoxification process of agrochemicals such as organophosphates carbamates and pyrethroids (Wheelock et al., 2005b; Abernathy and Casida, 1973). However,, other abiotic factors like toxic metals, i.e. zink and cadnium were shown to enhance CarE activity in carabid beetle *Poecilus cupreus*, (Wilczek et al., 2003) suggesting that some non-carboxylester compounds can lead to an increased CarE activity. Moreover, some stress situations, like physical stressors i.e. low temperature, and/or chemical stressor i.e. exposure to acid rain (Zhang et al., 2004) may increase esterase activity in living organisms. In addition, some metabolites of imidacloprid were reported to exhibit a high insecticide activity against aphids (Nauen et al., 1998), suggesting a probable existence of other imidacloprid-derived chemical stressors, which may trigger the process leading to increased CarE activity in TSSM. As possible pathway for enhancement of CarE activity, we suspect especially a general non-lethal stress effect of imidacloprid on TSSM., which any other insecticide or acaricides, at a certain concentration, might have on any non-target organism. In view of these results, no clear relationships can be established between higher CarE activity and the variation in oviposition, which was previously observed while studying the bionomics of TSSM exposed to imidacloprid (Ako et al., 2004). In that study, higher effects of imidacloprid were rather observed with drench application and not with foliar application like in the present essay with CarE activity.

SOD belongs to the group of endogenous enzymes of protective system (EEPS) and has been found in a number of living organisms (Fridovich, 1977). The increase in SOD activity with foliar application of imidacloprid, suggests a link between the contact activity of this insecticide and enhancement of oxidative stress in mites. SOD catalyzes the reduction of superoxide anions to hydrogen peroxide in order to avoid cell damage, which is induced by reactive oxygen species (ROS). The ROS are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them (Fiers et al., 1999; Nicholls and Budd, 2000). These are superoxide, hydrogen peroxide and hydroxyl radical. The metabolism of imidacloprid involves many processes of hydroxylation and loss of hydroxyl radical, i.e. the hydroxylation of the imidazolidine ring at position 4 or 5 leading to the mono- and dihydroxylated compounds and subsequent loss of hydroxyl radical under the form of water to yield olefin metabolite (Sur and Stork, 2003). The presence of hydroxyl radicals in the process of metabolism of imidacloprid following its application may partially explain the higher SOD activity in TSSM. It has been equally shown that the increased activities of EEPS were closely bound to the resistance of organisms to unfavorable environments (Packer, 1984) and recently, Zhang et al. (2004) reported significant increases in EEPS activity, including SOD in TSSM exposed to acid rain, which is mostly observed in regions with higher atmospheric pollution.

The increase in CarE and SOD activities following imidacloprid treatments, especially with foliar application suggests the occurrence of a certain unspecific stress effect in TSSM that may arise from contact and/or uptake of the product. Such processes would occur with any other insecticide, which must be treated by the mites catabolic system. Another eventuality could be an indirect effect imidacloprid on TSSM i.e. a reduction in feeding stimulation and feeding activity, when leaves are treated with imidacloprid. It is from this point clear that imidacloprid affects to some extent the physiology of TSSM, However, such moderate stress symptoms could again not explain the

observed field increases of TSSM and are much more in line with our observed negative influences (see chapter 2&3) of imidacloprid on TSSM fertility parameters.

In previous experiments, in which the influence of some important metabolites of imidacloprid on the variation of TSSM oviposition was investigated, the 6-Chloronicotinic acid was the only metabolite, which seemed to have influenced the oviposition in TSSM (Ako et al., 2004). The following experiment, run as a verification trial aimed at looking more in detail into the influence of this metabolite on the fecundity in TSSM.

CHAPTER V

5. Effects of 6-chloronicotinic acid, a metabolite of imidacloprid on the reproduction of *Tetranychus urticae* Koch (Acari: Tetranychidae)

5.1. Abstract

The present experiment aimed at quantifying 6-chloronicotinic acid (6-CNA), a metabolite of imidacloprid in French bean leaves and assessing its possible effects on the fecundity of TSSM. For this, the pure extract of 6-CNA (99%) was diluted to 1 and 10 ppm and applied as drench treatment to French bean plants. Thereafter, liquid extracts of leaves over a period of 16 days were subjected to High Performance Liquid Chromatography (HPLC) for the determination of 6-CNA in the leaves. In parallel to HPLC analysis, female mites were reared on 6-CNA-treated plants to determine the TSSM fecundity after different 6-CNA treatments. Results showed a quick uptake of 6-CNA, the concentration of which increased quickly and reached its maximum level in the leaves on day 6 AT (723.5 $\pm$ 91.3 ng/ml), followed by a rapid decrease from day 6 to day 10 (188.7 $\pm$ 31.6 ng/ml) and a relatively constant 6-CNA concentration from day 10 to day 16 (148.5 $\pm$ 22.8 ng/ml). No significant differences in TSSM oviposition were observed among 6-CNA treatments and the control. Moreover, the 6-CNA treatments did not affect the hatch rate of eggs nor the preimaginal survivorships in TSSM.

Nevertheless, the sex ratio (% females) was affected by both 6-CNA treatments with fewer numbers of females compared to control treatment (57.5 $\pm$ 1.5 and 52.8 $\pm$ 0.8 for 1ppm and 10ppm respectively) compared with the control treatment (73.0 $\pm$ 2.9)

5.2. Introduction

Imidacloprid is the first representative of neonicotinoid insecticides, which has been available on the market since 1991 for controlling several species of insect pests (Elbert, 1990; 1991). Following its application, imidacloprid is metabolised to several secondary products, of which monohydroxy-imidacloprid, olefine, guanidine and 6-chloronicotinic acid are the most commonly present in the treated plants (Nauen et al., 1999, Sur and Stork, 2003).

After root uptake, imidacloprid is translocated acropetally within the xylem and degraded quickly in the plants. Three principal metabolic pathways have been identified in various plant metabolism studies with different crops and types of application showing a nearly uniform quantitative and qualitative pattern in all test systems. The first pathway is the ethylene-bridge's hydroxylation of the imidazolidine ring and elimination of water, which leads to olefine and 4,5-dihydroxy-imidacloprid metabolites. The second one is the nitro-group reduction to nitrosimine and further loss of NO, leading to the formation of guanidine, triazinone and urea metabolites. Another pathway is the oxidative cleavage of the methylene bridge to form 6-chloropicolyl alcohol and subsequent oxidation, yielding 6-chloronicotinic acid (6-CAN) and gentiobioside metabolites (Sur and Stork, 2003).

Nauen et al. (1998, 1999) have shown in different types of bioassays that certain metabolites of imidacloprid, which have been described in the crop are active against the cotton whitefly (*Bemisia tabaci*), green peach aphid (*Myzus persicae*) and cotton aphid (*Aphis gossypii*). Some of them such as the olefine imidazoline can be highly active compared with imidacloprid. Moreover it is known that, whilst after foliar application of imidacloprid, most of the residues on the leaf surface remain as the unchanged parent compound, imidacloprid administered to the plant by soil application or seed treatment and systemically displaced is metabolised more or less completely.

Previous experiments showed a higher decrease of TSSM fecundity at field recommended dose rates (Ako et al., 2004) with drench application of

imidacloprid compared with its foliar application. In addition, among the metabolites of imidacloprid studied, only the 6-CAN showed a trend of correlating positively with the variation in oviposition in TSSM (Ako et al., 2004). Hence in this experiment we tried to figure out the potential effect of the pure 6-CAN on the variation in TSSM oviposition.

5.3. Material and methods

Plant and mite materials

The plant and mite materials were the same as described in previous experiments. We used French beans, *Phaseolus vulgaris* L., cv. Saxa, in this experiments. Plants were produced under greenhouse conditions at 25±1°C, 65±5% relative humidity (RH), and a photo period of 16:8 h (L:D). Seeds were sown in sterilized humus (Fruhstorfer Erde Type P" (Archut GmbH, Lauterbach-Wallenrod, Germany) in pots (7 cm in height and 5 cm in diameter) and used for the experiments two weeks later at 2 leave stage.

The strain GSS (German Susceptible Strain) of two spotted spider mites (TSSM) was used in the trials. GSS is an acaricide susceptible strain, which has been maintained in culture since 1965, without exposure to any acaricides.

Treatments and bioassays

A synchronised mite culture was established. Female mites arisen from a synchronised culture were transferred onto leaves in sticky rings as described in chapter 2 (2.3) Three treatments were applied i.e. control or water only treatment and two different doses rates of 6-CNA, 1 ppm and 10 ppm applied as drench treatments, corresponding to 1% and 10% of the mother compound imidacloprid respectively. The oviposition was monitored every two days for 16 days. Different life table parameters i.e. sex ratio, preimaginal survivorship and hatch rate of eggs were determined following the methodology described in chapter 2.3.6.

The drench application and the liquid extract of leaves.

To study possible relationships between the concentration of 6-CNA in the leaves and the oviposition pattern of TSSM, the pure 6-CNA (99%) was diluted at 1 and 10ppm and applied as drench treatment. The drench application was performed by applying both 6-CNA solutions directly to the soil. At two days intervals, starting from the treatment day (6 hours after drench application) 6-CAN was extracted from the leaves. Leaves were harvested with the aid of sterile scissors, which are cleaned with a solution of ethanol/water (6/4 v/v), after each leaf is cut. The leaves are cut at their basis. Per plant, four leaves were randomly sampled and weighted together. In the laboratory, the 4 leaves of each plant were cut into small pieces, which were ground in approximately 100ml of liquid N with a mortar (approximately 12g/100ml). The obtained leaf powder was subsequently transferred into a glass container, to which a solution of acetonitrile /water (8/2 v/v) was added at a rate of 4ml per g of fresh leaf. After one hour of stirring, the solution was centrifuged (Heraeus-Christ Labofuge A, Osterode, Germany) for five minutes at g = 5,000 and thereafter filtered, using a sterile 0.22µm filter (Millipore, Millex® - GS, Molsheim**,** France). The experiment consisted of four replicates per day and per replicate one plant (four leaves) was used. The liquid extracts of leaves were sent to the analytic department of Bayer CropScience for HPLC analysis.

High Performance Liquid Chromatography (HPLC) analysis

Following the filtration of the liquid extract of leaves, a quantity of 1500µl of the filtrate was injected per glass vial and subjected to ESI-MRM (Electrospray Ionization-Multiple Reaction Monitoring) mode of High Performance Liquid Chromatography-Tandem Mass Spectrometry (HCLP-MS/MS; HPLC: Model 1100 Binary, Agilent, Waldbronn, Germany; MS: model Quattro Ultima, Micromass Ltd, Manchester, UK) to determine the amount of 6-chloronicotinic acid in the leaf tissue.

Statistical analysis

The statistical analyses were performed using ANOVA (analysis of variance) through the general linear model (proc glm) procedure in SAS/STAT. The raw data on the fecundity, hatch rate of eggs, survival and sex ratio were first subjected to a descriptive analysis using box plot, which permitted the adjustment of the dependant variables to normal distribution by a log transformation for the fecundity and arcsin transformation for hatch rate of eggs, survival and sex ratio. Thereafter, the analysis of variance was run and the mean separation was preformed, using the least square means (lsmeans), adjusted to Bonferroni (Dunn) t test.

