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Riassunto

L'attività scientifica ha riguardato lo studio degli effetti dei prodotti fitosanitari sulle comunità di acari della vite, con particolare riferimento agli acari appartenenti alla famiglia dei Phytoseiidae. Lo studio si è articolato in una serie di esperimenti effettuati sia in laboratorio sia in pieno campo, di cui vengono di seguito esposti i principali risultati.

Resistenza in Tetranychus urticae in Italia

L'acaro fitofago *T. urticae* è stato scelto come primo modello di studio. Su diverse popolazioni dell'acaro tetranichide sono stati svolti dei test per valutare la tossicità acuta di alcuni acaricidi. I test hanno dimostrato che la resistenza agli acaricidi è diffusa e può manifestarsi anche nei confronti di formulati di recente introduzione. E' stato possibile individuare popolazioni resistenti e sensibili dell'acaro su cui effettuare una serie di saggi biochimici atti a valutare l'attività di diversi enzimi detossificanti potenzialmente correlati con la resistenza ai prodotti fitosanitari.

Effetti di prodotti fungicidi sulle comunità di acari in pieno campo

In questa seconda ricerca si sono valutati gli effetti di alcuni fungicidi (propineb, metiram, mancozeb, folpet e idrossido di rame) su una popolazione dell'acaro predatore *Kampimodromus aberrans* introdotta ex-novo in un vigneto in cui era già presente una popolazione autoctona di *Amblyseius andersoni*. L'acaro è stato inoculato nel vigneto sperimentale all'inizio della stagione vegetativa tramite l'apporto di femminelle prelevate da un vigneto "donatore". I fungicidi testati non sono apparsi selettivi nei confronti del predatore *Amblyseius andersoni*, probabilmente a causa dell'alta incidenza della peronospora nel testimone. La peronospora infatti rappresenta un alimento alternativo per tale predatore che ha dimostrato maggiori possibilità di sviluppo su piante non trattate. A differenza di *A. andersoni, K. aberrans* non è risultato influenzato dalla presenza della peronospora. Alcuni fungicidi (mancozeb e propineb) hanno esercitato effetti negativi significativi sulle popolazioni di *K. aberrans* a differenza di quanto verificato durante precedenti indagini su popolazioni stabili della stessa specie.

Effetto di insetticidi su Kampimodromus aberrans

Utilizzando materiale proveniente da diversi vigneti, sono stati allestiti allevamenti di *K. aberrans* in laboratorio. Su questi ceppi si è valutato l'effetto di alcuni insetticidi scelti sulla base del loro diverso modo di azione. In particolare si sono testati: esteri fosforici (chlorpyriphos), carbammati (indoxacarb), neonicotinoidi (thiamethoxam), chitino-inibitori (flufenoxuron), MAC

(methoxyfenozide), fenossiderivati (etofenprox), spinosine (spinosad). Le femmine dell'acaro predatore sono state sottoposte ad applicazioni di insetticidi alla dose di campo per valutare gli effetti su mortalità e fecondità delle femmine e schiusura delle uova deposte. L'impiego di etofenprox e spinosad è stato associato a effetti significativi sulla sopravvivenza e sulla fecondità di *K. aberrans*. Thiamethoxam ha causato una riduzione significativa della fecondità.

Resistenza di Kampimodromus aberrans a chlorpyriphos

Osservazioni condotte a Monteforte d'Alpone, S. Polo di Piave e S. Michele all'Adige hanno consentito di appurare un'elevata sopravvivenza nei confronti di chlorpyriphos da parte di *K. aberrans*. In seguito a tali osservazioni sono stati calcolati i parametri di tossicità acuta in queste popolazioni a confronto con altre non esposte a trattamenti insetticidi. Il calcolo dei parametri di tossicità acuta (DL_{50}, DL_{90}) è stato eseguito su due popolazioni raccolte su vite (Monteforte d'Alpone e S. Polo di Piave), su una popolazione raccolta su melo (S. Michele all'Adige) e su una popolazione prelevata su piante di bagolaro mai trattate con insetticidi. Quest'ultima ha esibito un'elevata sensibilità nei confronti di chlorpyriphos. Al contrario, i valori dei parametri di tossicità acuta nelle tre popolazioni raccolte su vite e melo sono risultati piuttosto elevati. I fattori di resistenza (RR), ottenuti dividendo la DL_{50} delle popolazioni resistenti per la DL_{50} della popolazione sensibile sono superiori a 100.000. E' possibile ipotizzare che i parametri della tossicità acuta si abbassino aumentando l'esposizione a chlorpyriphos (per via topica oltre che per via residuale) ma, in ogni caso, viene dimostrata per la prima volta la resistenza agli esteri fosforici in *K. aberrans*. I risultati ottenuti sono particolarmente significativi se si considerano le possibili implicazioni pratiche, in quanto le dosi letali superano ampiamente le dosi di pieno campo.

Effetti di diverse vie di esposizione a thiamethoxam in T. urticae e P. persimilis

Sono stati condotti esperimenti atti a valutare quale possa essere l'effetto degli insetticidi sugli acari in funzione di differenti modalità di esposizione. A tale scopo femmine del fitofago *T. urticae* e del predatore *P. persimilis* sono state esposte all'insetticida thiamethoxam per via topica (microimmersione), per via residuale (trattando solo il substrato fogliare) o per ingestione. In quest'ultima modalità, i predatori sono stati alimentati con prede (*T. urticae*) confinate su foglie di vite contaminate con thiamethoxam tramite applicazione al terreno. Infine, sono stati studiati gli effetti sugli acari delle possibili combinazioni tra i diversi modi di esposizione (topica + ingestione, topica + residuale, ingestione + residuale, topica + ingestione + residuale). La risposta delle diverse specie di acaro è stata valutata calcolando il tasso di sopravvivenza e il tasso di fecondità. In *T. urticae* e *P. persimilis* i trattamenti topici non hanno manifestato effetti letali, ma hanno ridotto in

modo significativo la fecondità. La combinazione di trattamenti fogliari e radicali ha avuto effetti negativi sia sulla mortalità sia sulla fecondità delle due specie.

Effetti di diverse vie di esposizione a thiamethoxam in Tetranychus urticae e Kampimodromus aberrans

Nel caso di *K. aberrans*, thiamethoxam non ha causato effetti negativi significativi a livello di sopravvivenza. Per quanto riguarda la fecondità, l'ingestione di prede contaminate è stata associata a effetti negativi più severi rispetto alle modalità di esposizione residuale e topica.

Interazioni tra K. aberrans – polline – insetticidi: prove di laboratorio

E' stata valutata l'influenza dell'apporto pollinico sulla sopravvivenza e sulla fecondità di acari predatori esposti o meno all'effetto di alcuni insetticidi impiegati in viticoltura e in frutticoltura (chlorpyriphos, etofenprox, indoxacarb e spinosad). Nel caso di chlorpyriphos, etofenprox e spinosad la sopravvivenza è stata inferiore rispetto a quella osservata nelle tesi testimone e indoxacarb. Il tasso di fuga è risultato più elevato su spinosad. Gli insetticidi hanno causato una riduzione significativa della fecondità di *K. aberrans* rispetto al testimone e a indoxacarb. I livelli più bassi di fecondità sono stati osservati nelle tesi etofenprox e spinosad, ma anche chlorpyriphos ha causato riduzioni significative della fecondità. La quantità e la frequenza delle applicazioni polliniche non sono state associate ad effetti significativi sulla sopravvivenza e sul tasso di fuga del predatore. Al contrario, la fecondità è aumentata sia con la dose sia con la frequenza di somministrazione di polline. Infine, è stata riscontrata un'interazione significativa insetticidi*frequenza di applicazione pollinica. A basse frequenze di somministrazione pollinica la fecondità è stata più elevata nel testimone che su chlorpyriphos, mentre a elevate frequenze l'insetticida non ha causato effetti sulla fecondità. Una tendenza simile a questa è stata riscontrata per indoxacarb. In conclusione, la disponibilità di alimenti alternativi può mitigare gli effetti deleteri degli insetticidi con importanti implicazioni di carattere applicativo.

Summary

The present study deals with the effect of pesticides on grape mites, with particular emphasis on predatory mites belonging to the family Phytoseiidae. This study comprises experiments carried out in laboratory and in open field. The main results are summarized below.

Resistance to acaricides in Tetranychus urticae *populations from Italy*
The two-spotted spider mite *Tetranychus urticae* was selected as study model. We conducted toxicological tests to assess the acute toxicity of several insecticides and acaricides on different spider mite populations. During these tests, resistance to acaricides in *T. urticae* was confirmed. This resistance can involve newly introduced formulations. The availability of susceptible and resistant populations of *T. urticae* allowed us to perform preliminary biochemical assays to assess the activity of detoxifying enzymes related to pesticide resistance.

Effects of some fungicides on predatory mites in North-Italian vineyards
We evaluated the effects of some fungicides (propineb, metiram, mancozeb, folpet and copper hydroxide) on the predatory mite *Kampimodromus aberrans* in a vineyard. The predatory mite was known to be poorly susceptible to ethylene-bis-dithiocarbamate (EBDCs) fungicides. In early season it was released in an experimental vineyard inhabited by another predatory mite, i.e. *Amblyseius andersoni*. Fungicides were applied following a randomized design. Fungicides proved to be not selective towards *A. andersoni* but this effect was influenced by the incidence of grape downy mildew *Plasmopara viticola*. In fact, this pathogenic fungus represents an alternative food for *A. andersoni*. *K. aberrans* was not influenced by the presence of *P. viticola*. EBDC fungicides caused significant effects on *K. aberrans* populations and induced spider mite increases.

Effects of a number of pesticides on the predatory mite Kampimodromus aberrans *in the laboratory*
To assess the effect of selected insecticides, we collected and reared some strains of *K. aberrans* native to different vineyards and apple orchards. We evaluated lethal and sub-lethal effects on predatory mite females. Spinosad and etofenprox were associated with significant effects on mite survival and fecundity. Thiamethoxam caused a significant reduction in fecundity.

Resistance to chlorpyriphos in the predatory mite Kampimodromus aberrans
Field investigations carried out in the Veneto region showed a potential resistance to chlorpyriphos in *K. aberrans* populations. Following these observations, we estimated acute toxicity (LC_{50} and

LC_{90}) on *K. aberrans* populations collected from vineyards (Monteforte d'Alpone and S. Polo di Piave), apple orchards (S. Michele all'Adige) or untreated hackberry trees (Legnaro). The latter exhibited a high susceptibility to chlorpyriphos. On the contrary, LC_{50} and LC_{90} in the three populations collected on grapes and apples were relatively high. Resistance factors (RR) were calculated by dividing LD_{50} of resistant strains by the LD_{50} of the susceptible strain. RR exceeded 100,000 for all strains being higher for Monteforte strain. These data demonstrated the resistance to organophosphates in *K. aberrans*. These results have significant implications because lethal concentrations were highest than recommended field doses.

Toxicity of thiamethoxam to Tetranychus urticae *and* Phytoseiulus persimilis *(Acari Tetranychidae, Phytoseiidae) through different routes of exposure*

The spider mite *Tetranychus urticae* and the predatory mite *Phytoseiulus persimilis* were exposed to topical (microimmersion bioassay), residual (treating leaves) or ingestion pesticide exposures. In the latter, the predators were fed with contaminated prey confined on treated leaves. We studied the effect of all possible exposure combinations on mites. Topical applications did not affect survival in *T. urticae* and *P. persimilis*, but showed significant effects on their fecundity.

Toxicity of thiamethoxam to Tetranychus urticae *and* Kampimodromus aberrans *(Acari Tetranychidae, Phytoseiidae) through different routes of exposure*

Similar experiments were conducted on *T. urticae* and the predatory mite *Kampimodromus aberrans*. Thiamethoxam did not affect *K. aberrans* survival. Regarding fecundity, feeding on contaminated prey was associated to more severe effects than residual and topical exposure.

Interactions between K. aberrans – pollen – insecticides: laboratory experiments

Field experiments suggested that the effects of pesticides on predatory mites were less pronounced where grass management induced a high pollen flow. We evaluated the influence of pollen availability on survival and fecundity of predatory mites exposed to some insecticides (chlorpyriphos, etofenprox, indoxacarb and spinosad). Survival on chlorpyriphos, etofenprox and spinosad was lower than that observed in the control and indoxacarb. The escape rate was higher on spinosad. Insecticides (exception made for indoxacarb) caused significant effects on the fecundity of *K. aberrans*. Etofenprox and spinosad showed the lowest levels of fecundity. The amount and the frequency of pollen application improved predatory mite fecundity. We found a significant interaction between insecticides and pollen frequency application. The availability of fresh pollen mitigated the effects of chlorpyriphos on predatory mite fecundity. In conclusion, the availability of

alternative foods can mitigate the effects of insecticide application with implications for Integrated Pest Management.

Chapter 1

Resistance to acaricides in *Tetranychus urticae* populations from Italy

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Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch, is one of the most important pests in agricultural systems, mainly annual crops, vegetables and ornamentals (Helle and Sabelis 1985). This species is characterised by high reproductive potential, short life cycle, and arrenotokous reproduction. These biological characteristics combined with frequent acaricides applications, facilities resistance build-up (Georghiou and Lagunes-Tejeda 1991; Van Leeuween et al. 2010). The application of some pesticides has been associated to an increase in *T. urticae* fecundity, and a number of pyrethroids and some herbicides, characterized by a repellent effect, induce population build-ups (Boller et al. 1985; Penman and Chapman 1988). Several products, toxic towards *T. urticae* natural enemies, reduce their potential allowing for spider mite population increasing (Helle and Sabelis 1985; Brandeburg and Kennedy 1987). A large number of synthetic pesticides with different modes of action have been used for many years to control of *T. urticae* that is currently considered as 'most resistant', in term of the total number of pesticides to which populations have became resistant (Van Leeuwen et al, 2009). In the last two decades problems connected with resistance to pesticides in *T. urticae* have stimulated a number of studies (e.g. Herron et al. 1994; Funayama and Takahashi 1995; Tsagkarakou et al.1996; Bruce-Oliver and Grafton-Cardwell 1997; Herron et al. 1997; Beers et al. 1998; Herron and Rophail 1998; Herron et al. 1998; Tsagkarakou et al. 1999; Nauen et al. 2001; Stumpf e Nauen 2001, 2002). Recently, Van Leeuwen et al. (2010) reviewed the most significant papers devoted to this topic giving special emphasis to insecticide/acaricide resistance mechanisms.