5.4. Results

The 6-CNA did not affect the general ovipositional pattern as observed in previous experiments with different imidacloprid treatments. After day 2 a rapid increase in egg laying was observed, with a maximum fecundity on day 6, and thereafter a constant decrease till day 16 (Figure 5.1). However, no differences in fecundity were observed among different 6-CNA treatments in terms of cumulative egg deposition during the 16-days period (Figure 5.2).

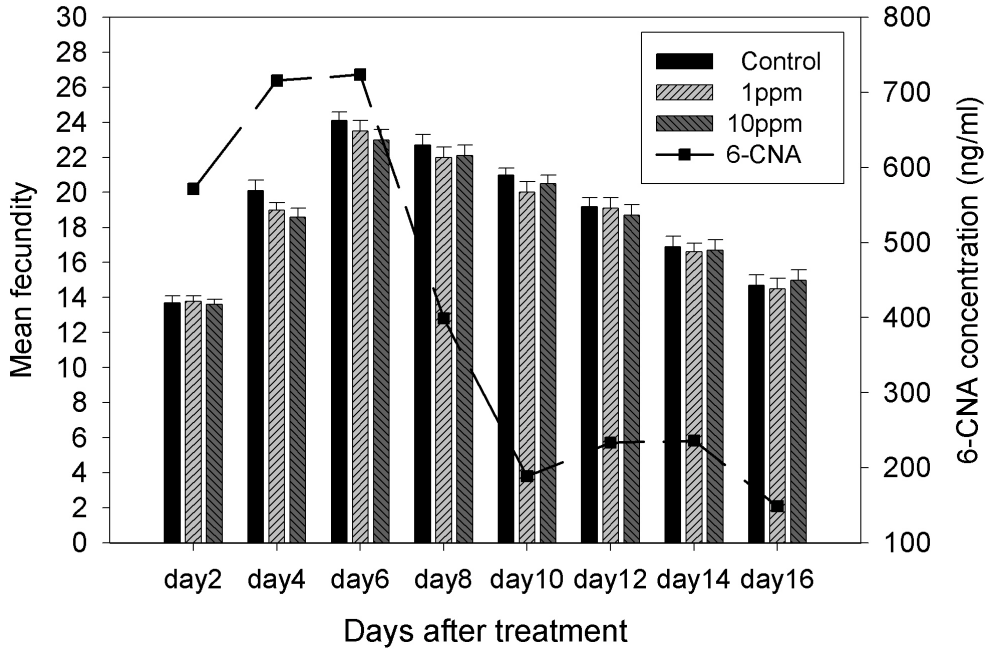

Figure 5.1. Concentrationof 6-CNA in leave extracts and fecundity of TSSM in relation to 6-CNA treatment over 16 days

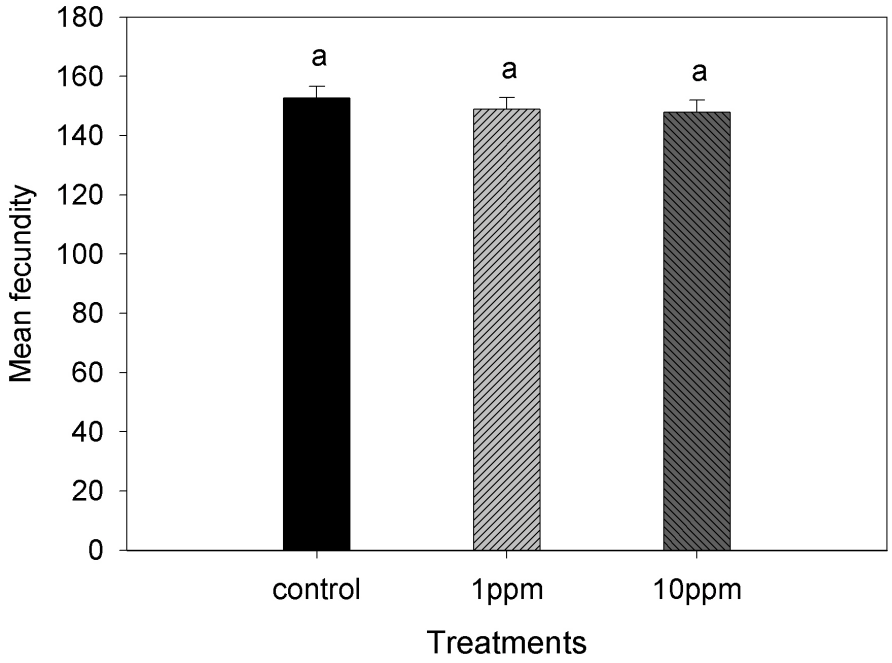

Figure 5.2. Effect of 6-CNA on the cumulative oviposition of TSSM after 16 days

The HPLC results showed that 6-CNA was already present in the leaves 2 days after its application as drench treatment. The highest concentration of 6-CNA was observed between day 4 and day 6 AT (723.5 ± 91.3 ng/ml), followed by a fast decrease from day 6 to day 10 (188.7 ± 31.6) and a relatively constant 6-CNA concentration from day 10 to day 16 (148.5 ± 22.8) AT (Figure 5.1). No differences in oviposition could be observed between the treatments. In addition, no effects of the 6-CNA were observed on the hatch rate of TSSM eggs as well as on the preimaginal survivorship (Table 5.1). However the sex ratio was affected by the 6-CNA with fewer numbers of females recorded in 6-CNA treatments (57.5 ± 1.5 and 52.8 ± 0.8 for 1ppm and 10ppm respectively) compared with the control treatment (73.0 ± 2.9) (Table 5.1)

Table 5.1. Mean (± SE) hatch rate of eggs, preimaginal survivorship and sex ratio (± SE) of strain GSS of *T. urticae* treated with 6-CNA at 1ppm and 10ppm

Treat.	Sex ratio (% females)	Survival (%)	Hatch rate of eggs (%)
C	73.0 ± 2.9a	74.7 ± 2.9a	90.4 ± 1.8a
1ppm	57.5 ± 1.5b	68.6 ± 3.0a	89.9 ± 1.7a
10ppm	52.8 ±0.84b	69.9 ± 3.8a	87.3 ± 1.8a

Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ with Bonferroni (Dunn) t tests. C, control; Treat., treatments; D, drench application; F, foliar application.

5.5. Discussion

In the present experiment we assayed the effects of 6-CNA, an important metabolite of imidacloprid, on the fecundity of TSSM. The objective of this experiment was to shade light on the possible influence that 6-CNA may have on the oviposition of TSSM, as previously showed in preliminary experiments,

in which different breakdown products of imidacloprid i.e. monohydroxy imidacloprid, guanidine, olefine and 6-CNA were studied in relation with the variation in fecundity in TSSM (Ako et al., 2004). The metabolisation of imidacloprid in different breakdown products after its application is well established and described in chapter I (figure 1.3, 1.4 &1.5) (Nauen et al., 1998; Sur and Stork 2003). Moreover the insecticidal potency of some metabolites of imidacloprid like olefine has been shown (Nauen et al., 1999).

6-CNA did not affect the fecundity in TSSM in this experiment. However the metabolite was found in relatively high concentration immideately after application in the leaves, which denotes the rapid uptake and dislocation of 6-CNA by the plants. The rapid decrease in the concentration of 6-CNA in leaves supports the instable nature of imidacloprid metabolites as shown by Sur and Stork (2003). The only parameter that was negatively affected by the 6-CNA was the sex ratio, which was higher male-biased compared with the normal situation where the female population represents 2 to 3 times that of the males. The male-biased sex ratio in TSSM observed with 6-CNA treatments could result from the arrhenotokous parthenogenesis in *T. urticae* where unmated females give rise to males only (Helle and Overmeer 1973). With that mechanism of sex determination, any interference of the metabolite with the reproductive biology of TSSM i.e. disturbance in mating behaviour may lead to an increased number of males in TSSM population. Moreover increasing male ratios in arrhenotokous populations often indicate adaptation to reduced quality of the resources (food quality and quantity) but also stress conditions (often sublethal).

In all the experiments conducted up to now, mites were restricted to sticky ring area for their activities. Although this is only practicable way to monitor the development and behaviour of this tiny mites on a individual level, this technique still creates an artificial situation and does not represent the normal field conditions, where different TSSM stages can move without space limitation on the plants. It can be hypothesized that the artificial experimental situation used could be one reason for different results from our studies and

the field experiments reported. It could be that under the optimized laboratory conditions (temperature, humidity, low intraspecific competition, optimal nutrition) level of reproduction is already at the upper limits.

For these reasons, a field-simulated greenhouse experiment is carried out, in which the influence of imidacloprid on abundance and within-plant distribution of TSSM was surveyed.

CHAPTER VI

6. Within-Plant dispersal and abundance of different *Tetranychus urticae* Koch (Acari: Tetranychidae) developmental stages as affected by imidacloprid [4]

6.1. Abstract

In the present greenhouse trial, the effect of field relevant dose rate of imidacloprid on abundance and within-plant dispersal of different *Tetranychus urticae* (TSSM) developmental stages i.e. eggs, immature stages and adults was assessed. The results showed an overall heterogeneous vertical repartition of different TSSM stages on French beans. On the lower part of the plant (lower canopy), significantly higher numbers of eggs were observed in the control treatment (75.8±4.7/leaf) in comparison with drench (57.9±4.0/leaf) and foliar (26.1±2.1/leaf) applications. Likewise, the control treatment showed significantly higher numbers of immature stages and adults (62.6±3.8/leaf) and (35.2±2.8/leaf) compared with drench (37.6±2.6/leaf) (30.0±2.3) and foliar (23.3±1.9) and (17.2±1.2) applications respectively. On lower canopy, eggs, immature stages and adults, recorded from drenched plants were all significantly higher compared with those observed on foliar treatment. On the upper part of the plant (upper canopy), no differences were observed among the treatments for different TSSM stages. However, the overall number of eggs, immature stages and adults recorded on upper canopy were far higher

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[4] To be published as Ako, Mouhoube, Hans-Michael Poehling and Ralf Nauen. Within-Plant Dispersal and Abundance of Different *Tetranychus urticae* Koch (Acari: Tetranychidae) Developmental Stages as Affected by Imidacloprid *in Pest Management Science*

compared with those observed on lower canopy. For eggs, the control, drench and foliar applications were 3, 4.5 and 10 folds higher respectively on upper canopy compared with lower canopy. For immature stages, the increases were 1.7, 3 and 4.5 fold, for adult TSSM 2.3, 3.4 and 5.5 folds respectively. The mean total number (lower + upper canopy) of eggs and adults did not show any variation, when the treatments were compared. However, the mean total number of immature stages was higher in the control treatment compared with foliar application but not with drench treatment.

6.2. Introduction

Two-spotted spider mite (TSSM) *Tetranychus urticae* Koch is an important mite pests feeding on a wide range of host plants including orchards and vegetables in tropical and temperate zones (van de Vrie et al., 1972). In apples and hops especially, TSSM has always been a major phytophagous pest in association with herbivorous insects pests like whiteflies and aphids. The simultaneous occurrence of insect and mite pests requires from farmers often intensive control measures and the use of both insecticides and acaricides to keep these herbivorous pests under control. Even in situations with low or naturally controlled mite populations a variety of insecticides are used to control pest insects. Of these neonicotinoids, a relatively new class of insecticide, with systemic and contact activities are widely used (Nauen et al., 2001a; Nauen and Bretschneider, 2002). Since their introduction into the market, neonicotinoids, namely imidacloprid, thiacloprid, acetamiprid and thiamethoxam have been successfully used against whiteflies and aphids and several species of beetles, flies, and moths (Elbert et al., 1990; Elbert et al., 1991). They are characterized as selective for insects regarding the arthropoda and as highly efficient against sucking pests (Elzen, 2001; Tomizawa &Casida, 2005).

The use of neonicotinoid insecticides, especially acetamiprid and imidacloprid to control aphids and whiteflies particularly in orchards and hops has become over the past few years a topic of concern in terms of spider mites control in

the general concept of IPM. Many laboratory and greenhouse trials considering the speculative information of field outbreaks of TSSM occurring following to the applications of neonicotinoid insecticides have yielded contradictory results. James and Price (2002) have reported in a laboratory trial that spray and systemic applications of imidacloprid at rates used in Washington hop yards lead to significantly increased fecundity in TSSM. The authors supported the hypothesis of hormoligosis as a possible factor leading to increased TSSM populations after neonicotinoids applications. Beers and al. (2005), examining the effect of neonicotinoids on integrated mite control in Washington apple reported important field increases in mite density with 4 or more applications of acetamiprid. However the same authors reported lower mite densities with the same insecticide, when applied only two times and the occurrence of elevated mite density was not constant but varied significantly from trial to trial with the same treatment, showing a certain instability in the occurrence of increased mite population in the field. On the other hand, Sclar et al. (1998) have found in greenhouse experiments no increase in TSSM population with soil application of imidacloprid, while the same experiment conducted in the field has shown increased mite populations and damages on Marigolds. The authors excluded the hormoligosis and phytotoxicity as possible reasons for increase in mite population. In a series of field-simulated greenhouse experiments undertaken to look into the effects of topical and drench applications of imidacloprid on the fecundity and population dynamics of TSSM, no clear evidence of increase in TSSM population has been found (Ako et al., 2004; 2006). In addition the biochemical investigations, in which stress enzymes' activities were investigated served only to corroborate the observed decreases in TSSM fecundity with imidacloprid treatments, without additional information about the potential effects of imidacloprid on the population outbreaks of TSSM. It was then decided to set up field-simulated greenhouse experiment, in which different TSSM stages could freely develop and reproduce on the plants without any spatial limitation through sticky rings like in previous trials, which would be more realistic compared to the field

situation. Therefore, the present experiment aimed at assessing the influence of imidacloprid on the intra-plant distribution and abundance of different TSSM developmental stages following different canopy levels.

6.3. Materials and methods

French bean plants and spider mites strain

In all experiments French beans, *Phaseolus vulgaris* L., cv. Saxa, were used. Plants were produced under greenhouse conditions at 25±1°C, 65±5% relative humidity (r.h.), and a photo period of 16:8 h (L:D). Seeds were sown in sterilized humus earth in pots (7 cm in height and 5 cm in diameter) and used for the experiments two weeks later at 2 leaves stage. One week prior to the start of the experiments, plants were transferred to the climatic chamber, where they were kept under the same conditions for an additional week.

The strain Wiesmor (WI) of two spotted spider mites (TSSM) was used in the trials. The strain is an organophosphate-selected maintained in the laboratories of Bayer CropScience (Monheim, Germany) since 1954 under a biannual selection with the organophosphate oxydemeton-methyl (Nauen et al., 2001b). The strain WI has been characterized as susceptible to numerous acaricides, except organophosphates (Nauen et al., 2001b).

Synchronized culture of *Tetranychus urticae*

To reduce the variability in test organisms, a synchronized mite culture was established using *T. urticae* specimens from the stock culture. The sticky ring technique was used (Stumpf and Nauen, 2001; Ako et al., 2004; 2006). The technique consists of applying a sticky substance in the form of a ring of approximately 3cm diameter on the upper side of a leaf to prevent mites from escaping. The sticky substance is made out of a mixture of two types of insect trap adhesive (Raupenleim Brunonia, F. Schacht GmbH & Co Kg, Braunschweig, Germany). For this, 20 young mated female mites were placed in a sticky ring on a bean leaf for 6 hours. Thereafter, the adults were removed and the offspring were kept under greenhouse conditions at 25±1°C and

65±5% r.h. The F_1 were maintained on the leaves until the first adults appeared, which under the before mentioned conditions took 12 days. Two days before the first adults appeared, five males from the stock culture were added to each sticky ring to increase the probability that young F_1 females would encounter a mating partner. This is important because of the arrhenotokous parthenogenesis in *T. urticae* where unmated females give rise to males only (Helle and Overmeer, 1973). Young females from the synchronized cultures were used in the experiments.

Bioassays

A solution of imidacloprid (Confidor® 200 SL) was prepared at 100 ppm in the laboratory using tap water. The solution was homogenized prior to its application. Prior to the treatments, young mated pre-ovipositional females from the synchronized culture were transferred with a soft brush onto French bean leaves at a rate of 2 females per leaf at two leaves stages. Three groups of treatments were tested:

Control treatment (water only): Plants were watered once a day (in the morning) with tap water until the end of the experiment.

*Drench applications***:** Plants were drenched with insecticide solution two days before the start of the experiment to assure optimum uptake of the insecticides. For the same reason, plants were not watered for two days after the insecticide treatment. One hundred ml of insecticide solution were applied on the top of the humus earth in the pot. Two days after the treatments until the end of the experiment, plants were watered daily with tap water.

*Foliar applications***:** Plants were sprayed using a manual pressure sprayer (Pico 3235, MESTO®, Freiberg, Germany) with an internal pressure of 3 bars. First, females were transferred onto leaves. Thereafter, both sides of the leaves were evenly sprayed with the Confidor® 200SL solution until the formation of suspended droplets at the edge of the leaves. To avoid mites being washed off, while spraying the nozzle was constantly kept at approximately 50 cm distance to the treated leaves.

Data collection using a mite-brushing machine

Data were collected 25 days after the treatments, which corresponds theoretically to two generations in our experimental conditions. Leaves were collected individually and brought to the laboratory for counting different TSSM stages i.e. eggs, immature stages and adults on each individual leaf. In order to assess the within-plant distribution of different TSSM stages, leaves were collected following two vertical levels of canopy: lower canopy and upper canopy. The lower canopy is defined as the 2nd and 3rd leaf level upwards and the upper canopy represents the 2 last leaf level before the fructification. The experiment was replicated three times and each replicate consisted of 10 leaves per canopy level and per treatment.

A mite-brushing machine made and calibrated by the engineering department of Bayer AG (Bayer AG, Leverkusen, Germany) was used to brush down all TSSM stages i.e. eggs, immature stages and adults of each individual leaf. The "brushing efficiency" of the machine was very high, with 100% for adults, 99,5% for immature stages and 98% for eggs. For the use of the mite-brushing machine, a single leaf is inserted between two cylindrical fine brushes of the machine, which were fixed near to each other following two horizontal axes. They turn in opposite directions in such a manner to brush everything that is on the leaf surface downwards on a transparent circular plate, which was coated with a very fine layer of a colorless sticky trap substance (Temmen-Insektenleim®, Temmen GmbH, Hattenshiem, Germany). The sticky substance allows the fixation of different TSSM stages to the plate in order to immobilize them to achive an easy the counting operation. A cylindrical plastic tube, which has the same diameter as the transparent plate was placed vertically (on the transparent plate), which permitted to direct all TSSM stages that were brushed from the leaf to the transparent plate. All TSSM stages are then brushed down and counted manually. The counting was performed under a stereomicroscope with the aid of a circular multi-compartmented plate, dividing the whole plate area into different small areas with different colors.

First, different TSSM stages were brushed down onto the transparent plate, which was coated with a sticky trap. Thereafter, the multi-compartmented plate (which has the same diameter as the transparent plate) was placed under the transparent plate; and because of its transparency the area of the plate is equally divided into different small areas following the design on the multi-compartmented plate. The system formed by both plates turns with constant speed, as the brushing operation was running, allowing an even distribution of different TSSM stages on the transparent plate.

Statistical analyses

Data on the number of eggs, immature stages and adults per leaf were subjected to analysis of variance (ANOVA), using the general linear model (GLM) procedure of SAS. Prior to ANOVA, a descriptive analysis performed with box-plot permitted to check and adjust the number of eggs, immature stages and adults' data to Gaussian distribution by applying a log transformation. In case of significant F-values, means were separated using the Least Square Mean (lsmeans) adjusted to Bonferroni (Dunn) method at a significance level of $\alpha = 0.05$.

6.4. Results

The within-plant distribution of different TSSM developmental stages has been highly heterogeneous and was significantly influenced by imidacloprid treatments, especially with foliar application on lower canopy.

Lower canopy

Important variations were observed among the treatments in the mean number of eggs, immature stages and adults on lower canopy. The highest number of TSSM eggs recorded on lower canopy was observed with control treatment (75.8±4.7/leaf), which was significantly different compared with both drench (57.9±4.0/leaf) and foliar (26.1±2.1/leaf) treatments of imidacloprid (Figure 6.1). Moreover, the number of eggs laid on plants treated with drench

application was significantly higher in comparison with the number of eggs that were observed on plants treated with foliar application (Figure 6.1). The same trend as observed with the distribution of eggs following different treatments was equally obtained with immature stages with the highest number in control treatment (62.6±3.8/leaf) compared with that observed in both drench (37.6±2.6/leaf) and foliar treatment (23.3±1.9/leaf) on lower canopy (Figure 6.2). The number of immature stages recorded on plants treated with foliar application was significantly lower compared with that recorded on drenched plants (Figure 6.2). On lower canopy, the number of adult TSSM recorded on controls plants (35.2±2.8/leaf) did not differ significantly in comparison with the number of adults TSSM, which were recorded on the plants that were treated with drench application (30.0±2.3/leaf) (Figure 6.3). However, both control and drench applications showed higher number of adults TSSM, when compared with the number of adult TSSM individuals, which were recorded on plants treated with foliar application (17.2±1.2/leaf) (Figure 6.3).