In Italy, *T. urticae* infestations are more common in greenhouses than in open field (e.g. vegetables, soybean and maize), and they occur sometimes in fruit orchards in correspondence with drought periods. Little is known on the spread of *T. urticae* resistant strains in Italy exception made for reported regarding dicofol and tetradifon (Rossi and Conti 1997). Nevertheless, problems with *T. urticae* are frequently reported on ornamental crops, in particular on protected roses where many acaricides recently introduced into the market show a progressive loss of effectiveness after few years from their use. This situation seems to reflect that reported in other countries. We have

*Paola Tirello contributed to all parts of the experimental work, data analysis and paper writing.

conducted laboratory trials to assess the response of some Italian strains of *T. urticae* to eight acaricides. Strains were selected on the base of their different origin and exposure to pesticides. Results of this study are here reported.

Materials and methods

Origin and rearing of spider mite strains

Three *T. urticae* strains were selected for this study: BOSA was collected from unsprayed vegetables near Bosa (Sardinia region) while PSE and SAN were collected from roses into commercial greenhouses located near Treviso (Veneto region) and Sanremo (Liguria region), respectively, where failures in acaricide use to control *T. urticae* were reported. These strains were maintained on the original host plants (beans or roses) in separated rearing boxes at the laboratories of Entomology, Department of Environmental Agronomy and Crop Science, University of Padua for a number of generations without exposing them to pesticides.

Acaricides

Eight acaricides were selected for trials due to their large use on ornamentals and other crops (Table 1). Doses used for the protection of roses or ornamentals were selected for trials. Spirodiclofen is not admitted on ornamental crops in Italy and thus doses admitted for other crops were considered. Some of these pesticides will be removed from the European market in the future. We used commercial formulations for each pesticide. Distilled water was used as a control during micro-immersion or leaf dipping.

Table 1. Characteristics of pesticides employed in laboratory trials.

Active Ingredient (A.I.)	Concentration of A.I.	Trade name	Recommended field dose
Abamectin	1.84 % ≡ 18 g/l	Vertimec 1.9 EC®	50 ml/hl ≡ 9 mg a.i/l
Bifenazate	22.65 % ≡ 240 g/l	Floramite 240 SC®	40ml/hl ≡ 96 mg a.i./l
Clofentezine	42 % ≡ 500 g/l	Apollo SC®	60ml/hl ≡ 300 mg a.i./l
Etoxazole	10.68 % ≡ 110 g/l	Borneo®	50ml/hl ≡ 55 mg a.i./l
Fenpyroximate	5.04% ≡ 51.26g/l	Miro®	200ml/hl ≡ 102.52 mg a.i./l
Flufenoxuron	4.7 % ≡ 50 g/l	Cascade 50 DC®	150ml/hl ≡ 75 mg a.i./l
Hexythiazox	24 % ≡ 257 g/l	Matacar FL®	20ml/hl ≡ 51.4 mg a.i/l
spirodiclofen	22.3% ≡ 240 g/l	Envidor 240 SC®	50ml/hl ≡ 120 mg a.i./l
Tebufenpyrad	25 %	Oscar®	65g/hl ≡ 162.5 mg a.i./l

Female bioassay

The acaricides primarily active on mite motile stages (e.g., abamectin, bifenazate fenpyroximate , flufenoxuron, tebufenpyrad) were tested on adult females of *T. urticae*. All trials were conducted using the micro-immersion bioassay (Dennehy 1993; Castagnoli et al. 2005, Duso et al. 2008). Mites were drawn into a small pipette tip, after which the acaricide solution was drawn up, immersing the mites for 30 s. The mites were ejected from the pipette, dried on filter paper and then transferred into holding cells with a fine brush. To combine topical and residual exposure, bean or rose leaves were dipped in the insecticide solution for 30 s before adding them to the holding cells as substrate for mite rearing. Five *T. urticae* coeval females were transferred into each cell. The experiment was carried out with five or more serially diluted concentrations covering the range of 0–100% mortality. The assessment of lethal toxicity involved at least 40 females per concentration. Lethal toxicity was evaluated 72 h after pesticide application by counting dead and alive mites. Mites were considered dead if they were unable to react when gently probed with a fine brush. All trials were conducted at 25 ± 1°C, 60±10% RH, and 16:8 h (L:D).

Egg bioassay

Lethal toxicities of acaricides with principal ovicidal activity (e.g., etoxazole, clofentezine, hexythiazox, spirodiclofen) were assessed on mite eggs using the leaf dip method (Castagnoli et al. 2005). Mite females were left on bean or rose leaves to oviposit for 24 h and then were removed. The leaves with eggs were dipped in the test solution for 30 s and then placed upside down on a wet cotton pad. Eggs were checked daily and hatching evaluated until the 90% of them hatched in the control. The effect of pesticides was evaluated on at least 100 one-day old eggs per dose. All trials were conducted at 25 ± 1°C, 60±10% RH, and 16:8 h (L:D).

Data analysis

In trials with females or eggs LC_{50} values and 95% fiducial limits were calculated from probit regressions using PriProbitNM (Sakuma 1998). Resistance ratios (RR_{50}) were calculated by dividing the LC_{50} value of the suspected resistant strain by the LC_{50} value of susceptible strain.

Kinetic enzymatic assay

For these trials, a number of *T. urticae* females were aspirated from cultures. Mass homogenates of freshly sampled mites were prepared by crushing 50 adult female mites in 175µl sodium phosphate buffer (0.1M, pH7.6) with a pestle in glass tubes. The homogenate was centrifugated at 10,000 × g

and 4ºC for 15 min. The resulting supernatant was used as an enzyme source for esterase assays. Protein concentration was determined with a commercially available Bradford kit (Bradford reagent, SIGMA® product number B6916) using bovine serum albumin as the standard. Esterase activities on different *T. urticae* strains were measured following the colorimetric enzymatic assay described by Van Leeuwen and Tirry (2007). 1-Naphthyl acetate (1-NA) esterase activity was measured by mixing 100µl of enzyme source, 60µl of phosphate buffer (0.1M, pH 7.6) and 20µl of Fast Blue RR salt (0,75mg/ml). The reaction was started by adding 20µl of a 1-NA (2.5mM, acetone 50% v/v). The formation of 2-naphtohol-fast blue RR dye complex was measured for 10min at 500nm and converted to specific activity a standard curve of 1-naphtol and fast blue RR.

Results and discussion

BOSA strain proved to be very susceptible to selected acaricides confirming the results of previous studies conducted with various insecticides (Duso et al. 2008). Lethal doses (LC_{50}) of different acaricides for females or eggs belonging to this strain were much lower than field doses. PSE and SAN strains showed to be resistant to some acaricides when compared to BOSA and their LC_{50} or LC_{90} values were often higher than field doses of these acaricides. The resistance ratios of these two strains did not follow a definite pattern but PSE appeared to be involved most frequently in higher RR values.

Regarding abamectin, PSE exhibited the highest resistant ratio and LC_{50} values resulted slightly higher than maximum field recommended dose (Table 1). SAN showed to be less susceptible than BOSA to abamectin (Table 1) but RR values were much lower than those of PSE. Resistance to abamectin in PSE and SAN strains was hypothesized on the base of field data. Both strains were reared in the laboratory without exposure to abamectin before to carry out these trials. Resistance to abamectin is unstable in the absence of selective pressure (Sato et al. 2005) and this phenomenon may be involved in the results obtained for SAN strain in the current study. On the other hand, data related to PSE strain confirm that resistance to abamectin can occur in Italian *T. urticae* populations inhabiting greenhouses as reported by rose growers. Abamectin has represented one of the most effective acaricides but its misuse has likely exacerbated problems with spider mites. Since resistance to abamectin in unstable its use could be re-established after some time depending on farm pesticide choice. Switching to biological control could reduce drastically acaricide use slowing down the occurrence of resistance phenomena. The compatibility between abamectin and predatory mites has been matter of study. Different authors reported that abamectin is substantially compatible with IPM in various systems (e.g. Trumble and Morse 1993; Cote et al. 2002). Resistance to abamectin has been reported in many studies conducted on *T. urticae* (Campos et al. 1995; Beers et

al. 1998; Stumpf and Nauen 2002; Sato et al. 2005; Nicastro et al. 2009) but few studies investigated the mechanisms of resistance. Stumpf and Nauen (2002) suggested the involvement of metabolic detoxification mediated by cytochrome P450-dependent monooxygenases and glutathione S-transferase. Others studies suggested a decrease in abamectin binding sites as a possible resistance mechanism (Clark et al. 1995).

Table 2 – Concentration probit-mortality data of eight acaricides applied to females of *T. urticae* strains with calculated resistance ratios.

Active ingredient	strain	LC_{50}	95% fiducial limit		LC_{90}	95% fiducial limit		Intercept ± SE	slope ± SE	RR_{50}	RR_{90}
			lower	upper		lower	upper				
abamectin	BOSA	0.01	0.00	0.01	0.46	0.24	1.33	0.98 ± 0.10	0.72 ± 0.10	-	-
	SAN	0.15	0.07	0.38	3.33	0.99	41.75	0.08 ± 0.17	0.95 ± 0.19	18.94	7.18
	PSE	10.13	7.05	14.12	78.60	48.01	172.20	-2.52 ± 0.40	1.44 ± 0.20	1294.15	169.29
bifenazate	BOSA	0.15	0.03	0.39	16.80	7.12	63.20	0.75 ± 0.11	0.63 ± 0.10	-	-
	SAN	3.70	2.02	7.02	100.87	34.90	919.78	-0.17 ± 0.12	0.89 ± 0.17	24.01	6.00
	PSE	5.15	3.77	7.29	72.42	39.69	175.98	-0.37 ± 0.09	1.12 ± 0.12	33.43	4.31
fenpyroximate	BOSA	154.47	113.21	213.08	1148.17	630.40	3872.33	-3.65 ± 0.71	1.471 ± 0.27	-	-
	SAN	11441.31	6066.11	39799.80	426795.89	90909.61	13042594.40	-3.55 ± 0.58	0.815 ± 0.16	74.07	371.72
	PSE	3996.59	2502.62	8857.22	54504.76	19464.96	389904.06	-4.4 ± 0.62	1.129 ± 0.19	25.87	47.47
tebufenpyrad	BOSA	2.58	2.09	3.22	14.06	9.39	26.72	-0.02 ± 0.09	1.74 ± 0.22	-	-
	SAN	124.79	83.86	170.60	838.88	548.43	1664.13	-2.63 ± 0.46	1.55 ± 0.23	59.76	89.36
	PSE	421.40	295.00	587.08	2406.28	1473.95	5729.75	-3.77 ± 0.66	1.69 ± 0.28	201.79	256.32

Figure 1: Concentration-mortality probit lines of *T. urticae* strains in response to four acaricides applied to females. Observed (obs) and expected (exp) values for each strain are reported.

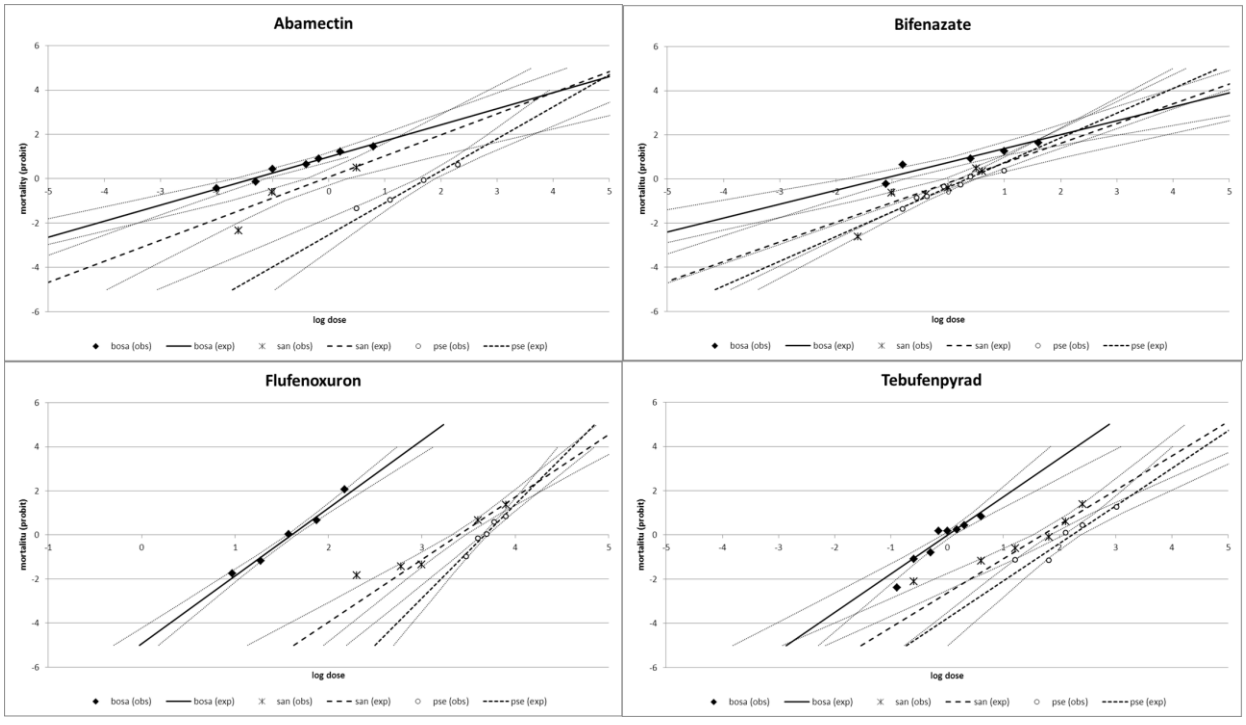

Table 3 – Concentration probit-mortality data of eight acaricides on eggs of *T. urticae* strains with calculated resistance ratios (RR).