Upper canopy

The population distribution of different TSSM developmental stages was different on upper canopy compared with that observed on lower canopy. The mean number of eggs did no vary significantly among the treatments (227.1±16.1, 261.7±18.9 and 262.6±15.2/leaf for control, drench and foliar applications respectively) though the lowest absolute mean value was observed

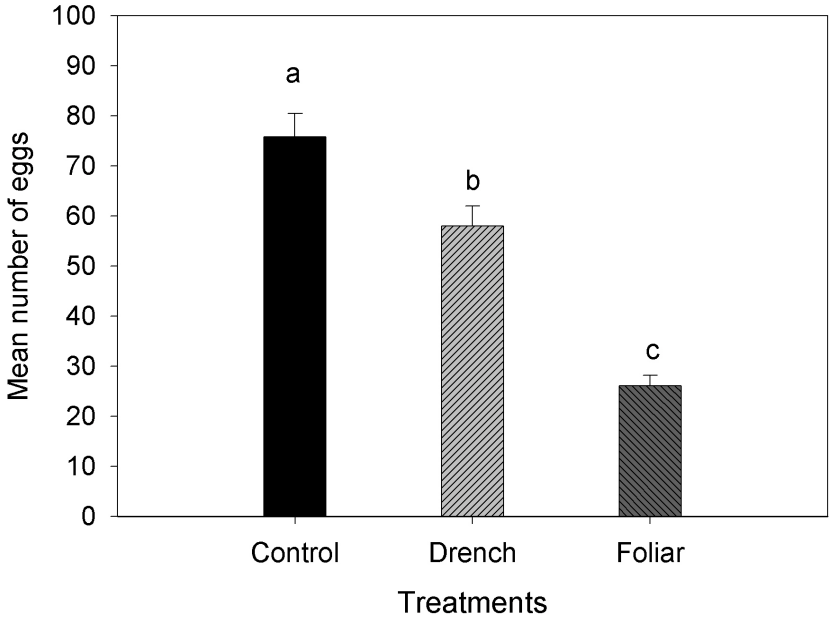

Figure 6.1. Effect of imidacloprid on the mean number of TSSM eggs per leaf on lower canopy

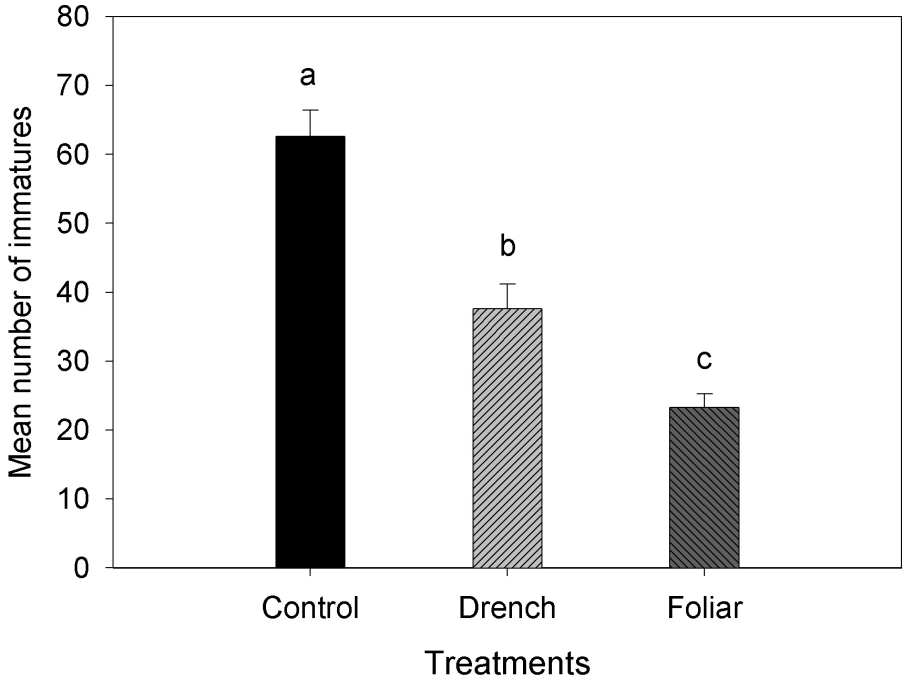

Figure 6.2. Effect of imidacloprid on the mean number of TSSM immature stages per leaf on lower canopy

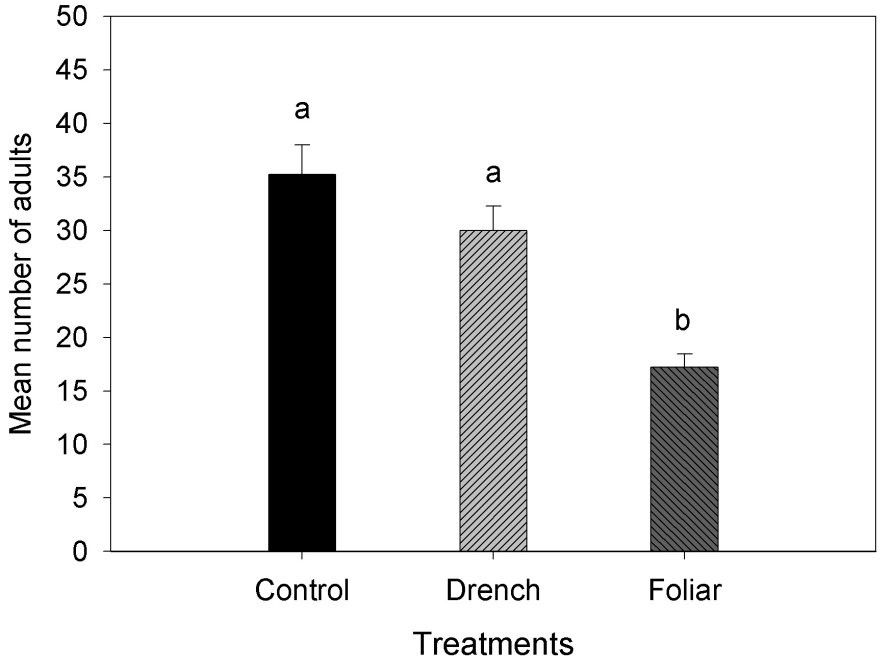

Figure 6.3. Effect of imidacloprid on the mean number of adults TSSM per leaf on lower canopy

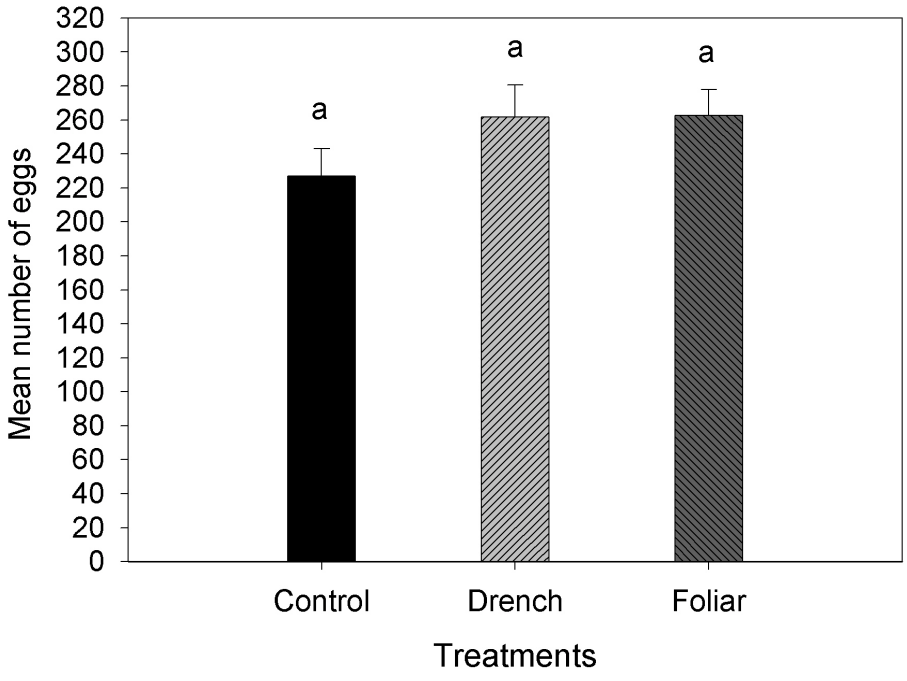

Figure 6.4. Effect of imidacloprid on the mean number of TSSM eggs per leaf on upper canopy

in control treatment (Figure 6.4). Moreover, the number of immature stages and adults TSSM followed the same trend as observed with the number of eggs, with no important variations observed among the treatments (Figures 6.5 & 6.6). However, the overall number of eggs, immature stages and adults recorded on upper canopy were far higher compared with lower canopy. For eggs, the control, drench and foliar applications were respectively 3, 4.5 and 10 folds higher on upper canopy compared with lower canopy. For immature stages, the differences were 1.7, 3 and 4.5 folds higher and 2.3, 3.4 and 5.5 folds higher for adults TSSM respectively.

Total mean number of eggs, immature stages and adults per plant and per treatment

The mean total number of eggs, immature stages and adults TSSM per plant and per treatment were obtained by combining the total number of eggs immature stages and adults that were recorded on upper and lower canopies. The mean total number of eggs (302.8±18.3, 319.6±18.3 and 288.7±15.4 for control, drench and foliar applications respectively) as well as the mean total number of adults (117.0±4.4, 125.2±6.4 and 108.4±5.7 for control, drench and foliar application respectively) did not show any significant variation, when the treatments were compared (Figures 6.7&6.9). For the mean total number of immature stages (Figure 6.8), significant differences were observed between the control treatment (169.0±7.4) and foliar application (138.1±7.7), with higher number of immature stages in the control treatment. However, the mean number of immature stages recorded on drench-treated plants (155.2±6.3) did not differ significantly from that observed in control treatment nor in foliar application (Figure 6.8).

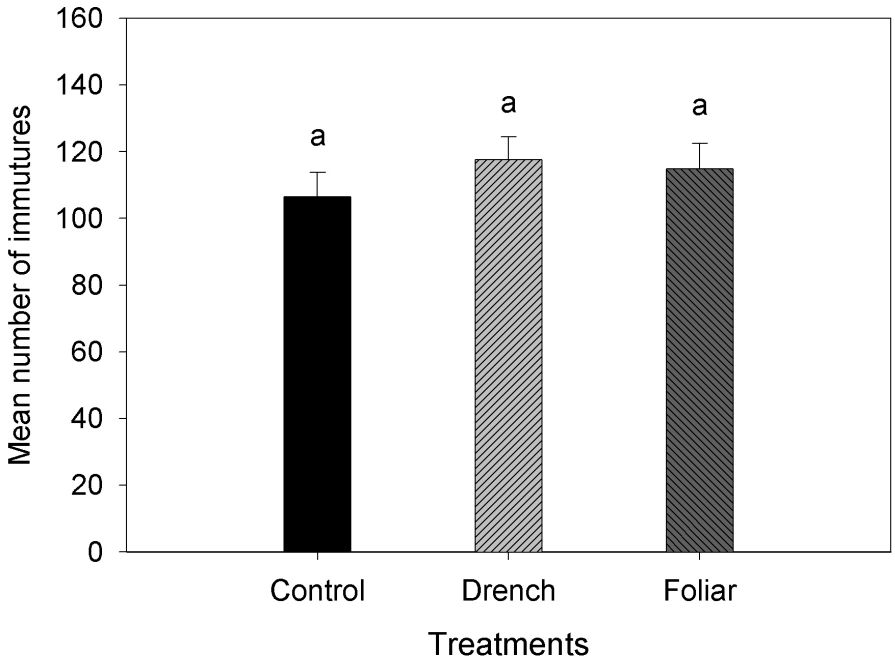

Figure 6.5. Effect of imidacloprid on the mean number of TSSM immature stages per leaf on upper canopy

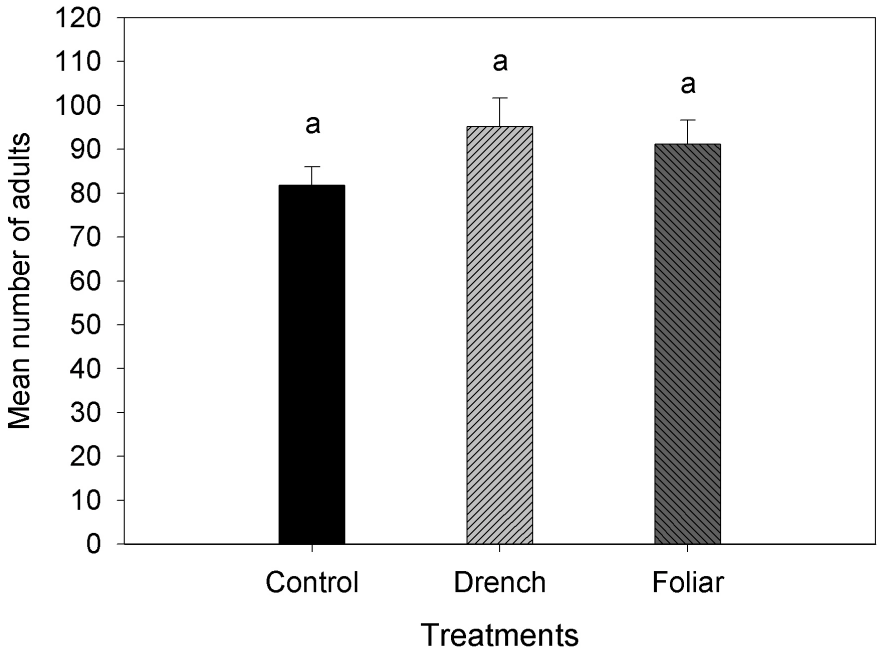

Figure 6.6. Effect of imidacloprid on the mean number of adults TSSM per leaf on upper canopy

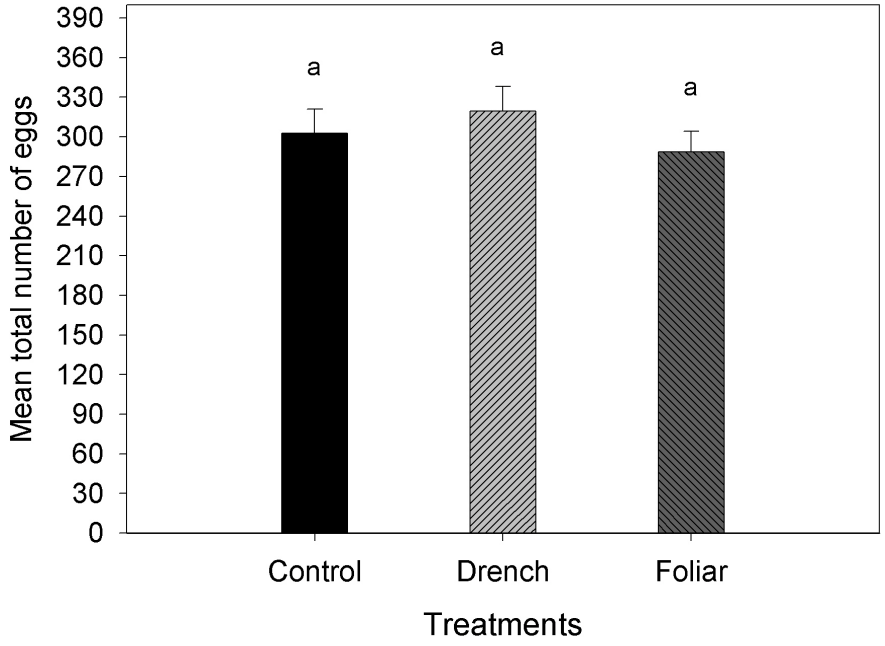

Figure 6.7. Effect of imidacloprid on the mean total number of TSSM eggs (lower + upper canopies)

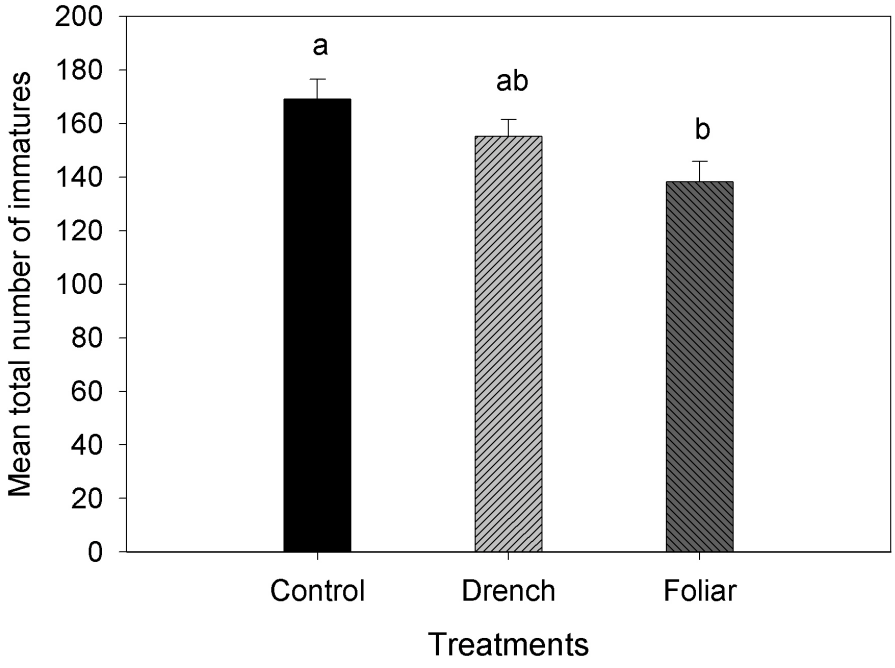

Figure 6.8. Effect of imidacloprid on the mean total number of TSSM immature stages (lower + upper canopies)

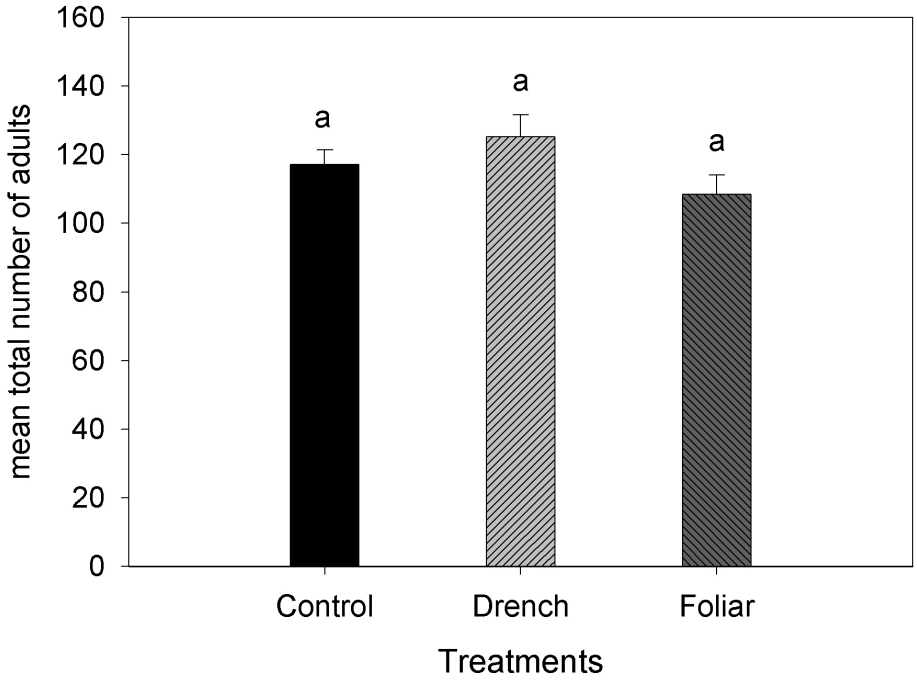

Figure 6.9. Effect of imidacloprid on the mean total number of adults TSSM (lower + upper canopies)

6.5. Discussion

In this study, the influence of a field relevant dose rate of imidacloprid on abundance and within-plant distribution of different TSSM stages i.e. eggs, immature stages and adults on French was assessed. The results revealed an overall heterogeneous vertical repartition of different TSSM stages, with higher population densities on the upper part of the plants (upper canopy), while relatively lower density of TSSM population stages were recorded on the lower parts of the same plants (lower canopy). Similar within-plant distributions of TSSM and other herbivorous mites populations have been reported in several studies. Helle (1962) postulated that during the short pre-oviposition period the females TSSM migrate to fresh leaves higher up the host plant. Nihoul et al. (1991) found that TSSM populations showed a greater tendency to increase on the upper ten leaves of a tomato plant than on the lower ones. Similarly, a study conducted by Nachman et al. (1990) indicated that the cassava green

mite *(Mononychellus tanajoa)* is more abundant on the upper than on the lower leaves. Similar results have been reported by Yaninek et al. (1989) showing a dispersal of spider mites upwards to colonize the youngest leaves. Likewise, Kielkiewicz (1996), studying the dispersal of *Tetranychus cinnabarinus* on various tomato cultivars concluded that the mobile stages of the mites move and aggregate in the middle and upper part of the tomato plant when lower leaves (older leaves) getting necrotised. According to Kielkiewicz (1996) and Karban & English-Loeb, (1988), many factors i.e. leaf surface, food availability and quality, leaf exploitation, predation, mite density, temperature, light, gravity, and humidity can modify intra-plant mite dispersal. Of these factors, the food availability and quality, which is related to mite density would be the most determinant factor for mass migration of young TSSM stages towards newer leaves.

In this study, lower number of eggs, immature stages and adults of TSSM were observed on the lower part of the plants, which were subjected to foliar application of imidacloprid at two leaves stages, suggesting a mass migration of mobile TSSM stages upwards, towards newer leaves, probably looking for untreated leaves, when leaves are treated. Likewise, Raupp et al. (2004) showed that terminals on imidacloprid-treated hemlocks were approximately nine times more likely to have severe needle damage due to mites infestation than untreated trees. The hypothesis of mass migration of mobile TSSM stages towards new leaves is particularly supportable if it is assumed that following the leaves' treatment, the residual effects of imidacloprid on the leaf surface interfere in one way or another with the feeding behavior of mites and that the new untreated leaves of previously treated plants are freet of any residual traces of the insecticide. In addition, Oslon et al., (2004), studying the distribution of imidacloprid in potato have shown that the concentration of imidacloprid was lower in the younger tissues of the upper leaves and higher in the older leaves, corroborating the hypothesis of mass migration of TSSM towards younger and less contaminated leaves. Moreover, the fact that higher numbers of different TSSM stages were observed on the upper canopy of the

plants, which were subjected to soil application, serves to corroborate the hypothesis that mobile stages of mites are massively migrating towards upper part of the plant when the leaves are treated.