Active ingredient	strain	LC_{50}	95% fiducial limit		LC_{90}	95% fiducial limit		Intercept ± SE	slope ± SE	RR_{50}	RR_{90}
			lower	upper		lower	upper				
clofentezine	BOSA	1.61	13.49	1.90	5.46	4.36	7.39	1.19 ± 0.13	2.42 ± 0.24	-	-
	SAN	107147.58	89515.00	130685.00	414560.00	295135.00	710550.00	-9.45 ± 1.21	2.18 ± 0.28	66472.64	75975.44
	PSE	275175.12	221220.00	348550.00	1891050.00	1177600.00	4169150.00	-7.26 ± 1.00	1.53 ± 0.21	170714.23	346568.31
etoxazole	BOSA	0.006	0.005	0.008	0.035	0.026	0.051	3.89 ± 0.35	1.73 ± 0.15	-	-
	SAN	0.017	0.011	0.024	0.291	0.173	0.585	1.88 ± 0.17	1.04 ± 0.10	2.67	8.41
	PSE	0.402	0.322	0.516	3.053	1.967	5.767	0.64 ± 0.11	1.46 ± 0.15	63.86	88.35
flufenoxuron	BOSA	20.05	18.00	22.33	52.37	44.76	63.73	-4.93 ± 0.36	3.07 ± 0.22	-	-
	SAN	1241.25	1042.45	1449.05	3522.70	2884.65	4623.70	-9.60 ± 1.06	2.83 ± 0.30	61.92	67.27
	PSE	2364.61	2212.15	2520.90	4733.65	4173.70	5681.00	-15.62 ± 1.62	4.25 ± 0.44	117.96	90.39
hexythiazox	BOSA	0.25	0.18	0.34	3.10	2.13	4.94	1.19 ± 0.10	1.18 ± 0.10	-	-
	SAN	17767.28	14386.60	21446.91	123000.20	90749.27	186633.40	-5.86 ± 0.57	1.53 ± 0.14	70243.93	39740.93
	PSE	40341.61	36740.72	44882.48	145061.08	115590.89	197288.62	-9.67 ± 0.78	2.31 ± 0.19	159492.82	46868.72
spirodiclofen	BOSA	0.76	0.65	0.90	3.12	2.33	4.67	1.04 ± 0.14	2.09 ± 0.18	-	-
	SAN	1.24	1.08	1.42	4.68	3.80	6.11	0.64 ± 0.08	2.23 ± 0.17	1.63	1.50
	PSE	1.45	1.28	1.64	4.76	3.94	6.06	0.54 ± 0.08	2.48 ± 0.19	1.91	1.52

Figure 2: Concentration-mortality probit lines of *T. urticae* strains in response to four acaricides applied to eggs. Observed (obs) and expected (exp) values for each strain are reported.

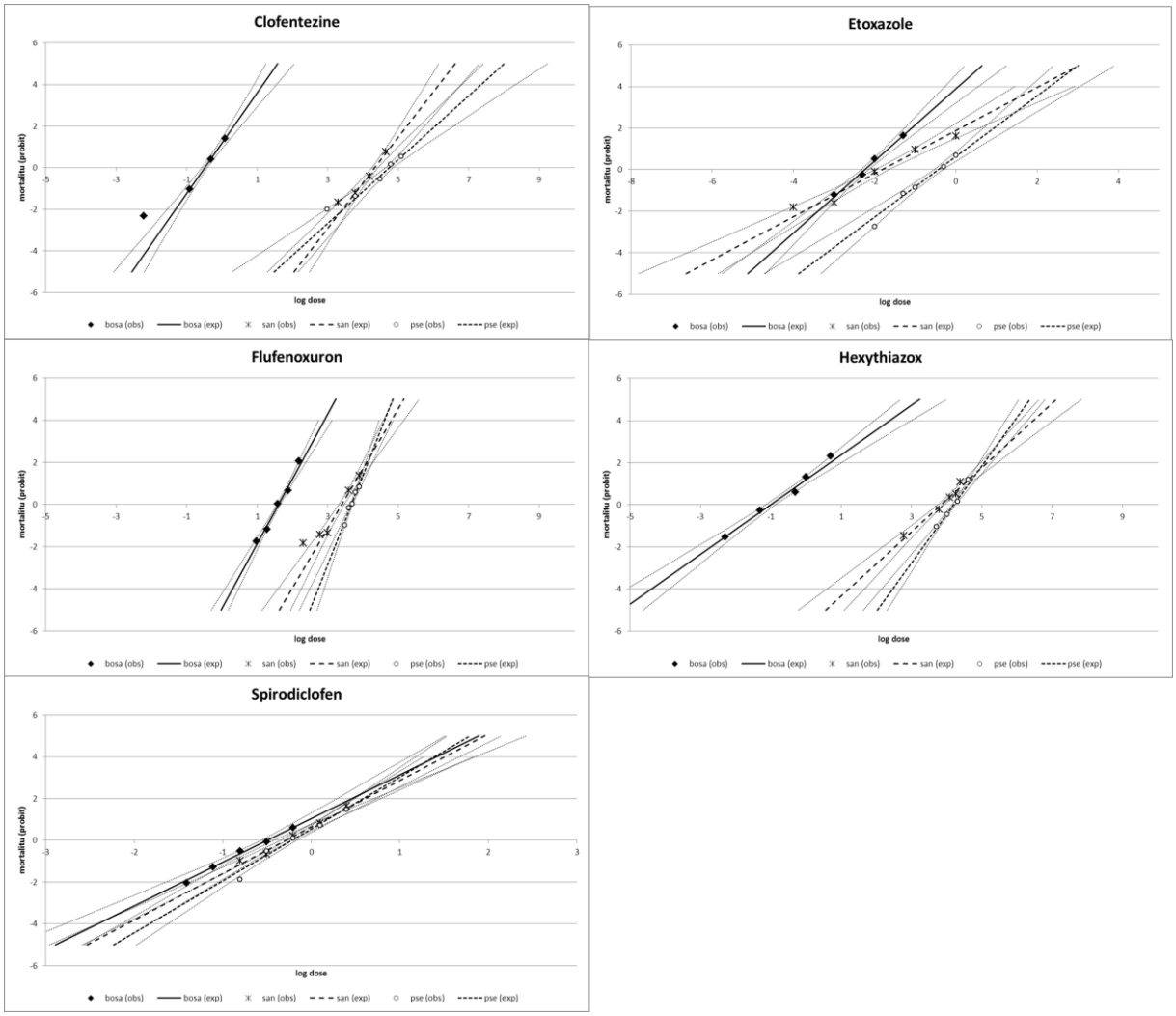

Table 3 – Hydrolysis activity (nmol/min/mg protein) of α-Nin susceptible (BOSA) and resistant strains (SAN, PSE) of *T. urticae* and relative ratios between resistant and susceptible strains (R/S)

Strain	EST ± SD * nmol/min/mg	R/S
BOSA	206.72 ± 34.73 a	-
PSE	268.23 ± 41.47 a	1.30
SAN	349.02 ± 137.02 b	1.70

*Mean following by the same letter are not significantly different ($p < 0.05$; Student's *t* test).

BOSA females were susceptible to tebufenpyrad in contrast to PSE and SAN females (Table 2). Relatively high RR_{50} values were found for the latter. Similar results were obtained with fenpyroximate belonging to the same pesticide class (Table 2). LC_{50} values of PSE and SAN populations exceeded field doses explaining the failure of these METI compounds in the control of spider mites on protected roses. Resistance to METIs has been reported in a number of regions and crops (Van Leeuwen et al. 2009) and mechanisms seem to be due to oxidative degradation (Stumpf and Nauen 2001; Kim et al. 2004). Genetic studies (Van Pottelberge et al. 2009c) suggested that resistance to fenpyroximate was under monogenic control, while resistance to tebufenpyrad was under the control of more genes.

Bifenazate belongs to the recently discovered acaricide group of hydrazine derivatives and is nowadays used worldwide for the control of spider mites on several crops (Dekeyser 2005). BOSA was susceptible to bifenazate, and its LC_{50} was almost 100-fold less than the recommended field dose (Table 2). Moderate RR_{50} values were calculated for PSE and SAN (33.43 and 24.01, respectively) and their LC_{90} values were closed to field dose. The use of bifenazate has been associated to good results in its early use and failures in controlling *T. urticae* are not frequently reported in Italy. However, our results suggest that resistance can develop easily. Moreover, a high level of resistance has been developed by strains selected with bifenazate for a number of generations (Van Leeuwen et al. 2006). Cytochrome b, a protein encoded by the mitochondrial genome (gene cytb) has been recently proposed as the target site for bifenazate (Van Leeuwen et al. 2008).

Egg bioassays showed the susceptibility of BOSA eggs to growth inhibitors hexythiazox and clofentezine (Table 3). However, PSE and SAN strains exhibited high resistance levels to these pesticides (RR_{50}> 150000 for PSE and RR_{50} > 60000 for SAN). These acaricides have been successfully used for many years but failures have been recently reported. Nevertheless, they are still used in rotation with other compounds because of their ovicidal activity. Lethal doses were much higher than field doses confirming the poor results obtained when these acaricides are employed on protected ornamentals. Resistance to clofentezine and hexythiazox has been reported in Australia and in Europe (see Van Leeuwen et al. 2009). Herron and Rophail (1993) found that hexythiazox resistance is under monogenic control. More recently, Asahara et al (2008) demonstrated that more loci are involved in hexythiazox resistance in *T. urticae*. Cross resistance between clofentezine and hexythiazox has often been reported (Herron et al. 1993; Nauen et al. 2001; Pree et al. 2002).

Etoxazole caused high mortality to the three strains. LC_{90} values were much lower than field doses in accordance with the good results obtained in greenhouses with this acaricide. Resistance to

etoxazole was found in population of *T. urticae* collected from apple orchards in Japan (Kobayashi et al. 2001; Usagi et al. 2002) and from rose greenhouses in Korea Republic (Lee et al. 2004). In Canada (Pree et al. 2005) resistance to etoxazole was detected in *Panonychus ulmi* (Koch) populations resistant to clofentezine and hexythiazox. Also Asahara et al. (2008) demonstrated cross-resistance between hexythiazox and etoxazole. In Europe, etoxazole resistant strains have not been found. However, the use of this pesticide should be limited to reduce risk especially where resistance to clofentezine and hexythiazox occurs.

Flufenoxuron affected BOSA eggs while lethal values for SAN and PSE strains were higher than field doses. Resistance to flufenoxuron is suggested when toxicities are compared to that of the susceptible strain (RR_{50} of 61.92 and 117.96, respectively). On the other hand flufenoxuron is associated with failures in Italian greenhouses.

The effects of spirodiclofen on eggs of the three strains were significant. However its use in greenhouse ornamentals is not admitted in Italy. Some authors (Rauch and Nauen 2002; Van Pottelberge et al. 2009b) reported spirodiclofen resistance in selected *T. urticae* strains. They suggested that ester cleavage and oxidative detoxification were the main degradation pathway of this pesticide in *T. urticae*. Van Pottelberge et al. (2009a) stressed that the detection of spirodiclofen resistance should not be limited to mortality bioassays with eggs or larvae, but should be combined with inhibitory studies on female fecundity. Moreover, without selection pressure, resistance was unstable (Rauch and Nauen 2002), probably due to the intermediate and polygenic mode of inheritance (Van Pottelberge et al. 2009b).

In the present study we investigated whether increased esterase activities can be linked to resistance in *T. urticae*. Only PSE strain presented a significant difference on hydrolysis of α-naphthyl acetate. Ratios between resistant and susceptible values in terms of esterase activity were 1.30 and 1.70, for SAN and PSE respectively. Esterases have been associated with insecticide resistance in various arthropods. While many organophosphates are irreversible inhibitors of carboxylesterases, other organophosphates, but also carbamates, pyrethroids, organochlorines, benzoylphenylureas and juvenile hormone analogues are susceptible to esterase hydrolysis (Van Leeuwen et al. 2009). Further biochemical studies with alternative substrates and use of synergists in bioassays will be conducted to elucidate this topic.

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Chapter 2

Effects of some fungicides on predatory mites in North-Italian vineyards

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Introduction

Compatibility of pesticides with beneficial arthropods is a key aspect of Integrated Pest Management (IPM). Fungicides used in viticulture can affect beneficial mites (Phytoseiidae) with implications for spider mite outbreaks (Ivancich Gambaro 1972, 1973; Girolami 1981, 1987). Ethylene-bis-dithiocarbamate fungicides (EBDCs) have been frequently involved in these problems. Predatory mites have been included as non-target organisms in trials required to register pesticides to be used in viticulture. Information on such effects is fundamental for IPM.

In the 1980s, the frequent application of mancozeb (belonging to EBDCs) instead of copper fungicides was associated with a dramatic decline in phytoseiids, mainly *Kampimodromus aberrans* (Oudemans) and *Typhlodromus pyri* Scheuten in European vineyards (Girolami 1981; Benciolini 1982; Baillod et al. 1982; Duso et al. 1983; Girolami and Duso 1984; Ivancich Gambaro 1984). On the other hand the presence of *K. aberrans* strains capable to tolerate the repeated use of EBDCs has been reported (Vettorello and Girolami 1992; Posenato 1994). More recently, resistance to EBDCs has been demonstrated in the laboratory for a strain of *K. aberrans* inhabiting French vineyards (Auger et al. 2004, 2005) and a strain of *Amblyseius andersoni* (Chant) inhabiting apple orchards (Angeli and Ioriatti 1994).

In the current study we compared the effects of a number of fungicides on predatory mite populations in a vineyard located in Northern Italy. We investigated these effects on native predatory mite populations of *A. andersoni* and on released populations of *K. aberrans* known for their field resistance to EBDCs. Relationships between plant pathogen incidence (i.e. *Plasmospara viticola* Berk. et Curtis ex. de Bary) and predatory mite population abundance were also considered.

Materials and methods

Observations were carried out in 2008 in a vineyard comprising the Merlot cultivar and located at Conegliano (Treviso province, North-eastern Italy). Five fungicide lines were compared: (1) ‘‘control” – untreated; (2) ‘‘metiram” where metiram 71.2% (Poliram DF) was applied at 200 g/hl;

(3) “propineb” where propineb 70% (Antracol WG) was applied at 200 g/hl; (4) “mancozeb” where mancozeb 75% (Dithane DG Neotech) was applied at 200 g/hl; (5) ‘‘folpet - Cu hydroxide” where folpet 80% (Folpan 80 WDG) was applied at 150 g/hl in the first dates and copper hydroxide (Kocide 3000) was applied at 300 g/hl in the subsequent dates. The last was considered as “non toxic reference”.

Different fungicides were applied on four plots (replicates) each of nine plants (Figure 1). In early season, two plants per plot were treated with pyrethrins to eliminate virtually native predatory mite populations. Then a strain of *K. aberrans* field resistant to mancozeb and originating from a vineyard located in the Veneto region (Monteforte d’Alpone, Verona province) was released on four plants of each plot in early May. Approximately, 100 *K. aberrans* motile forms were released per plant by using suckers. Therefore, the original plots were divided into subplots to compare the effect of fungicides on released (*K. aberrans*) or native predatory mites. Fungicides were applied every 7–10 days from May 6th to July 7th; from July 15th onwards all plots were treated with copper hydroxide. Insecticides were not applied.

Sampling was made approximately every 15 days from May to early September by collecting 32 leaves for each treatment. On each sampling, leaves were returned to the laboratory where mites were identified at family level and counted under a dissecting microscope. Phytoseiids were mounted on slides, in Hoyer’s medium, and identified under a phase contrast microscope. At the same time we estimated *P. viticola* incidence as a surface (cm^2) of leaves showing sporulations/lesions. Sampling was performed during the fungicide application period plus a month afterward.

Data analysis

We evaluated the effects of fungicides on predatory mite abundance and GDM foliar symptoms with a Repeated Measure Analysis of Variance model performed with the SAS MIXED procedure (SAS Institute Inc. 1999). In this analysis, treatment, time and their interactions were considered as sources of variation and F tests were used to evaluate their effects (a = 0.05). Degrees of freedom were estimated using the Kenward-Roger method (Littell et al. 1996). The results were analyzed with SLICE option of LSMEANS (SAS Institute Inc. 1999), considering two different phases: (a) the period of fungicide application plus an additional month (Blümel et al. 2000) and (b) the period subsequent fungicide application.

Results

*Effect of fungicides on “*K. aberrans *release” plots*

Kampimodromus aberrans releases were successful and this species was dominant over the native *A. andersoni* (Figure 1). Differences among treatments were significant (Table 1). In the evaluation phase (a) *K. aberrans* densities were higher on the control and "folpet - Cu hydroxide" treatments than on "mancozeb", "propineb" and "metiram" treatments. There were no differences among treatments when evaluation phase (b) was considered (Table 1).

Table 1 – Effect of fungicides on the abundance of *K. aberrans* on "*K. aberrans* release" plots.

Effects	F	d.f.	P
Treatment	4.80	4; 96.1	0.001
Date	12.74	6; 95.2	< 0.001
Treatment x Date	2.55	24; 95.2	< 0.001
During period a (fungicide application + one month)			
control vs. mancozeb	14.59	1; 95.2	< 0.001
control vs. propineb	18.48	1; 95.2	< 0.001
control vs. metiram	4.79	1; 95.2	0.031
control vs. folpet - Cu hydroxide	0.91	1; 96.8	0.343
mancozeb vs. propineb	0.23	1; 95.2	0.632
mancozeb vs. metiram	2.66	1; 95.2	0.106
mancozeb vs. folpet - Cu hydroxide	19.81	1; 96.8	< 0.001
propineb vs. metiram	4.45	1; 95.2	0.037
propineb vs. folpet - Cu hydroxide	23.91	1; 96.8	< 0.001
metiram vs. folpet - Cu hydroxide	8.74	1; 96.8	0.004
During period b (after fungicide application)			
control vs. mancozeb	0.55	1; 95.2	0.460
control vs. propineb	0.02	1; 95.2	0.879
control vs. metiram	0.10	1; 95.2	0.758
control vs. folpet - Cu hydroxide	2.45	1; 96.5	0.120
mancozeb vs. propineb	0.35	1; 95.2	0.557
mancozeb vs. metiram	0.19	1; 95.2	0.666
mancozeb vs. folpet - Cu hydroxide	0.78	1; 96.5	0.378
propineb vs. metiram	0.02	1; 95.2	0.875
propineb vs. folpet - Cu hydroxide	2.03	1; 96.5	0.157
metiram vs. folpet - Cu hydroxide	1.64	1; 96.5	0.203

Amblyseius andersoni populations reached low levels in "*K. aberrans* release" plots (Figure 1). There were no differences among treatments (Table 2).

Table 2 – Effect of fungicides on the abundance of *A. andersoni* on "*K. aberrans* release" plots.

Effects	F	d.f.	P
Treatment	1.97	4; 96.4	0.106
Date	1.73	6; 95.3	0.122
Treatment x Date	0.88	24; 95.3	0.632

Densities of *Panonychus ulmi* (Koch) increased in July (Figure 2). Differences among treatments were found only when evaluation phase (b) was considered (Table 3). Spider mites reached higher densities on "propineb" and "mancozeb" treatments than in the control and "folpet – Cu hydroxide" treatments (Table 3).

Symptoms by *P. viticola* were detected in June (Figure 1). During evaluation period (a) its incidence on leaves was higher in the control plots than on remaining treatments (Table 4). There were no differences among treatments in the period (b).

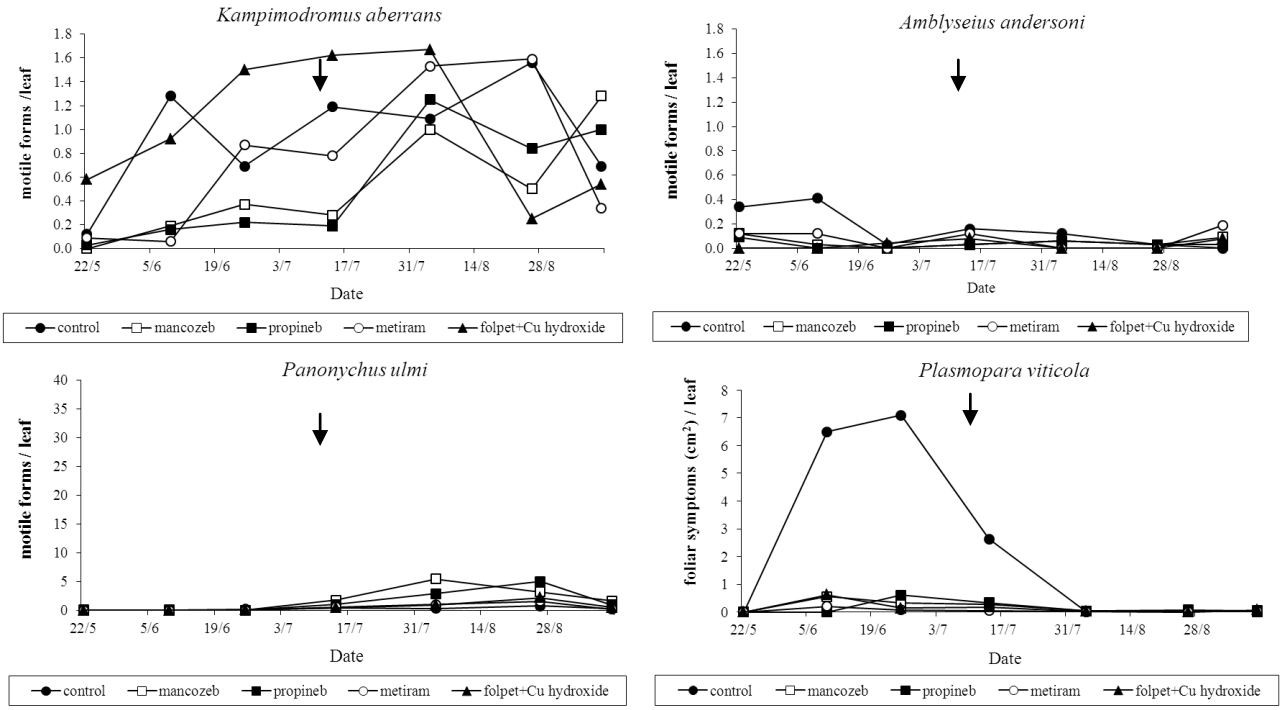

Figure 1 – Population dynamics of predatory mites, *P. ulmi* and incidence of *P. viticola* on "*K. aberrans* release" plots. Arrows indicate the last fungicide application.

Table 3 – Effect of fungicides on the abundance of *P. ulmi* on "*K. aberrans* release" plots.

Effect	F	d.f.	P
Treatment	5.15	4; 96	< 0.001
Date	21.77	6; 94.6	< 0.001
Treatment x Date	1.33	24; 94.6	0.169
During period a (fungicide application + one month)			
control vs. mancozeb	0.07	94.6	0.792
control vs. propineb	0.01	94.6	0.927
control vs. metiram	0.66	94.6	0.417
control vs. folpet - Cu hydroxide	1.04	97.1	0.310
mancozeb vs. propineb	0.03	94.6	0.862
mancozeb vs. metiram	1.16	94.6	0.283
mancozeb vs. folpet - Cu hydroxide	1.59	97.1	0.210
propineb vs. metiram	0.82	94.6	0.367
propineb vs. folpet - Cu hydroxide	1.22	97.1	0.272
metiram vs. folpet - Cu hydroxide	0.08	97.1	0.784
During period b (after fungicide application)			
control vs. mancozeb	22.84	94.6	< 0.001
control vs. propineb	20.73	94.6	< 0.001
control vs. metiram	2.03	94.6	0.158
control vs. folpet - Cu hydroxide	2.82	96.7	0.096
mancozeb vs. propineb	0.05	94.6	0.821
mancozeb vs. metiram	11.26	94.6	0.001
mancozeb vs. folpet - Cu hydroxide	7.33	96.7	0.008
propineb vs. metiram	9.80	94.6	0.002
propineb vs. folpet - Cu hydroxide	6.24	96.7	0.014
metiram vs. folpet - Cu hydroxide	0.14	96.7	0.709

Table 4 – Effect of fungicides on the incidence of *P. viticola* in "*K. aberrans* releases" plots.

Effect	F	d.f.	P
Treatment	34.68	4; 95.7	< 0.001
Date	23.33	6; 94.8	< 0.001
Treatment x Date	9.97	24; 94.8	< 0.001
during period a (fungicide application + one month)			
control vs. mancozeb	146.54	1; 94.8	< 0.001

control vs. propineb	154.62	1; 94.8	< 0. 001
control vs. metiram	185.17	1; 94.8	< 0.001
control vs. folpet - Cu hydroxide	138.34	1; 96.3	< 0.001
mancozeb vs. propineb	0.11	1; 94.8	0.742
mancozeb vs. metiram	2.26	1; 94.8	0.136
mancozeb vs. folpet - Cu hydroxide	0.46	1; 96.3	0.497
propineb vs. metiram	1.38	1; 94.8	0.243
propineb vs. folpet - Cu hydroxide	0.14	1; 96.3	0.705
metiram vs. folpet - Cu hydroxide	0.48	1; 96.3	0.488
During period b (after fungicide application)			
control vs. mancozeb	0.37	1; 94.8	0.546
control vs. propineb	0.52	1; 94.8	0.472
control vs. metiram	0.03	1; 94.8	0.855
control vs. folpet - Cu hydroxide	0.19	1; 96	0.661
mancozeb vs. propineb	0.01	1; 94.8	0.908
mancozeb vs. metiram	0.18	1; 94.8	0.673
mancozeb vs. folpet - Cu hydroxide	0.01	1; 96	0.906
propineb vs. metiram	0.29	1; 94.8	0.592
propineb vs. folpet - Cu hydroxide	0.05	1; 96	0.824
metiram vs. folpet - Cu hydroxide	0.07	1; 96	0.787

Effect of fungicides on "no release" plots

The occurrence of *K. aberrans* was detected also in these plots but at lower densities than on release plots (Figure 2). Native predatory mites were represented by *A. andersoni*. Its populations increased in late summer (Figure 2).

The effect of fungicides on *K. aberrans* populations was significant (Table 5). In the evaluation period (a) there were more predatory mites on "folpet - Cu hydroxide" than on "propineb" treatments (Table 5). In the period (b) predatory mites were more abundant on "folpet - Cu hydroxide" than on the remaining treatments (Table 5).

Fungicide applications determined significant effects even on *A. andersoni* populations (Table 6). Considering the period (a), *A. andersoni* densities were higher in the control than on the remaining treatments (Table 6). Regarding the period (b), there were more predatory mites on "mancozeb" and "propineb" than on other treatments (Table 6).

The abundance of *P. ulmi* increased over the season (Figure 2). Differences among treatments were significant but this depended on data related to period (b) (Table 7). Spider mite densities were higher on "mancozeb" and "propineb" than on the remaining treatments; higher populations levels were reached on "metiram" than in the control and on "folpet – Cu hydroxide" (Table 7).

Plasmopara viticola showed a higher incidence in the control (Figure 2, Table 4).

Table 5 – Effect of fungicides on the abundance of *K. aberrans* in "no release" plots.