The speculative information linking the field increases in spider mite populations to the use of neonicotinoid insecticides to control aphids and whiteflies has been for the few past years a topic, which has drown the attention of entomologists. In order to have an approximate description of the population growth potential of TSSM as affected by imidacloprid treatments, we determined the total number of different TSSM stages on each individual plant by combining the number of eggs, immature stages and adults that were recorded on both upper and lower canopy of each plant, following different treatments. Results showed overall no clear evidence of a potential population increase, which may arise from either spray or drench application of imidacloprid in these greenhouse experiments. In previous greenhouse trials, in which the effect of four different neonicotinoid insecticides on detailed bionomics of TSSM were studied, it was even found that the soil application of imidacloprid and acetamiprid using field relevant doses rates can negatively affect the female TSSM by reducing their fecundity on French beans plants and at the same time leading to a male-biased sex ratio (Ako et al, 2004). Likewise, Sclar et al. (1998) observed no effects on TSSM populations or damage caused by mites in greenhouse trials although they reported significantly greater damage by *T. urticae* in the field after a soil application of imidacloprid. However, James and Price (2002) indicated in a laboratory trial that spray and systemic applications of imidacloprid at rates used in Washington hop yards have led to significant increases in TSSM fecundity. In addition Raupp et al. (2004) reported greater infestations of spider mite in imidacloprid-treated hemlocks. According to the same authors, a survey of hemlocks in gardens, parks, and residential landscapes revealed that hemlocks treated with imidacloprid were more likely to be infested with spider mites. The biases results observed in the population dynamics of TSSM between greenhouse experiments (Sclar et al., 1998; Ako et al., 2004; 2006)

and field trials (Beers et al., 2005; Raupp et al., 2004) support the hypothesis following that, many field factors, including food availability, climate, habitat management, inorganic soil fertilization, pests management strategies, pest resistance, etc. (Kielkiewicz, 1996; Karban & English-Loeb, 1988; Ako et al., 2006) may interact in a complex way to determine the population size of a specific pest at a certain time, when the conditions are particularly favorable for the development of this pest (Cohen, 2006). A broad example of conditions for optimal spider mite development may include (1) the absence on the host plant of other herbivorous competitors like insects, which are perfectly controlled by neonicotinoid insecticides (Elbert et al., 1991; 1998; van Iersel et al., 2000; Diaz & McLeod, 2005; Weichel & Nauen, 2004; Ishaaya et al., 2001; Nauen et al., 1996), (2) a reduced toxicity of these insecticides to spider mites and their predators, which is very well established for neonicotinoid (Elbert et al., 1990; 1991; Elzen, 2001; James, 1997; Hough-Goldstein and Whalen, 1993), (3) an increase in food availability for herbivorous pest and at the same time a reduced plant resistance against pests, a situation observed with the use/overuse of inorganic nitrogen fertilizers (Henneberry, 1962; Barker, 1975; Altieri et al., 1998; Altieri and Nicholls, 2003; Letourneau et al., 1996; Brodbeck et al., 2001) (4) an optimal temperature (25°-30°C) and relative humidity (50% and more) for spider mite reproduction (Sabelis, 1981; Jeppson et al., 1975; Laing, 1969), etc. same as (3) has nothing to do with imda problem A simultaneous occurrence of these favorable conditions may result in a boost in a specific pest population like TSSM.

The mass migration of mobile TSSM stages towards the upper parts of the plant as an effect of foliar application of field relevant dose rate of imidacloprid remains the most important finding in this study. The interference of residual effects of imidacloprid with the feeding behaviour of spider mites on the treated leaves is assumed to be the principal reason, though not yet studied. The overall number of different TSSM stages recorded in different treatments, once again, does not corroborate a potential increase in spider mite populations in the present greenhouse trials.

So far we considered only a single factor, i.e. neonicotinoid insecticides. However from the experimental point of view it is quite difficult to consider multiple field relevant factor in combination. But as a first attempt with a more complex situation in the following trial we focused, on the combined effect of inorganic nitrogen and imidacloprid on the fecundity of TSSM.

CHAPTER VII

7. Impact assessment of inorganic nitrogen fertilization combined with imidacloprid treatments on the reproduction of *Tetranychus urticae* Koch (Acari: Tetranychidae) populations on tomato [5]

7.1. Abstract

Abnormal increases in TSSM populations in the fields following the use of neonicotinoid insecticides is thought to result not only from the insecticide utilisation but also from several other fields factors including nitrogen fertilisation in combination with the insecticides. In the present greenhouse experiment, the effects of combined nitrogen and imidacloprid on the fecundity, the number of immature stages and adults TSSM were assessed on tomato plants. Two different levels of nitrogen Ni30 and Ni60 (144g and 288g/plant respectively) and the field recommended dose rate of imidacloprid (100ppm) (Imi) and their combinations Imi+Ni30, Imi+Ni60 were tested. Results showed no significant variations in the fecundity and the number of immature stages TSSM between the control treatment (C) and imidacloprid only treatment (Imi) (119.00 ± 10.48 and 112.40 ±9.15 eggs/leaf; 105.46 ± 7.24 and 108.80 ± 10.60 immature/leaf respectively). Both nitrogen only treatments Ni30 and Ni60 positively affected the fecundity (204.86 ± 19.72 and 213.73 ± 19.46 respectively) compared with C and Imi. The same effect of Ni30 and Ni60 was observed on the number of immature stages (156.33 ± 11.59 and 165.53 ±12.16) in comparison with C and Imi. However, The combinations Imi/Ni30

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[5] To be published as Ako, Mouhoube, Hans-Michael Poehling and Ralf Nauen. Impact Assessment of Inorganic Nitrogen Fertilization Combined with Imidacloprid Treatments on the Reproduction of *Tetranychus urticae* Koch (Acari: Tetranychidae) Populations on Tomato in *Entomologia Experimentalis et Applicata*

and Imi/Ni60 did not vary significantly in the fecundity (216.00 ± 15.17 and 189.10 ± 20.16 respectively) and the number of immature stages (138.20 ± 11.69 and 142.66 ± 10.81 respectively) compared with Ni30 and Ni60, suggesting that the increased fecundity and number of immature stages were due to nitrogen effects. No differences were observed between both nitrogen levels for fecundity and the number of immature stages. In two generations, the number of adults TSSM did not vary among the treatments. However the increased number of eggs and immature stages with nitrogen treatments suggests significant variations in the number of adults from the third adults' generation onwards.

7.2. Introduction

Common cultural practices such as inorganic soil amendments and pest management methods in intensive agro-ecosystems are nowadays clearly established in their role as major factors affecting crop susceptibility to agricultural pests (Henneberry, 1962; Barker, 1975; Altieri et al., 1998; Altieri and Nicholls, 2003; Letourneau et al., 1996; Brodbeck et al., 2001). Fertilization and especially the intensive use of inorganic nitrogen has been shown to affect all three categories of resistance i.e. preference, antibiosis, and tolerance (Altieri and Nicholls, 2003). The obvious morphological responses of crops to fertilizers, such as changes in growth rates, accelerated or delayed maturity, size of plant parts, and thickness and hardness of epicuticle, also influence the success of many pest species in utilizing the host (Altieri and Nicholls, 2003; Busch and Phelan, 1999). The indirect effects of fertilization practices on crop susceptibility to pests act through changes in the nutrient composition of the crop (Altieri and Nicholls, 2003). Among the nutritional factors that influence the level of arthropod damage in a crop, total nitrogen has been considered critical for both plants and their consumers (Mattson, 1980; Scriber, 1984; Slansky and Rodriguez, 1987). In many studies, in which the response of aphids and mites to N fertilization was evaluated, results showed that increases in N rates dramatically increased

aphid and mite numbers. According to van Emden (1966) increases in fecundity and developmental rates of the green peach aphid, *Myzus persicae*, were highly correlated to increased levels of soluble N in leaf tissue. Luna, (1988) has also indicated increased aphid and mite populations from N fertilization.

Recently, increased mites populations have been reported to occur field trials when neonicotinoid insecticides were used for controlling aphids and whiteflies in orchards and hops (Beers et al., 2005). In order to elucidate the relationships between the field increase in spider mites and the used of neonicotinoids, several laboratory experiments were carried out (Sclar et al., 1998; Ako et al., 2004; 2006, see chapter 2). Detailed studies on the reproductive biology of mites in relation to imidacloprid treatment revealed no evidences of mite population increases, while field experiments have clearly established the relationships between mite increases and the use of acetamiprid and imidacloprid in the field. These differences in the results between filed and laboratory experiments could be explained by the fact that, in the laboratory, the plants are protected from most abiotic stress factors and in term of nutrition, they are growing under the best conditions with a surplus e.g. of nitrogen, because of the absence of other plants competitors. In this case, the influence of nitrogen on the reproduction of TSSM being highly expressed, the eventual additional effects of the insecticide would not be important since the upper level of the reproduction is reached. According to the results discussed in chapter 4, it is very unlikely that a stress induced by the insecticide is responsible for the population increase in the field trials and that other factors not considered in the lab experiments may be interfering. In previous experiments, only the organic nitrogen contained in the humus earth used as plant growing media was available for plants. In the present experiment an evaluation of the influence of inorganic nitrogen and imidacloprid on the reproduction of TSSM in the laboratory will be done.

7.3. Materials and methods

Plant and mite materials

Contrary to previous experiments, in which French bean were used as host plant, we used tomato plants (*Solanum lycopersicum L*) var. Suso obtained from the horticultural seeds company Sperli (Carl Sperling &Co, Lüneburg, Germany) as we are evaluating the effect of nitrogen and in this case bean plants, which are able to fix and use atmospheric nitrogen would not be the right host plant. Tomato plants were produced in greenhouse in pots filled with humus earth (Fruhstorfer Erde Type P, Archut GmbH, Lauterbach-Wallenrod, Germany), at 25±1°C and 65±5% r.h. and used in the experiment at 6 leaves stage. Only one plant was produced per pot.

The strain Wiesmor (WI) of two-spotted spider mite was used in this experiment. The strain is an organophosphate-selected maintained in the laboratories of Bayer CropScience (Monheim, Germany) since 1954 under a biannual selection with the organophosphate oxydemeton-methyl (Nauen et al., 2001b). The strain WI has been characterized as susceptible to numerous acaricides, except organophosphates (Nauen et al., 2001b).

Nitrogen fertilization and bioassays

As inorganic nitrogen source, ammonium nitrate (granular formulation) (27%N) containing 13.5% ammonium and 13.5% nitrate was used. Two nitrogen levels were employed resembling a fertilization level of 30kgN/ha (Ni30) and 60kgN/ha (Ni60). Regarding a population of 20800 plants/ha (60cm x 80cm) recommended for the tomato cultivars used in this experiment this levels correspond to 1.44g and 2.88 g N/plant (here per pot) respectively for 30kgN/ha and 60kgN/ha. Different levels of fertilizer were incorporated to the soil in pot, a week prior to imidacloprid treatments. The experiment consisted of following treatments:

- *Control or 0 amendment* (C). Plants were watered daily with tape water,

- *Nitrogen only treatment*. There were two nitrogen levels: 1.44g (Ni30) and 2.88g/plant (Ni60).
- *Imidacloprid only treatment* (Imi). Imidacloprid was applied as drench treatment at 100ppm was applied. Per plant 100 ml of imidacloprid solution were applied.
- *Imidacloprid combined with nitrogen* (Imi+Ni30 and Imi+Ni60). Imidacloprid was combined with the two nitrogen levels. Imidacloprid solution was applied as above (100ml of 100ppm solution) as drench a week after the application of different fertilizer levels.

The experiment was replicated 3 times overtime and each replicate consisted of 5 plants

Once the treatments were complete plants were infested with female TSSM arisen from a synchronized culture. Each tomato plant leaflet received two females and the plants were kept in a climatic chamber at 25±1°C and 65±5% RH for 24 days after which different TSSM developmental stages i.e. eggs, immature stages and adults were recorded following different treatments.