Effect	F	d.f.	P
Treatment	9.68	4; 96.3	< 0.001
Date	2.55	6; 95.2	0.024
Treatment x Date	1.32	24; 95.2	0.170
During period a (fungicide application + one month)			
control vs. mancozeb	0.24	1; 95.2	0.626
control vs. propineb	0.54	1; 95.2	0.465
control vs. metiram	0.83	1; 95.2	0.363
control vs. folpet - Cu hydroxide	2.33	1; 97.1	0.129
mancozeb vs. propineb	0.06	1; 95.2	0.807
mancozeb vs. metiram	1.96	1; 95.2	0.164
mancozeb vs. folpet - Cu hydroxide	3.90	1; 97.1	0.051
propineb vs. metiram	2.71	1; 95.2	0.103
propineb vs. folpet - Cu hydroxide	4.84	1; 97.1	0.030
metiram vs. folpet - Cu hydroxide	0.48	1; 97.1	0.490
During period b (after fungicide application)			
control vs. mancozeb	1.99	1; 95.2	0.161
control vs. propineb	4.38	1; 95.2	0.039
control vs. metiram	0.85	1; 95.2	0.359
control vs. folpet - Cu hydroxide	20.06	1; 96.7	< 0.001
mancozeb vs. propineb	0.46	1; 95.2	0.497
mancozeb vs. metiram	0.24	1; 95.2	0.626
mancozeb vs. folpet - Cu hydroxide	33.35	1; 96.7	< 0.001
propineb vs. metiram	1.37	1; 95.2	0.244
propineb vs. folpet - Cu hydroxide	40.97	1; 96.7	< 0.001
metiram vs. folpet - Cu hydroxide	28.36	1; 96.7	< 0.001

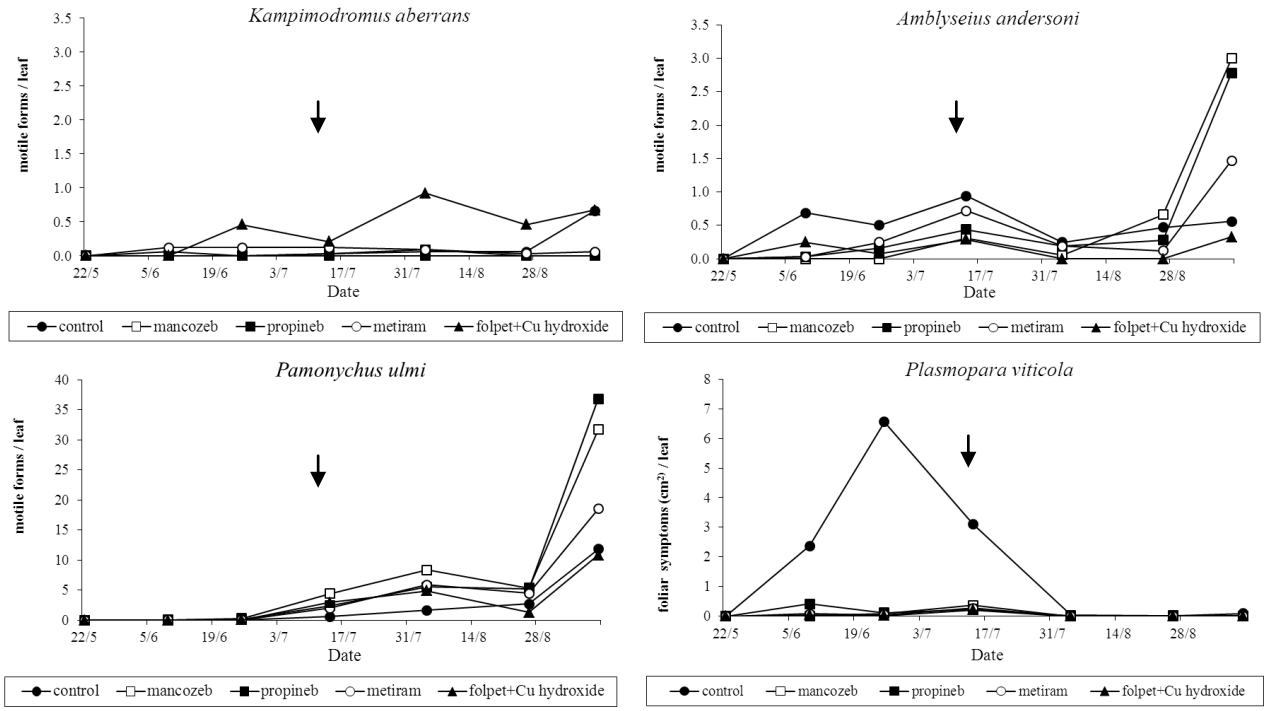

Figure 2 – Population dynamics of predatory mites, *P. ulmi* and incidence of *P. viticola* on "no release" plots. Arrow indicates the last fungicide application.

Table 6 – Effect of fungicides on the abundance of *A. andersoni* in "no release" plots.

Effect	F	d.f.	P
Treatment	7.03	4; 96	< 0.001
Date	37.96	6; 95.1	< 0.001
Treatment x Date	5.06	24; 95.1	< 0.001
During period a (fungicide application + one month)			
control vs. mancozeb	26.32	1; 95.1	< 0.001
control vs. propineb	12.75	1; 95.1	< 0.001
control vs. metiram	7.39	1; 95.1	0.007
control vs. folpet - Cu hydroxide	12.24	1; 96.5	< 0.001
mancozeb vs. propineb	2.43	1; 95.1	0.122
mancozeb vs. metiram	5.82	1; 95.1	0.017
mancozeb vs. folpet - Cu hydroxide	1.44	1; 96.5	0.232
propineb vs. metiram	0.73	1; 95.1	0.395
propineb vs. folpet - Cu hydroxide	0.05	1; 96.5	0.820
metiram vs. folpet - Cu hydroxide	1.02	1; 96.5	0.315
During period b (after fungicide application)			
control vs. mancozeb	8.49	1; 95.1	0.004

control vs. propineb	6.38	1; 95.1	0.013
control vs. metiram	0.12	1; 95.1	0.725
control vs. folpet - Cu hydroxide	14.68	1; 96.2	< 0.001
mancozeb vs. propineb	0.15	1; 95.1	0.699
mancozeb vs. metiram	6.56	1; 95.1	0.012
mancozeb vs. folpet - Cu hydroxide	42.35	1; 96.2	< 0.001
propineb vs. metiram	4.73	1; 95.1	0.032
propineb vs. folpet - Cu hydroxide	37.85	1; 96.2	< 0.001
metiram vs. folpet - Cu hydroxide	17.27	1; 96.2	< 0.001

Table 7 – Effects of fungicides on the abundance of *P. ulmi* in "no release" plots.

Effect	F	d.f.	P
Treatment	8.05	4; 95.6	< 0.001
Date	81.86	6; 94.9	< 0.001
Treatment x Date	1.86	24; 94.9	0.018
During period a (fungicide application + one month)			
control vs. mancozeb	2.47	1; 94.9	0.119
control vs. propineb	0.68	1; 94.9	0.412
control vs. metiram	0.68	1; 94.9	0.412
control vs. folpet - Cu hydroxide	0.08	1; 96	0.776
mancozeb vs. propineb	0.56	1; 94.9	0.456
mancozeb vs. metiram	0.56	1; 94.9	0.456
mancozeb vs. folpet - Cu hydroxide	1.33	1; 96	0.251
propineb vs. metiram	0.00	1; 94.9	1.000
propineb vs. folpet - Cu hydroxide	0.22	1; 96	0.639
metiram vs. folpet - Cu hydroxide	0.22	1; 96	0.639
During period b (after fungicide application)			
control vs. mancozeb	25.01	1; 94.9	< 0.001
control vs. propineb	22.65	1; 94.9	< 0.001
control vs. metiram	8.08	1; 94.9	0.005
control vs. folpet - Cu hydroxide	0.22	1; 95.8	0.638
mancozeb vs. propineb	0.06	1; 94.9	0.809
mancozeb vs. metiram	4.65	1; 94.9	0.033
mancozeb vs. folpet - Cu hydroxide	25.63	1; 95.8	< 0.001
propineb vs. metiram	3.67	1; 94.9	0.058
propineb vs. folpet - Cu hydroxide	23.43	1; 95.8	< 0.001
metiram vs. folpet - Cu hydroxide	9.50	1; 95.8	0.002

Table 8 – Effect of fungicides on the incidence of *P. viticola* on "no release" plots.

Effect	F	d.f.	P
Treatment	48.54	4; 95.7	< 0.001
Date	27.22	6; 94.8	< 0.001
Treatment x Date	10.88	24; 94.8	< 0.001
During period a (fungicide application + one month)	F	d.f.	P
control vs. mancozeb	195.01	1; 94.8	< 0.001
control vs. propineb	165.24	1; 94.8	< 0.001
control vs. metiram	194.35	1; 94.8	< 0.001
control vs. folpet - Cu hydroxide	171.59	1; 96.3	< 0.001
mancozeb vs. propineb	1.23	1; 94.8	0.269
mancozeb vs. metiram	0.00	1; 94.8	0.981
mancozeb vs. folpet - Cu hydroxide	0.10	1; 96.3	0.751
propineb vs. metiram	1.18	1; 94.8	0.280
propineb vs. folpet - Cu hydroxide	1.78	1; 96.3	0.185
metiram vs. folpet - Cu hydroxide	0.12	1; 96.3	0.734
During period b (fungicide application + one month)	F	d.f.	P
control vs. mancozeb	1.10	1; 94.8	0.296
control vs. propineb	0.19	1; 94.8	0.665
control vs. metiram	1.79	1; 94.8	0.183
control vs. folpet - Cu hydroxide	1.18	1; 96	0.281
mancozeb vs. propineb	0.38	1; 94.8	0.539
mancozeb vs. metiram	0.08	1; 94.8	0.772
mancozeb vs. folpet - Cu hydroxide	0.01	1; 96	0.903
propineb vs. metiram	0.82	1; 94.8	0.367
propineb vs. folpet - Cu hydroxide	0.47	1; 96	0.494
metiram vs. folpet - Cu hydroxide	0.02	1; 96	0.884

Discussion

All fungicides employed were not selective to *A. andersoni* but this result was likely influenced by the presence of *P. viticola* symptoms. In fact, this pathogen represents an alternative food for *A. andersoni* (Duso et al. 2003; Pozzebon and Duso 2008). This effect was observed with a lower magnitude for *K. aberrans.* Grape downy mildew mediated effects of pesticides on predatory mites have implications in field evaluation of pesticide side-effects on non-target arthropods. These effects could be included in pesticide risk assessment evaluation (Pozzebon et al. 2010).

Regarding *K. aberrans*, mancozeb and propineb caused significant but not dramatic effects on its populations. The repeated use of these fungicides (and other dithiocarbamates) was associated to the decline of *K. aberrans* populations in vineyards and subsequent spider mite infestations (Duso et al. 1983; Duso and Girolami 1984; Marchesini 1989). The *K. aberrans* strain used in this study was considered to be field resistant to mancozeb (Posenato 1994). However, its response to EBDC fungicides in our trials did not support high resistance levels. Propineb effect was similar to that of mancozeb while metiram appeared slightly less detrimental than other EBDCs. Spider mites increased significantly on EBDC plots confirming that predatory mites are fundamental for their control. Folpet confirmed to be selective for predatory mites (Girolami and Duso 1985; Duso and Girolami 1985; Camporese et al. 1993; Girolami et al. 1989, 1999).

Recently, the use of EBDCs has been limited to few applications per year and they are frequently mixed with other fungicides. Thus the real impact of these fungicides on predatory mites should be lower than that observed in this research. It should be stressed that other factors affect the impact of EBDCs on *K. aberrans*, for example leaf morphology (Pozzebon et al. 2002). Moreover, in this study *K. aberrans* was released in early season. Predatory mite colonization was just started when fungicides were applied. Therefore the results of this trial was obtained in a worst scenario. Resistance to mancozeb in *K. aberrans* has been reported and demonstrated in France (Auger et al. 2004) but little is known on propineb and metiram. Additional studies will be addressed to investigate on this topic.

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Chapter 3

Effects of a number of pesticides on the predatory mite *Kampimodromus aberrans* in the laboratory

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Introduction

Kampimodromus aberrans is a common predatory mite occurring in South-European vineyards. Its effectiveness in controlling phytophagous mite populations has been proved in a number of studies (Ivancich Gambaro 1973; Duso et al. 1983; Duso 1989; Girolami et al. 1992). Among factors affecting positively *K. aberrans* performance we can mention the capacity to persist in condition of prey scarcity and the tolerance to relatively high temperatures and dry conditions. However, its potential as biocontrol agent is often limited by its susceptibility to several pesticides (e.g. Ivancich Gambaro 1973; Duso et al. 1983; Ragusa 1983; Marchesini 1989). In European vineyards, predatory mites are exposed to fungicides and insecticides. The latter are required to control berry moths, leafhoppers and minor pests. Studied conducted in the last decades showed the detrimental effects of a number of pesticides (e.g. organophosphates, carbamates and pyrethroids) on predatory mites included *K. aberrans* (e.g. Marchesini 1989; Duso et al. 1992). However, strains of *K. aberrans* apparently resistant to dithiocarbamates (e.g. mancozeb) and organophosphates (OPs) insecticides were reported to occur in North-Italian vineyards (Corino 1986; Vettorello and Girolami 1992; Posenato 1994) and in France (Auger et al. 2004). These strains were released in vineyards located in other areas with successful results despite the repeated use of OPs (Facchin 1996; Tirello, this thesis). Few toxicological tests have been conducted on *K. aberrans* in laboratory due to difficulties in rearing this species (e.g. Auger et al. 2004).

Recently, we were able to rear *K. aberrans* in laboratory for several generations. The availability of predatory mite cultures gave the possibility to point out a toxicological test procedure and to perform a series of studies on *K. aberrans.* We evaluated the effect of insecticides (i.e. chlorpyriphos, etofenprox, flufenoxuron, indoxacarb, methoxyfenozide, spinosad and thiamethoxam) frequently used in European vineyards on two *K. aberrans* strains. Insecticides characterised by different modes of action were selected for trials.

*Paola Tirello contributed to all parts of the experimental work, data analysis and paper writing.

Material and methods

Predatory mites strains

This study was performed on two *K. aberrans* strains collected in the Veneto region, North-Eastern Italy. PO strain was collected from a commercial vineyard located in Monteforte d'Alpone (Verona province) where predatory mites showed a low susceptibility to OPs (Posenato 1994). In this farm OPs (i.e. fenitrothion and chlorpyriphos) were used approximately 1-2 times per year in the last decade. SP strain was collected from a commercial vineyard (S. Polo di Piave, Treviso province) where PO had been introduced in 1996. In this farm OPs were used only two times in the late 1990s.

Predatory mites were mass-reared on artificial arenas using a grape leaf placed on a wet cotton layer as a substrate. A cotton barrier was created on leaves edges to prevent mite escape. *Kampimodromus aberrans* females were collected from leaves and transferred onto rearing arenas using a fine brush. Small pieces of PVC were placed on each arena as oviposition sites. *Typha latifolia* pollen was used as food source and was replenished every two days.

Bioassay

We selected seven insecticides characterized by different mode of action, in particular organophosphates (chlorpyriphos), pyrethroids (etofenprox), chitin-inhibitors (flufenoxuron), carbamates (indoxacarb), moulting accelerating compounds (methoxyfenozide), spinosyns (spinosad) and neonicotinoids (thiamethoxam). Insecticides were tested at the highest recommended field dose (Table 1). We evaluated the lethal and sub-lethal effects for each pesticides tested.

Table 1 – Pesticides employed in laboratory trials.

Trade name	Active ingredient (a.i.)	Dose	Chemical class	Mode of action
Dursban ®	Chlorpyriphos	525 mg a.i/L	Organophosphate	Acetylcholine esterase inhibitor
Trebon ® star	Etofenprox	158 mg a.i./L	Pyrethroids	Sodium channel modulators
Cascade ®	Flufenoxuron	75 mg a.i/L	Benzoylureas	Insecticides Growth Regulators – IGR
Steward ®	Indoxacarb	49.5 mg a.i/L	-	Voltage-dependent sodium channel blockers
Prodigy ®	Methoxyfenozide	96 mg a.i/L	Diacylhydrazines	Moulting Accelerating Compounds - MAC
Laser ®	Spinosad	96 mg a.i./L	Spinosyns	Nicotinic Acetylcholine receptor allosteric activators
Actara ®	Thiamethoxam	50 mg a.i/L	Neonicotinoids	Nicotinic Acetylcholine receptor agonists

Pesticide formulations were diluted into distilled water before toxicological test procedures. The latter were performed by using rectangular leaf sections (approximately 6 cm^2). They were immersed in the insecticide solution for 30", and distilled water was used in control treatments. When pesticide residues completely dried out, leaf sections were put on a wet cotton pad and cotton barriers were created along their perimeter to avoid predatory mite escape. Two mated *K. aberrans* females (about 12 d old) were gently transferred on each leaf section and fresh pollen was provided every two days as food. The experimental units were maintained in a climatic chamber at 25 ± 2° C, 70 ± 10% relative humidity and 16L:8D photoperiod. Female mortality was assessed 72 h after treatments and fecundity was recorded daily for four additional days. After seven days, the surviving females and young stages were removed and the eggs were monitored until they hatched out completely in the control. Females drowned or escaped were removed from the initial tested number. In total we assessed 30-40 females per active ingredient and strain.

Data analysis

We used one-way ANOVA to analyse the effects of pesticides on mite survival, fecundity and egg hatching. Treatment means were separated by the Tukey test or the Tukey–Kramer test (a = 0.05). The data on survival were arcsin-transformed while data on fecundity were square-root transformed, prior to the analyses, in order to meet the ANOVA assumptions. Possible changes in the number of females present on the test units during the reproduction period and the hatching of larvae from eggs between the assessment dates were taken into account by using the Blümel and Hausdorf (2002) formula. The overall toxicity of each pesticide was expressed as:

$$E = 100\% - (100\% - M) \cdot R$$

where E is the coefficient of toxicity; M is the percentage of mortality calculated according to Abbott (1925); R is the ratio between the average number of hatched eggs produced by treated females and the average number of hatched eggs produced by females in the control group. Data were discussed considering IOBC toxicity classes (Table 2).

Results

Results of toxicological tests are reported in Tables 3 and 4. Etofenprox and spinosad showed to be the most detrimental pesticides. Etofenprox caused 73.94% and 86.11% mortality, for PO and SP strains respectively. Mortality caused by spinosad exceeded 90% for both strains. The remaining pesticides did not affect female survival significantly. Fecundity of surviving *K. aberrans* females

was dramatically reduced by etofenprox and spinosad. Oviposition also decreased significantly after treatments with thiamethoxam (PO strain) and chlorpyriphos (SP strain). In term of overall toxicity, spinosad and etofenprox resulted the most toxic insecticides, while chlorpyriphos, flufenoxuron and indoxacarb were associated with moderate toxicity levels. Regarding thiamethoxam there were remarkable differences between the two strains. Methoxyfenozide did not affect markedly predatory mite demographic parameters.

Table 2 – IOBC classification of pesticide side-effects.

Classes	**Effect (E)**	**Toxicity level**
Class 1	< 30%	harmless
Class 2	30% ÷ 79%	slightly harmful
Class 3	80% ÷ 99%	moderately harmful
Class 4	> 99%	harmful

Table 3 – Effects of selected pesticides on survival and fecundity on PO *K. aberrans* strain; the coefficient of toxicity is also reported.

	Females							
	Survival * (%)		Abbott mortality (%)	Eggs/female/day * (mean ± SE)				E (%)
control	97.83	a	0.00	0.87	±	0.03	a	-
chlorpyriphos	94.12	a	3.79	0.49	±	0.09	ab	45.59
etofenprox	25.49	b	73.94	0.04	±	0.02	c	98.89
flufenoxuron	95.83	a	2.04	0.51	±	0.06	ab	42.43
indoxacarb	98.15	a	-0.33	0.53	±	0.05	ab	38.21
methoxyfenozide	100.00	a	-2.22	0.85	±	0.07	a	0.43
spinosad	7.69	b	92.14	0.00	±	0.00	c	100.00
thiamethoxam	94.44	a	3.46	0.26	±	0.04	bc	70.57

* Means followed by the same letter are not significantly different (Tukey test, p = 0.05).

Table 4 - Effects of selected pesticides on survival and fecundity on SP *K. aberrans* strain; the coefficient of toxicity is also reported.

	Females							
	Survival * (%)		Abbott mortality (%)	Eggs/female/day * (mean ± SE)				E (%)
control	100.00	a	0.00	1.22	±	0.07	a	-
chlorpyriphos	100.00	a	0.00	0.58	±	0.19	b	52.28
etofenprox	37.50	b	62.50	0.08	±	0.03	c	97.63
flufenoxuron	100.00	a	0.00	0.98	±	0.10	ab	19.62
indoxacarb	100.00	a	0.00	0.92	±	0.05	ab	24.49
methoxyfenozide	100.00	a	0.00	0.76	±	0.16	ab	38.06
spinosad	5.00	b	95.00	0.10	±	0.06	c	99.61
thiamethoxam	100.00	a	0.00	0.83	±	0.13	ab	32.15

* Means followed by the same letter are not significantly different (Tukey test, p = 0.05).

Discussion

In the present study, spinosad and etofenprox have been associated with significant effects on *K. aberrans* survival and fecundity. The use of these pesticides could be associated with high risks in vineyards inhabited by *K. aberrans* populations and probably by other predatory mite species. In fact, field application of etofenprox caused dramatic reductions of *K. aberrans* population densities (Girolami et al. 2000; Tosi et al. 2006). Miles and Dutton (2003) also showed dramatic effects of spinosad on juveniles of *Typhlodromus pyri* Scheuten in laboratory, even at lower concentrations than that considered in our study (9.6 g a. i../hl). These authors reported the results of a number of field studies. One application of spinosad, at the same concentration used in our trials, reduced *T. pyri* by 43% compared to the untreated control. The application of spinosad at 4.8 g a.i./hl did not exert significant effects on a population of *K. aberrans*. In other studies where multiple applications were tested on these species, the impact of spinosad was not remarkable. Tosi et al. (2006) compared the effects of several pesticides on a population of *K. aberrans* in North-Italian vineyards. Pesticides were applied once in early July. Spinosad caused 54-60% of population reduction compared to the control 10 and 20 days after treatment, respectively. Additional data from literature on the effects of spinosad on other predatory mite species gave contrasting results (e.g. Williams et al. 2003; Kim et al. 2005; Van Driesche et al. 2006; Villanueva and Walgenbach 2005). Significant effects (47% of mortality and 57% of fecundity reduction) were found in a laboratory study where spinosad was applied at 36 g a.i../hl to *P. persimilis* females (Duso et al. 2008) and lower doses (50 ppm) caused similar effects on another *P. persimilis* strain (Yoo & Kim 2000; Ahn et al. 2004). However, no significant effects were observed in semi-field studies with *P. persimilis* even at doses

of 36 g a.i./hl. In conclusion, the effects of spinosad on predatory mites in laboratory trial are much more significant than those observed in field one.

In the present study chlorpyriphos caused a lower toxicity to predatory mites compared to spinosad. The two populations proved to be resistant to chlorpyriphos if compared to a susceptible strain (chapter 4, this thesis). Apparently there were no differences in survival and fecundity rates of predatory mites belonging to SP and PO strains despite the lower use of OPs in the vineyard where SP strain was collected. The reduction in fecundity observed in the present study confirm that sub-lethal effects may involve predatory mite resistant populations (Duso et al. 1992). Field studies on another strain of *K. aberrans* (Tosi et al. 2006) showed that the effects of chlorpyriphos were detrimental compared to the control (52-54% population reduction 10 and 20 days after treatment, respectively) but similar to those of spinosad. In other experiments the impact of chlorpyriphos depended on pesticide experienced by *K. aberrans* strains (Mori et al. 1999; Girolami et al. 2000).

In our trials thiamethoxam was associated with significant effects on *K. aberrans* fecundity (PO strain) while survival was not affected. Thiamethoxam showed remarkable effects on *Neoseiulus cucumeris, Neoseiulus fallacis*, *P. persimilis* and *Galendromus occidentalis* fecundity when exposed to residues or subjected to direct application of the pesticide (Kim et al. 2005; Villanueva and Walgenbach 2005; Duso et al. 2008; Bostanian et al. 2009). Additional sub-lethal effects of thiamethoxam were reported by Poletti et al. (2007). The pesticide slightly reduced *Neoseiulus californicus* and *Phytoseiulus macropilis* survival but affected spider mite eggs consumption (Poletti et al. 2007). In vineyards, thiamethoxam did not show significant effects on *K. aberrans* population densities (Tosi et al. 2006). Trials conducted on *A. andersoni* in vineyards and apple orchards showed low to moderate effects of thiamethoxam (Baldessari et al. 2010).

Flufenoxuron and indoxacarb showed low effects on both *K. aberrans* strains, in particular a slight effect in fecundity was noted. In laboratory and field trials flufenoxuron and indoxacarb were associated to low effects on *K. aberrans* (Mori et al. 1999) and other predatory mite species (Park and Kim 1996; Rodriguez et al. 2004).

Methoxyfenozide was not associated to detrimental effects on *K. aberrans* except for a slight reduction in fecundity in SP strain. No effects of this pesticide were observed in field experiments carried out with the same species (Tosi et al. 2006; Sancassani 2006; Scannavini et al. 2006).

The present study shed light on lethal and sub-lethal effects of pesticides frequently used in vineyards. For most pesticides results were consistent with those of field trials available in the literature. In contrast, the results of laboratory tests on spinosad were not useful to predict its impact in realistic conditions and this discrepancy needs to be studied in depth.

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Chapter 4

Resistance to chlorpyriphos in the predatory mite *Kampimodromus aberrans*

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Introduction

Predatory mites of the family Phytoseiidae are of great interest for controlling phytophagous mites on various crops worldwide (McMurtry and Croft 1997). In European vineyards the most common species are *Typhlodromus pyri* Scheuten, *Kampimodromus aberrans* (Oud.), *Amblyseius andersoni* (Chant), *Typhlodromus exhilaratus* Ragusa and *Phytoseius finitimus* (Ribaga) (Kreiter et al. 1989, 1993, 2000; Duso 2005). Among them, *K. aberrans* proved to be effective in controlling spider mite populations in vineyards of Northern Italy treated with selective pesticides (Ivancich Gambaro 1972; Duso et al. 1983; Girolami 1987; Duso 1989; Girolami et al. 1992). Moreover, *K. aberrans* showed a great ability to outcompete predatory mite species naturally occurring or artificially introduced in vineyards (Duso and Pasqualetto 1993; Camporese and Duso 1996; Duso and Vettorazzo 1999). The susceptibility of *K. aberrans* to common pesticides, i.e Ethylen-bis-dithiocarbamate (EBDC) fungicides and organophosphate (OPs) insecticides, has been considered a key factor preventing the performance by this predatory mite. EBDCs are widely used to control grape downy mildew *Plasmopara viticola* (Berk and Curtis ex de Bary) while OPs are required to control a number of grape pests (berry moths, mealybugs and leafhoppers). Populations of *K. aberrans* apparently resistant to EBDCs (e.g. mancozeb) and organophosphates insecticides were reported to occur in North-Italian vineyards (Corino 1986; Vettorello and Girolami 1992, Posenato 1994). Among them, a strain originating from the Verona area was released in vineyards located in other areas with successful results despite the repeated use of OPs (Facchin 1996; Duso et al. 2010). The resistance of *K. aberrans* to mancozeb has been demonstrated for French strains (Auger et al. 2004, 2005). Nevertheless, little is known on the acute toxicity of OPs towards *K. aberrans* populations showing different degrees of susceptibility to these pesticides. OPs act by contact, ingestion and vapor action. They inhibit the activity of acetylcholinesterase causing convulsion and paralysis to contaminated arthropods. OPs have been widely used in agriculture due to their broad-spectrum activity and rapid action towards arthropod pests. However, most of OPs have been recently removed from the European market due to their unfavorable effects to human health. Currently, the use of few OPs active ingredients is allowed in Europe. Among them, chlorpyriphos

*Paola Tirello contributed to all parts of the experimental work, data analysis and paper writing.

is largely used in viticulture to control berry moths, leafhoppers, scales and mealybugs. Field and laboratory studies revealed that the effects of chlorpyriphos on predatory mites can vary greatly depending on pesticide history and species (e.g. Ioriatti and Balloid 1987; Angeli et al. 1997; Fitzgerald et al. 1999; Barbar et al. 2007; Bonafos et al. 2008). Resistance to OPs has been shown for a number of predatory mite species potentially occurring in European vineyards, such as *T. pyri* and *A. andersoni* (Overmeer and van Zon 1983; Baillod et al. 1985; Van de Baan et al. 1985; Maixner 1990; Dunley et al. 1991; Duso et al. 1992; Vidal and Kreiter 1995; Bonafos et al. 2007, 2008). Resistance to OPs in *K. aberrans* has been suggested from field observations (e.g., Posenato 1994; Mori et al. 1999) but studies on their acute toxicity on resistant and susceptible strains in the laboratory are lacking. In the present study we evaluated the effects of chlorpyriphos on different strains of *K. aberrans* to measure and show definitely the resistance to OPs in this predatory mite species. We investigated the dose-response effect of chlorpyriphos in four *K. aberrans* strains characterized by a differential level of exposure to OPs insecticides in the past: from never to frequently exposed.