Data collection

The number of eggs, immature stages and adults TSSM per plant were recorded using a mite-brushing machine as described in chapter 6 (paragraph 6.3.4) at the difference to experiment in chapter 6 that in this case, all the leaves of each experimental plant were harvested and thoroughly brushed.

Statistical analysis

The statistical differences among the treatments were checked using ANOVA (analysis of variance) through the general linear model (proc glm) procedure in SAS/STAT. The raw data on the number of eggs, immature stages and adults TSSM were first subjected to a descriptive analysis using box plot, which allowed the adjustment of the dependant variables to normal distribution through a log transformation. Thereafter, the analysis of variance was run and

the mean separation was preformed, in case of significant F values, using the least square means (lsmeans), adjusted to Bonferroni (Dunn) ttest.

7.4. Results

After two TSSM generations, the number of eggs, immature stages and adults were differently affected by the nitrogen level and by the combination of N supply with imidacloprid. No variations were observed in the number of eggs recorded between C and imidacloprid only treatment (Imi) (Figure 7.1). Both nitrogen levels have positively affected the fecundity with on average 71% and 79% more eggs respectively for Ni30 and Ni60 compared with C. Compared with Imi, the differences were on average 81% and 90% more eggs in Ni30 and Ni60 respectively (Figure 7.1). The combination of nitrogen and imidacloprid has shown almost the same results on the number of eggs as have shown both nitrogen only treatments, Ni30 and Ni60. In comparison with C treatment, the differences in fecundity were on average 81% and 58% more eggs for Imi+Ni30 and Imi+Ni60 respectively and compared with Imi, the differences in fecundity were on average 92% and 86% for Imi+Ni30 and Imi+Ni60 respectively (Figure 7.1). No differences in the fecundity were observed when comparing Ni30 and Ni60 with their corresponding combinations with imidacloprid Imi+Ni30 and Imi+Ni60 (Figure 7.1), suggesting that the increased fecundity observed in TSSM is likely to be a nitrogen effect. The numbers of TSSM immature stages were equally positively affected by Ni30 and Ni60 treatments but to a lesser extent compared with the fecundity. For Ni30 and Ni60 respectively, on average 48% and 57% more immature

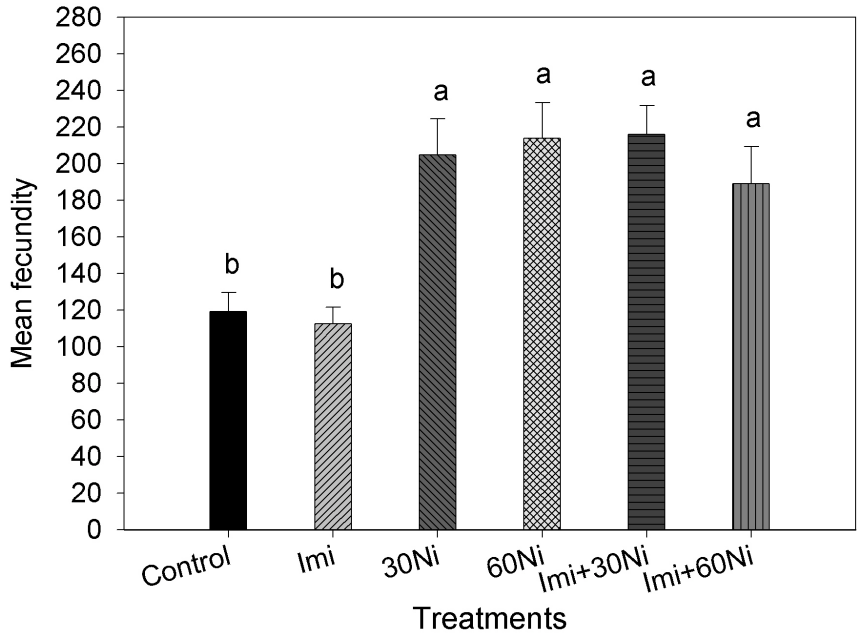

Figure 7.1. Effects of imidacloprid and nitrogen on the number of TSSM eggs per leaf

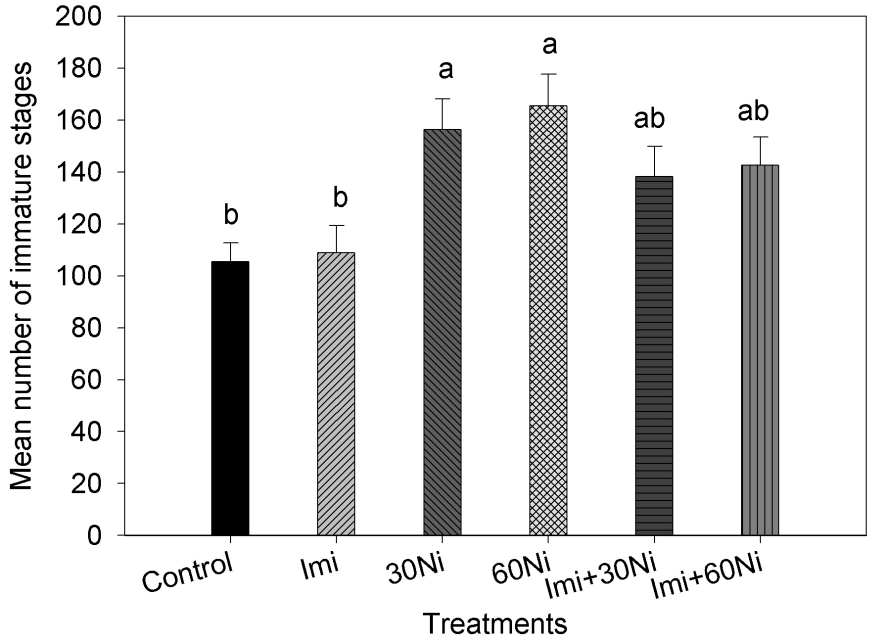

Figure 7.2. Effects of imidacloprid and nitrogen on the number of TSSM immature stages per leaf

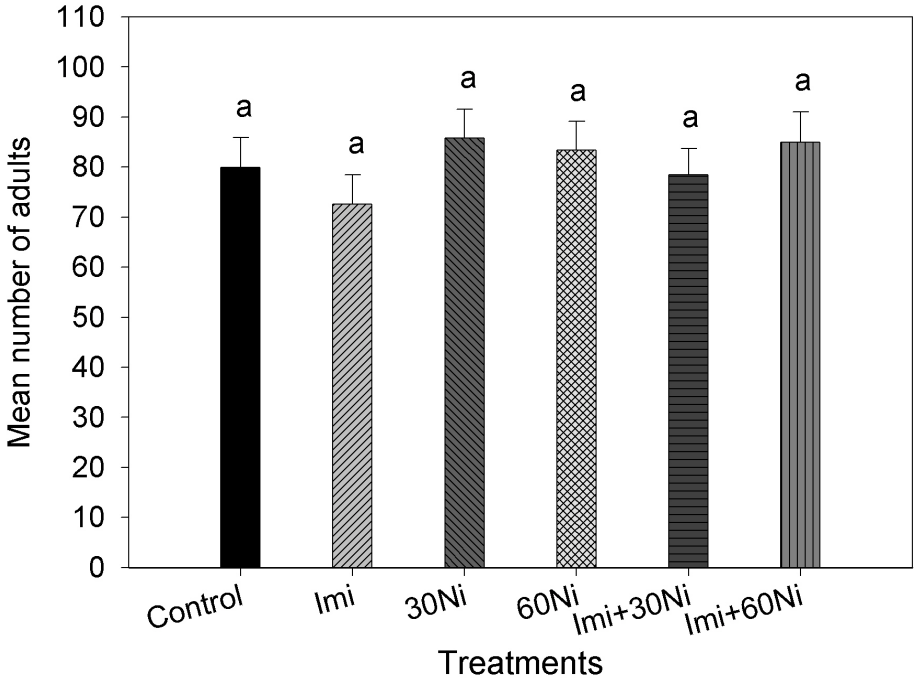

Figure 7.3. Effects of imidacloprid and nitrogen on the number of adults TSSM per leaf

stages were recorded compared with C and in comparison with Imi, the differences in the number of immature stages were on average 44% and 52% for Ni30 and Ni60 respectively (Figure 7.2). Regarding the number of immature stages, Imi+Ni30 and Imi+Ni60 did not differ significantly compared with C and Imi nor compared with Ni30 and Ni60 (Figure 7.2). In addition, the number of immature stages did not vary significantly between C and Imi, suggesting that the increased number of immature stages in two TSSM generations is more likely to be nitrogen-dependant.

For two generations TSSM developmental period, there were no significant differences in the number of adults TSSM among all the treatments (Figure 7.3).

7.5. Discussion

In the present experiment the combined effect of imidacloprid - which has been thoroughly essayed in the laboratory for its potential influence on TMMS population increases - and inorganic nitrogen - one of the most important factor not only for plant growth but also for increasing host susceptibility for herbivorous pests - have been tested to find out the effect of each of both factors in the reported increases in TSSM populations in the field. The results have clearly established the positive influence of nitrogen fertilization on the fecundity and the number of TSSM immature stages, with higher numbers of these TSSM developmental stages recorded on nitrogen-fertilized tomato plants compared with the control and imidacloprid only-treated plants. Similar effects of nitrogen fertilization on TSSM and other herbivorous arthropod pests have been reported. Wermelinger et al., (1991), studying the response of TSSM to different nutrients i.e N, P and K reported the existence of a positive correlation between the oviposition and the pre-imaginal developmental rate with the nitrogen and carbohydrate content of the apple leaves. Likewise, Wilson et al. (1998) reported significantly increased fecundity and shorter immature developmental time in *Tetranychus pacificus* with increasing foliar N concentrations. Moreover, Nukenine et al. (2000) reported a positive correlation of N concentration in cassava leaves and the population density of cassava green mite *Mononychellus tanajoa* Bondar. Several other investigations focusing on the role of plant nutrients on population dynamics of TSSM and other mite species have emphasized the increasing plant susceptibility to mite damages with increasing nitrogen concentrations of plant tissues (Tulisalo, 1971; Van de Vrie and Delver, 1979; Fritzsche et al., 1980; Jackson and Hunter, 1983; Wermelinger et al., 1985; Sharma and Pande, 1986; Wilson et al., 1988; Wermelinger, 1989; Yaninek et al., 1989; Wermelinger and Delucchi,1990). Considering the exponential pattern of TSSM population growth (Carey, 1982), an increase in the fecundity of more that 70% as recorded on nitrogen–fertilized plants could lead to tremendous changes in TSSM population density over only few generations. The increased

plant susceptibility to spider mites with higher concentrations of nitrogen in plant tissues has been attributed to an increase in food availability and quality for mites, combined with a decrease in plant defence compounds (Hoffland et al., 2000; Herms, 2002). Although the rates of N fertilization in this trial were lower than the rates employed in the field conditions (90-200 kg/ha against 30-60kg/ha in this experiment), it have been possible to put in evidence the reported effect of N on TSSM in a relatively short time corresponding to two generation of TSSM developmental time.