Materials and methods

Predatory mites populations

The study was performed on four *K. aberrans* strains collected in North-eastern Italy: PO (1) was collected from a commercial vineyard located at Monteforte d'Alpone (Verona province, Veneto region), where predatory mites showed a low susceptibility to OPs (Posenato 1994); populations SP (2) and SM (3) were collected from a commercial vineyard (San Polo di Piave, Treviso province, Veneto region) or an apple orchard (San Michele all'Adige, Trento province, Trentino-Alto Adige region) where PO population was introduced in 1996 and 1997, respectively. From 1997 to 2008, OPs (i.e., fenitrothion, chlorpyriphos) were used every year at Monteforte d'Alpone but much less frequently (1-2 times in the last 1990s) at San Polo di Piave and San Michele all'Adige. Another strain (LE) was collected from untreated hackberry trees (*Celtis australis* L.) at Legnaro (Padova province, Veneto region). We assumed this population as reference for its susceptibility. These populations were collected in 2007 and reared for several generations in separate boxes in the laboratory at the Department of Environmental Agronomy and Crop Science, University of Padua. Grapevine leaves were used as substrate for predatory mites. They were settled on a pad of wet cotton where small pieces of PVC were placed for shelter and oviposition. *Typha latifolia* pollen was provided every two days as food.

Bioassays

Bioassays were conducted with a chlorpyriphos formulation (Dursban® 75 WG, 75% a.i., Dow AgroSciences) widely used in vineyards to control leafhoppers (e.g., *Scaphoideus titanus* Ball. *Empoasca vitis* Göthe), grape berry months (*Lobesia botrana* Den. & Schiff. and *Eupoecilia ambiguella* Hübner) and mealybugs (e.g. *Planococcus ficus* Signoret). This formulation was diluted into distilled water before testing procedures. Toxicological trials were performed by using rectangular leaf sections (6 cm^2). They were immersed in the insecticide solution for 30" and then left to dry. Control leaf units were immersed into distilled water for 30". Leaf sections were put on a wet cotton pad and cotton barriers were created along their perimeter to avoid predatory mite escape. Two mated *K. aberrans* females (about 12 d old) were gently transferred on each leaf section using a fine brush and fresh pollen was provided every two days as food. Units were kept into a climatic chamber at 25 ± 2° C, 70 ± 10% relative humidity and 16L:8D photoperiod. In order to calculate LC_{50} values, bioassays were repeated with at least 6-9 serially diluted concentrations covering the range of 0–100% mortality. Female mortality was assessed 72 h after treatments. Females drowned or escaped were removed from the initial tested number.

Data analysis

LC_{50} values and 95% fiducial limits were calculated from probit regressions using PriProbitNM (Sakuma 1998). Resistance factors (RR_{50}) were calculated by dividing the LC_{50} value of the strains collected in vineyards or orchards by the LC_{50} value of LE (susceptible) strain.

The susceptibility of mite strains to chlorpyriphos was compared using the Lethal dose ratio method (Robertson et al, 2007). It is based on the ratios between LC_x at specific response levels of the two strains under comparison and their 95% confidence limit. The 95% confidence limits for each ratio is based on estimates for intercepts and the slopes of two probit lines and estimates of their variance–covariance matrices. Intercepts, slopes of probit lines and estimates of their variance–covariance matrixes were produced in the PriProbitNM output. We tested parallelism of dose-response lines with a pairwise Likelihood Ratio Test ($\alpha = 0.05$).

Results

Tested chlorpyriphos concentrations were different among the four strains ranging from 0.525 to 5.25 x 10^{-6} mg a.i./l for LE, from 131.25 to 3675 mg a.i/l for SP, from 675 to 2625 mg a.i./l for SM, and from 1050 to 16800 mg a.i./l for PO. The susceptibility to chlorpyriphos was significantly different among the four strains (Table 1). LE strain was highly susceptible while PO, SM and SP strains exhibited relatively high LC_{50} and RR values (Table 1). A variation in acute toxicity levels

was observed for the latter strains: PO evidenced the highest resistance level, while SM the lowest (Table 1). Dose response lines of PO and SM were parallel, but different compared to LE and SP (Table 1, Figure 2). Different slopes were observed between the latter strains (Table 1, Figure 2).

Table 1 – Lethal concentrations of chlorpyriphos and resistance factors in four strains of *Kampimodromus aberrans*.

strain	LC_{50} [a]		95% fiducial limit		LC_{90} [a]		95% fiducial limit		Intercept ± SE			Slope ± SE [b]				RR_{50}	RR_{90}
	(mg a.i./l)		Lower	Uper	(mg a.i./l)		Lower	Upper									
LE	0.006653	d	0.003218	0.014672	0.138158	d	0.049217	0.870225	2.97	±	0.54	0.97	±	0.16	c	-	-
PO	3589.70	a	2870.60	4583.10	10113.00	b	7157.85	19558.50	-7.64	±	1.37	2.85	±	0.51	a	539602	73199
SM	968.67	c	871.15	1075.58	2059.50	c	1719.90	2751.38	-8.26	±	1.11	3.91	±	0.52	a	145609	14907
SP	1071.07	b	856.34	1350.83	6369.53	a	4247.33	11852.25	-3.57	±	0.43	1.66	±	0.20	b	161003	46103

[a] LC values followed by the same letter are not significantly different (α = 0.05) at Dose Ratios method (Robertson et al, 2007).

[b] Slopes with different letters are significant different at Likelihood Ratio Test (α = 0.05).

Figure 1. Dose-mortality curves of *K. aberrans* strains for chlorpyriphos.

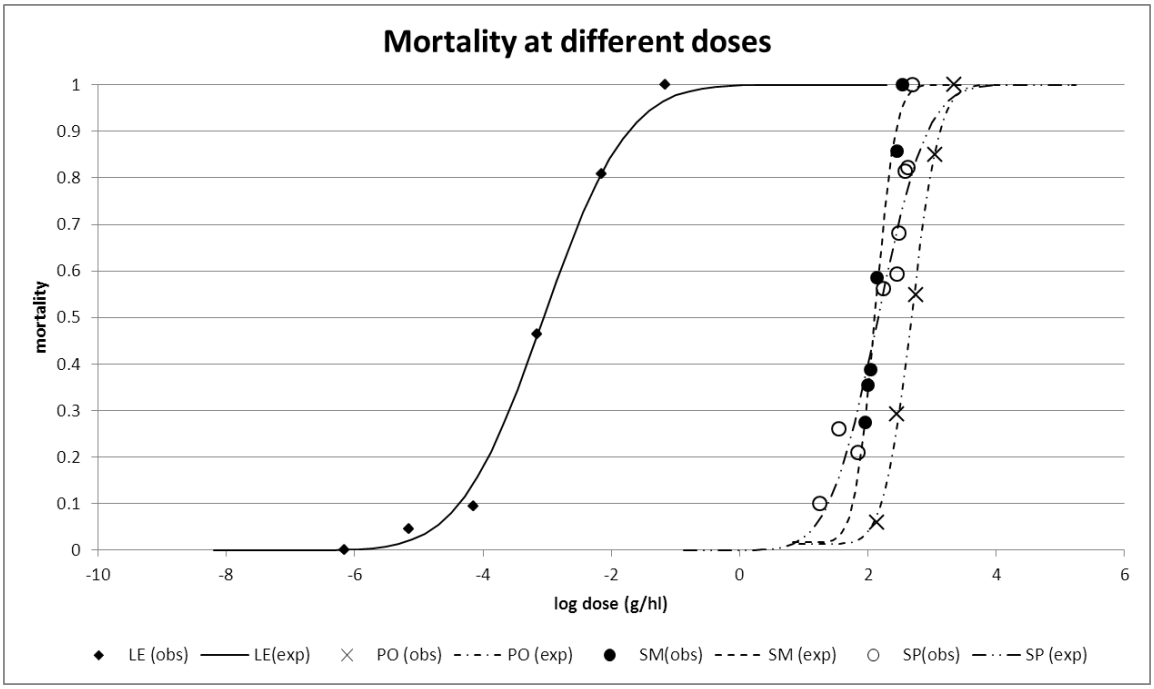

Figure 2: Concentration-mortality probit lines of *K. aberrans* strains in response to chlorpyriphos. Observed (obs) and expected (exp) values for each strain are reported.

Discussion

Up to date, resistance to chlorpyriphos in *K. aberrans* was suggested only by field experiments (Posenato 1994; Mori et al. 1999). In the present study this phenomenon is demonstrated for the first time in the laboratory. The availability of a highly susceptible strain led us to calculate resistance factors for the three strains collected from commercial vineyards or orchards. PO strain originated SP and SM strains but remained the most resistant to chlorpyriphos. This could be due to a different pesticide pressure exerted in the different farms. OPs were used at least once per year at Monteforte d'Alpone (PO), but occasionally in farms located at San Polo di Piave and San Michele all'Adige. Nevertheless, it should be stressed that LC_{50} values for PO, SP and SM strains were 6.83, 2.04 and 1.85 times respectively, the recommended field dose of chlorpyriphos (525 mg a.i./l) in vineyards. These resistance levels can explain the response by predatory mites in field conditions.

Test of parallelism among dose-response lines evidenced differences among resistant strains. Biologically, changes in slopes of regression lines may reflect the quality of enzymes involved in detoxification (Robertson et al. 2007). Hence, PO and SM population appear to share the same mechanism of detoxification, but quantitative differences in detoxification enzymes are likely present. On the other hand mechanism of detoxification in PO and SM strains appeared different than that of SP strain. Detoxification mechanisms in OPs resistant strains of predatory mites are not well studied. Previous research evidenced that cytochrome P450-dependent monooxygenase

activities can determine OPs resistance in *Amblyseius womersleyi* Schicha (Sato et al. 2006; Sato et al. 2007), but Vidal and Kreiter (1995) found a low activity of hydrolitic and oxidative mechanisms in *T. pyri* and suggested a role of modification in pesticide target, particularly acetylcholinesterase (AChE). Among other mite species, resistance to OPs has been detailed studied in *Tetranychus urticae* Koch (Van Leeuwen et al. 2010), where principal resistance mechanism has been associated to target site modification (Khajehali et al. 2010). Results obtained in this study represent a starting point for further investigation on biochemical aspects of *K. aberrans* resistance to insecticides.

Our trials were based on residual exposure only, while in field conditions topical and ingestion exposures could also be involved leading to higher toxicity values in predatory mites (Pozzebon et al. 2011). Nevertheless, residual toxicity component is very important in determining the overall effects of a pesticide on predatory mites. The use of chlorpyriphos has become frequent since control measures against *S. titanus* in vineyards have become mandatory in Italy, France and elsewhere. Resistance to OPs in *K. aberrans* is a positive aspect in this context. Knowledge of the distribution of resistant populations in Northern Italian vineyards will give us useful information for pesticide use in vineyards.

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Chapter 5

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Toxicity of thiamethoxam to *Tetranychus urticae* Koch and *Phytoseiulus persimilis* Athias-Henriot (Acari Tetranychidae, Phytoseiidae) through different routes of exposure

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Abstract

BACKGROUND: Knowledge of the impact of insecticides on *Tetranychus urticae* Koch and its predator *Phytoseiulus persimilis* Athias-Henriot is crucial for IPM. This study evaluates the effect of thiamethoxam on *T. urticae* and its predator by considering different routes of exposure (topical, residual and contaminated food exposures) and their combinations.

RESULTS: Thiamethoxam effects on *T. urticae* were higher when residual and contaminated food exposures were considered. The total effect was higher than 90% where contaminated food exposure was involved. On *P. persimilis*, the total effect was higher in residual and contaminated prey exposures compared with topical exposure, and all combinations of routes of exposure attained a total effect higher than 90%.

CONCLUSION: Thiamethoxam was found to be toxic to *T. urticae* and *P. persimilis*; however, the impact of the insecticide depended on the routes of exposure and their combinations. Lethal and sublethal effects occurred in residual and contaminated food exposures, while only sublethal effects occurred in topical exposure of predators and prey. The toxicity of thiamethoxam on prey and predator increased with the number of exposure routes involved. By limiting exposure to thiamethoxam to ingestion of contaminated food only, the impact of the pesticide was more favourable to *P. persimilis* than to its prey.

Keywords: two-spotted spider mite; predatory mites; biological control; integrated pest management; neonicotinoids; sublethal effects

1 INTRODUCTION

The two-spotted spider mite *Tetranychus urticae* Koch is an important pest in various agricultural systems.[1] This spider mite feeds on plants by inserting its stylets into the plant epidermis and sucking out the cellular content of cells in the mesophyll layer.[2] The use of pesticides less toxic to the herbivore than to its natural enemies is considered one of the most important factors causing outbreaks of *T. urticae*.[3] As resistance to acaricides in *T. urticae* spreads rapidly, biological control tactics are considered crucial to manage spider mite populations.[4,5]

The specialised predator of *T. urticae*, *Phytoseiulus persimilis* Athias-Henriot, is a native of the Mediterranean area and has been used worldwide to control spider mites on several crops.[6,7] With their mouth stylets, predatory mites pierce their prey and extract the body fluids, which are sucked into the digestive tract.[8] Pesticides used to control arthropod pests may be toxic to *P. persimilis*, posing major limitations to the success of biocontrol strategies against *T. urticae*.

In recent years, neonicotinoids have been the fastest-growing class of insecticides. Their unique chemical and biological properties, excellent systemic characteristics and favourable safety profile to human and non-target organisms are considered the key to their success.[9] Among this class of insecticides, thiamethoxam is considered as a second-generation neonicotinoid that provides control of a broad range of pests of various crops, including whitefly, aphids and thrips.[10,11] This insecticide is characterised by contact, stomach and systemic activity.[12] It has been developed both for soil and foliar use as well as for seed treatments.[10] Some authors have suggested that this insecticide's mode of action presents some differences compared with other neonicotinoids (i.e. imidacloprid).[13]

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Arthropods colonising plants can be exposed to pesticides through different routes of exposure. These include direct exposure to pesticide droplets (topical exposure), exposure to pesticide residues and exposure to contaminated food sources.[14] Depending on the pesticide application method and the timing of application, the exposure of an arthropod to a pesticide may involve different combinations of routes of exposure that can determine variable effects. Moreover, pesticides with systemic translocation represent a special case that can induce the exposure to systemically contaminated food.[15]

The evaluation of pesticides under laboratory conditions is useful to obtain information on their potential impact on arthropods. In the laboratory it is possible to perform bioassays under controlled conditions, aimed at assessing lethal and sublethal effects.[16–19] Differential effects can be induced by different routes of exposure, but this is usually not considered in standard laboratory bioassays.[20]

The application of thiamethoxam can entail combinations of different routes of exposure, and their effect on predator–prey relationships has never been explored specifically. The purpose of this study was to quantify the relative importance of each of the different routes of exposure to thiamethoxam on *T. urticae* and *P. persimilis* mortality and fecundity. Here, topical exposure, residual exposure and systemically contaminated food exposure were considered. As combinations of multiple routes of exposure can occur, in this study a factorial experimental design approach was used to partition the effect of single routes and to evaluate the effect of the interactions among different routes.