The combination of field relevant dose rate of imidacloprid and two different nitrogen levels has neither affected significantly the fecundity nor the number of immature stages compared with the treatments where the nitrogen was added alone. Moreover, no important variations were observed between the control (0 amendment) and imidacloprid alone treatment on tomato plants, corroborating the previous observations made on TSSM while testing the influence of imidacloprid on the reproduction of TSSM in the laboratory (Sclar et al., 1998, Ako et al., 2004; 2006). Nevertheless, an interrogation remains whether the observed effects of nitrogen in the laboratory conditions are transposable to the field insofar as the reproduction of TSSM and other spider mite species were reported to fluctuate highly with environmental conditions (Wermelinger et al., 1991), which are in turn often variable from seasons to seasons for the same location. Moreover some investigations showed that the influence of N on plants' susceptibility to mites depends on not only the concentration of N in the plant tissues but also on the proportion of other minerals such as phosphorus and sulphur, their lack or availability (Busch and Phelan, 1999). In conclusion, the effects of N on the nutritional quality of plants are themselves dependant on other factors and consequently, the same N concentration in leaves may result in different responses in TSSM development on the same host plant in different agro-ecosystems. Likewise, neonicotinoids may affect differently the reproduction of TSSM depending on many other factors that are still unknown.

It is nevertheless known that during the metabolism process of imidacloprid, the nitro-group reduction to nitrosimine and further loss of NO results in guanidine, which could in turn yield a derivate of urea (Sur and Stork, 2003). Urea being known as a nitrogen fertilizer, the suspicions that imidacloprid could provoke an increase in spider mites' populations in the field could be justified, in case it is demonstrated that the metabolite urea could be produced in enough quantity to trigger the already known effects of N on the reproduction of spider mites. For this, the extent to which the imidacloprid-derived nitrogen (urea) could affect the reproduction of spider mites has to be clarified, since the metabolite urea - like most metabolites - appears like trace compared with the mother compound and the conditions and delay for this metabolite to occur in the field conditions are not fully studied. However the effects of this metabolite were not observable in greenhouse conditions, where imidacloprid treatments did not differ from the control in for the fecundity and the number of immature stages.

In these circumstances, the most realistic and expectable situation, which could help to explain the increasing spider mites damages observed in the past few years, is more likely a synergistic effect of many agricultural factors than a single effect (Cohen, 2006).

CHAPTER VIII

8. General discussion

Two-spotted spider mite is a major cosmopolitan herbivorous pest occurring in the field as well as in greenhouse environment. The damage due to this pest is particularly remarkable in orchards and hops, where they share the same hosts with other important herbivorous insects like aphids and whiteflies. All this pests have to be controlled from an economical point of view but in an integrated approach with different measures. Whereas spider mites can be regulated, particularly in permanent crops like orchards with improved natural enemy populations (e.g. predatory mites), aphid and whitefly control often requires the intensive use of insecticides to keep these pests under economical thresholds. Insecticides of common use in the last decade, applied against aphids and whiteflies in orchards and hops are neonicotinoid insecticides. Neonicotinoids belong to the newest class of insecticides exhibiting an interesting mode of action (Nicotinic acethylcholine receptor agonist/antogonist). They are not only efficient against OPs or pyrethroid resistant pest strains but they are highly selective for insects (Elbert et al., 1991, Shimomura, 2005; Matsuda et al., 2005) making them more or less harmless to other arthropods like spider mites and predatory mites (Elbert et al., 1991), which is particularly important in an integrated control approach.

The use of neonicotinoid insecticides has recently retained the attention of producers and entomologists by the fact that sporadic abnormal increases in TSSM have been observed in the field where neonicotinoids, especially acetamiprid and imidacloprid were used for the control of insect pests. Since then, scientists have been drawing a particular attention to this phenomenon by undertaking field, but mainly greenhouse experiments in the sense of shading light on what could be called the “positive effect” of neonicotinoid

insecticides on the reproduction of TSSM. There exist up to now a relatively small body of literature on the phenomenon TSSM/neonicotinoids. However one of the first papers describing the effects of neonicotinoids on TSSM was published by Sclar al. in 1989, nearly a decade after the introduction of neonicotinoids into the market. In this paper, the authors (Sclar et al., 1989) reported significantly greater damage by *T. urticae* in the field after a soil application of imidacloprid, although they observed no effects on *T. urticae* populations or damage caused by mites in greenhouse trials. The authors rejected the hypothesis of hormolygosis and/or phytotoxicity and concluded that the adverse effect of the insecticide on the natural enemies may be a possible reason for spiter mite population increases. Contrary to these results, James and Price (2002) showed in laboratory experiments, in which leaf discs placed on water-saturated were used, that topical (spray) and systemic (drench) applications of imidacloprid at rates used in Washington hop yards have laid to significant increases in fecundity in TSSM. However, the leaf cut technique used by James and Prices (2002) was for many technical reasons not reproducible when we undertook our studies on the effect of 4 main neonicotinoids' active ingredients i.e. imidacloprid, thiacloprid acetamiprid and thiamethoxam, on the bionomics of TSSM (Ako et al., 2004). Moreover we found this technique not convenient. Hence the sticky ring technique (Nauen et al., 2001; Stumpf and Nauen, 2001), which has the advantage of allowing the use of living plants, which are more appropriate for testing systemic insecticides, was used. The results showed no evidences of a potential increase of TSSM in greenhouse as has already shown by Sclar et al. (1998).

On account of these controversial results, we hypothesized that the observed field increases of TSSM populations following neonicotinoids may be strain-dependant, insofar as the strains used in different trials were not the same and may probably bear different pesticide resistance backgrounds because of they originated from different agro-ecosystems. Therefore, we set up the first experiment (Chapter 2), with the objective to test the reproductive response to imidacloprid treatments of different TSSM strains exhibiting different acaricide

resistance background. The acaricide resistance was shown to affect the response of TSSM strains to imidacloprid in the sense that the acaricide susceptible strains were more affected by imidacloprid treatment than the resistance ones. However, none of the tested TSSM strains exhibited an increased fecundity. In addition, the fertility life table parameters were determined in relation to imidacloprid (Chapter 3) but the population dynamic parameters such as intrinsic rate of increase and finite rate of increase did not show, as hypothesized from reported field trials, any trends of a potential increase of TSSM populations. The fertility life table parameters showed on the opposite a potential decrease in TSSM population especially with soil application of imidacloprid, corroborating the results reported in previous experiments (Ako et al., 2004; 2006, see chapter 2). In addition, the increase in stress enzymes' activity observed in TSSM with imidacloprid treatment (Chapter 4) fits to the observed depressive effect of imidacloprid on the fecundity and can be interpreted as a kind of fitness costs, expressed by higher stress enzymes activity. Similar reactions have been reported from other stress situations, e.g. Zhang et al. (2004) described significant increases in stress enzyme activities in TSSM exposed to acid rain. Moreover, the mass migration of mobile TSSM stages from the imidacloprid-treated leaves to untreated leaves upward, when the leaves are treated with imidacloprid (Chapter 6) may attest non-lethal stress that imidacloprid treatment may have on TSSM as shown by increase enzym activities.

Different laboratory experiments ranging from bionomic to enzymatic activities have not given any evidence for the potential of imidacloprid to trigger abnormal mite increases. However the potential of imidacloprid on outbreaks of TSSM populations may be related to other factors not considered in the laboratory situation. Nevertheless, it would be difficult to predict the response of the same TSSM strains in the field conditions, as field factors are extremely variable and unpredictable. In order to obtain very first information about the effects of combined imidacloprid and one important field factor in terms of inorganic soil management on spider mite population outbreaks, we undertook

to study the combination of nitrogen fertilization and imidacloprid treatments (Chapter 7). N-fertilization is well known for its role of increasing the host plant susceptibility to herbivorous pests (Tulisalo, 1971; Van de Vrie and Delver, 1979; Fritzsche et al., 1980; Jackson and Hunter, 1983; Wermelinger et al., 1985; Sharma and Pande, 1986; Wilson et al., 1988; Wermelinger, 1989; Yaninek et al., 1989; Wermelinger and Delucchi, 1990). As already shown by Wermelinger et al. (1991), Wilson et al. (1998) and several other authors, nitrogen fertilization has led to higher TSSM fecundity and number of immature stages on tomato plants. We hypothesized that different fertilisation levels in the field and laboratory experiments may influence the magnitude or even direct spider mite response to imidacloprid. However, again we could not find any positive effect of imidaclorpid unter different fertilization levels on the reproduction of TSSM. An indirect positive effect of imidacloprid on TSSM population outbreaks could be imaginable if the metabolite urea, which is a breakdown product of imidacloprid (Sur and Stork, 2003), was shown to occur in enough quantity in field conditions to be able to reproduce the already known effect of nitrogen. However, the precise conditions, in which the metabolism of imidacloprid could lead to the formation of urea are still unknown, and it is very doubtful that some field conditions may eventually favor the formation of urea in quantity enough to act like a nitrogen fertilizer. Even then from our results we do not expect any significant effect.

Another pathway, which could trigger increased TSSM populations as a result of the use of neonicotinoid insecticides, would be a reduced inter-specific competition between insects – which are eliminated by insecticide treatments - and mites – which are not harmed by neonicotinoids. An increase in spider mite reproduction would be nearly evident if the insects, the principal concurrent of spider mites for food and space are eliminated. Likewise, an eventual disruption of biological equilibrium by eliminating or reducing the efficacy of natural enemies that would regulate TSSM population (Sclar et al., 1998; Hardin, 1995) would be an additional important synergistic factor, which

could result in increased TSSM populations in the fields, where neonicotinoids are used.

Experiments conducted in this project have confirmed the fact that the population outbreaks of spider mites consecutive to the use of neonicotinoid insecticides remain a complex field phenomenon. In order to shade additional light on the factors, which are involved in this phenomenon, additional stepwise field experiments allowing a multi-component statistical evaluation would be necessary.

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Acknowledgements

This research project was financially and technically supported by the Department of Insect Toxicology and Resistance of Bayer CropScience AG in collaboration with the Institute of Plant Disease and Plant Protection of Hannover University (IPP).

I'm most indebted to my supervisors Prof. Dr. Hans-Michael Poehling of Institute of Plant Diseases and Plant protection and Dr. Ralf Nauen of Bayer CropScience AG who followed my progress through valuable discussions, suggestions and comments. The same gratitude goes to Prof. Dr. Christian Borgemeister, the actual Deputy Director General of ICIPE, Kenya, who was my first academic supervisor in this project before joining ICIPE.

I'm grateful to Dr. David James of Washington State University for kindly providing me with his *T. urticae* strain, which I used in many experiments. I'm very much thankful to Dr. Martin Kaussman of the Analytic Department of Bayer CropScience AG, who has kindly accepted to help for HPLC analysis. Many thanks to M. Harald Köhler, Ms. Brunhild Lawson and all laboratory assistants working at Insect Toxicology and Resistance laboratory for their willingness to assist me in diverse experiments, which I conducted in this laboratory.

My sincere gratitude goes to all my colleagues and members of Entomology Working Group in IPP for valuable comments and discussions and for the very friendly working atmosphere, which has positively influenced the success of this project.

My sincere gratitude goes to my parents and specially my wife Essodjelinam Telou for the diverse encouragements and moral supports for the successful completion of my studies.

Many thanks to all physical or moral persons who have brought their contributions for the success of this project.

Thank you! ***Danke!*** ***Merci!***

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