2 EXPERIMENTAL METHODS

2.1 Stock cultures

The strains of *P. persimilis* and *T. urticae* used here were collected from unsprayed plants in the Sardinia region (Tinnura, Oristano, Italy, latitude 40° 16′ 16″ N/longitude 8° 33′ 1″ E) and reared for several years in the laboratory of the Department of Environmental Agronomy and Crop Science, University of Padova, Italy [22 ± 1 °C, 60 ± 10% relative humidity (RH) and a 16 : 8 h light : dark photoperiod]. *Tetranychus urticae* was reared on French bean (*Phaseolus vulgaris* L.) plants, while *P. persimilis* was reared on leaves detached from French bean plants infested with spider mites. In previous investigations, these strains appeared to be relatively susceptible to some insecticides.[21]

2.2 Toxicological methods

To evaluate the effects of thiamethoxam on *T. urticae* and *P. persimilis* survival and hatching rate, holding cells similar to those described by Dennehy *et al.*[22] were used, with a few changes.[23] Detached French bean leaves were used as a substrate for mite rearing. In the experiments, the first developed leaves of coeval French bean plants (2 weeks old) potted in 1.15 L pots and grown in the greenhouse at the Department of Environmental Agronomy and Crop Science, University of Padua, Italy, were used. Batches of five *T. urticae* or 2–3 *P. persimilis* females were transferred into each holding cell. Trials were conducted using coeval mite females. In experiments involving *P. persimilis*, the procedure described by Duso *et al.*[23] was followed. Prior to *P. persimilis* females being transferred into holding cells, *T. urticae* females (20 per predator female) were transferred into cells as food for predators. An equal number of *T. urticae* females were added every 48 h until experiments were completed. Before adding *T. urticae* females, the cells were placed in a refrigerator (4 °C) for 5 min in order to chill the predatory mites and prevent their escape during the experiment.

For both predators and prey, mortality was assessed 72 h after mite transfer by counting dead and alive individuals and the number of eggs laid. Surviving female fecundity was assessed for an additional 4 days after the mites were removed. One predator female was left in each cell after the assessment of mortality (72 h from the beginning of trials) to reduce cannibalism and to furnish prey food supply easily. Eggs laid during the experiment were checked daily for an additional 5 days. During assessment, dead and immature mites were counted and removed from the cells. The escaping rate was calculated as the number of mites that were not found (living or dead) at the end of trials over the initial number of mites. During experiments, holding cells were held under controlled conditions (25 ± 2 °C, 70 ± 5% RH, 16 : 8 h light : dark). In all experiments, an aqueous dispersion of thiamethoxam 250 g kg^{-1} WG (Actara® 25 WG; Syngenta Crop Protection spa, Milan, Italy) containing 95 mg AI L^{-1} was used.

2.3 Systemic toxicity assessment on *Tetranychus urticae*

Prior to the different routes of exposure experiments, a preliminary experiment was set up to evaluate the variation in the systemic toxicity of thiamethoxam on *T. urticae* at different times after insecticide application. Plants were drenched with thiamethoxam dispersion (95 mg AI L^{-1}; 100 mL) and were not watered for 3 days after the insecticide treatment. Leaves were detached from drenched plants and used in holding cells after 0, 3, 6 and 9 days after insecticide application in order to examine the effect of time from application on mite toxicity. The effect on survival was evaluated at 72 h from the beginning of this experiment on 50 *T. urticae* females (ten replicates) per time after treatment. Fecundity of surviving females was assessed for 168 h from the beginning of the experiment on 40 *T. urticae* females per time after treatment.

A logistic regression model was used to evaluate the relationship between time from drench application of thiamethoxam and survival and egg hatching of *T. urticae*. The number of live mites and hatched eggs were considered as response variables with binomially distributed errors.[24] The analyses were performed using the GENMOD procedure of SAS.[25] The effect of time was evaluated with a likelihood–ratio G test ($\alpha = 0.05$). With the use of the LSMEAN statement, a Wald χ^2 test was performed on the pairwise comparison of different times from application ($\alpha = 0.05$).[25] Data on fecundity were analysed using a restricted maximum likelihood (REML) analysis of variance using the PROC MIXED of SAS.[25] The variation in *T. urticae* fecundity with time was evaluated by an F-test ($\alpha = 0.05$), and a pairwise t-test ($\alpha = 0.05$) was also applied to the differences between least-square means of fecundity observed at each time from application.[26] Data on fecundity were checked for normality and homoscedasticity prior to the analyses, and were log ($x + 1$) transformed to meet REML assumption.

2.4 Effect of different routes of exposure on *Tetranychus urticae* and *Phytoseiulus persimilis*

The effect of the different routes of exposure to thiamethoxam on *T. urticae* and *P. persimilis* survival, fecundity and egg hatching was evaluated by a three-factorial design experiment. The exposure to the insecticide through the three routes of exposure (topical, residual and systemically contaminated food) were considered as experimental factors. The experimental design comprised

Table 1. Description of treatments used in factorial experiments with *Tetranychus urticae* and *Phytoseiulus persimilis* to examine the influence of different routes of exposure to thiamethoxam. The symbol (+) denotes that the route of exposure was involved, while the symbol (−) indicates that the route of exposure was excluded in each treatment

Treatment	Topical exposure	Residual exposure	Systemically contamined food exposure[a]
1	−	−	−
2	−	−	+
3	−	+	−
4	−	+	+
5	+	−	−
6	+	−	+
7	+	+	−
8	+	+	+

[a] In experiments with *Phytoseiulus persimilis*, predatory mites were exposed to contaminated prey.

eight treatments obtained by all routes of exposure and their combinations (Table 1). Microimmersion bioassay[22,23,27] was used for insecticide treatment in topical exposure. Mites were drawn into a small pipette tip, after which the insecticide solution was drawn up, immersing the mites for 30 s. The mites were ejected from the pipette, dried on filter paper and then transferred into holding cells. In residual exposure, foliar residues were used. Bean leaves were dipped in the insecticide solution for 30 s before adding them to the holding cells as substrate for mite rearing. To obtain systemic contamination of food, drench applications of thiamethoxam were used. Preliminary systemic toxicity assessment on *T. urticae* showed that the maximum effect on survival and fecundity peaked 6 days after drench application of thiamethoxam, with no significant increases in the subsequent period. Experiments were performed using 80 *T. urticae* (16 replicates) and 32 *P. persimilis* (16 replicates) females per treatment.

Factorial logistic regression models were used to evaluate the relationship between the different routes of exposure to thiamethoxam and the survival and hatching rates of *T. urticae* and *P. persimilis* observed during the experiments. The analyses were performed using the GENMOD procedure of SAS.[25] The effect of each experimental factor was evaluated with a likelihood–ratio G test ($\alpha = 0.05$). It was also of interest to study interactions among the different routes of exposure. With the use of the LSMEAN statement, a Wald test was performed to partition the effect of a single route of exposure when combined with the others.[25] Data on fecundity were analysed using a REML analysis of variance. The effect of each experimental factor and their interactions was evaluated with an F-test, while significant interactions were investigated with t-tests applied to the differences between least-square means of treatments with a Bonferroni adjustment ($\alpha = 0.05$).[26] To meet REML assumptions, data on fecundity were log $(x + 1)$ transformed prior to the analyses.

The total effects (E) of thiamethoxam observed in each route of exposure and their combinations were calculated using the Overmeer–Van Zon formula:[28]

$$E(\%) = 100(\%) - (100\% - M) \times R$$

where M is the percentage mortality,[29] and R is the ratio between the average number of hatched eggs produced by treated females and the average number of hatched eggs produced by females in the control group.

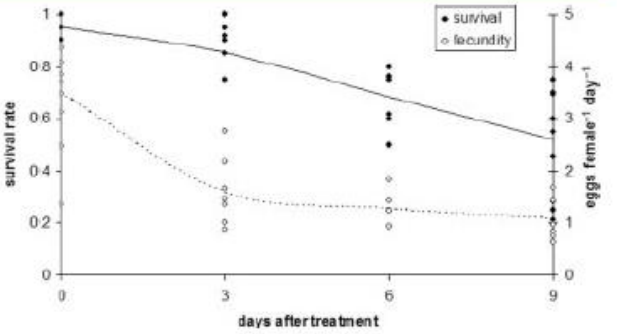

Figure 1. Survival at 72 h and fecundity at 168 h of *Tetranychus urticae* females observed on French bean leaves detached from plants at 0, 3, 6 and 9 days after a drench application of thiamethoxam. Observed values are denoted by circles, while lines indicate fitted values (solid lines for survival, dashed lines for fecundity).

3 RESULTS

3.1 Systemic toxicity assessment on *Tetranychus urticae*

The survival of *T. urticae* confined on leaves removed from plants that had received a drench application of thiamethoxam decreased over time after treatment ($G = 36.06$; $P < 0.001$) (Fig. 1). No differences were observed between *T. urticae* leaves detached after 0 days and 3 days ($\chi^2 = 2.85$; $P = 0.09$) and after 6 days and 9 days ($\chi^2 = 2.76$; $P = 0.09$). Survival of spider mites was higher on leaves detached after 0 days compared with 6 days ($\chi^2 = 10.60$; $P = 0.001$) and 9 days ($\chi^2 = 19.79$; $P < 0.001$). A higher reduction in *T. urticae* survival was also found after 6 days ($\chi^2 = 4.03$; $P = 0.04$) and 9 days ($\chi^2 = 13.26$; $P < 0.001$) compared with 3 days.

The effect of thiamethoxam on *T. urticae* fecundity depended on time from drench application ($F_{3,29} = 13.89$; $P < 0.001$) (Fig. 1). Fecundity was higher at 0 days compared with 3 days ($t_{29} = 4.44$; $P = 0.001$), 6 days ($t_{29} = 4.66$; $P < 0.001$) and 9 days ($t_{29} = 5.98$; $P < 0.001$). No differences were observed after 3 days compared with 6 days ($t_{29} = 0.22$; $P = 0.829$) and 9 days ($t_{29} = 1.50$; $P = 0.145$), and between 6 days and 9 days ($t_{29} = 1.25$; $P = 0.211$). Egg hatching rate was not affected by thiamethoxam ($\chi^2 = 0.73$; $P = 0.393$).

3.2 Effect of different routes of exposure on *Tetranychus urticae*

Exposure to thiamethoxam through residual exposure and contaminated food exposure resulted in a reduced survival of *T. urticae*, while no effect of topical exposure was observed (Table 2, Fig. 2). A significant 'residual exposure × contaminated food exposure' interaction was found (Table 2). The combined exposure of residual and contaminated food determined a higher mortality compared with contaminated food exposure ($\chi^2 = 6.97$; $P = 0.01$) (Fig. 2) and residual exposure ($\chi^2 = 4.8$; df = 1; $P = 0.028$) (Fig. 2) considered alone. A significant effect of thiamethoxam through all routes of exposure was observed on *T. urticae* fecundity (Table 2, Fig. 3), while no effect of thiamethoxam was observed on hatching rate (Table 2, data not shown). Among single routes of exposure, higher overall effects were observed for residual and contaminated food exposure applications than for topical

Table 2. Results of factorial logistic regression of survival and egg hatching, and REML (restricted maximum likelihood) factorial analysis of variance of fecundity expressed by *Tetranychus urticae* and *Phytoseiulus persimilis* during experiments

	Survival		Fecundity			Egg hatching[a]	
Source of variation	*G*	*P*	*F*	df	*P*	*G*	*P*
	Tetranychus urticae						
Residual exposure	62.08	<0.0001	27.87	1, 46	<0.0001	2.38	0.1226
Contaminated food exposure	61.21	<0.0001	32.47	1, 46	<0.0001	0.59	0.4442
Topical exposure	2.22	0.1364	10.93	1, 46	0.0018	0	0.9909
Residual exp.* residual exp.	25.67	<0.0001	0.09	1, 46	0.7613	0.93	0.3351
Topical exp.* contaminated food exp.	3.39	0.0654	0.70	1, 46	0.4082	0.72	0.3951
Topical exposure* residual exposure* contaminated food exp.	0.18	0.6726	0.00	1, 46	0.9470	0.52	0.4722
	Phytoseiulus persimilis						
Residual exposure	30.28	<0.0001	31.37	1, 82	<0.0001	–	
Contaminated prey exposure	41.42	<0.0001	27.11	1, 82	<0.0001	–	
Topical exposure	3.04	0.0812	14.00	1, 82	0.0003	–	
Residual exp.* contaminated prey exp.	11.82	0.0006	3.39	1, 82	0.0691	–	
Topical exp.* residual exp.	0.85	0.3568	0.10	1, 82	0.7527	–	
Topical exp.* contaminated prey exp.	1.24	0.2659	0.51	1, 82	0.4771	–	
Topical exp.* residual exp.* contaminated prey exposure	1.94	0.1632	3.55	1, 82	0.0632	–	

[a] Data on *Phytoseiulus persimilis* egg hatching were not analysed because the hatch rate of eggs was 100% in all treatments.

exposure. Comparing multiple routes of exposure combinations, total effects were higher than 90% where contaminated food exposure was involved (Table 3). Escaping rate was lower than 5% in all treatments.

3.3 Effect of different routes of exposure on *Phytoseiulus persimilis*

Predatory mite survival was significantly reduced by the exposure to thiamethoxam residues and by the ingestion of contaminated prey, while no effect of topical exposure was observed (Table 2, Fig. 4). The 'residual exposure × contaminated prey exposure' interaction was significant (Table 2). The survival observed in treatments where residual and contaminated prey exposures were combined was similar to that observed in residual exposure treatments ($\chi^2 = 2.40$; df = 1; $P = 0.121$) (Fig. 4) but lower compared with contaminated prey treatments ($\chi^2 = 4.92$; df = 1; $P = 0.026$) (Fig. 4). A reduction in *P. persimilis* fecundity was observed for all routes of exposure (Table 2, Fig. 5). Egg hatching rates reached 100% in all treatments. Among single routes of exposure, a higher overall effect was observed for residual and contaminated prey exposure than for topical exposure. However, exposure to multiple routes resulted in higher than 90% reductions in all combinations (Table 3). Escaping rate was lower than 5% in all treatments.

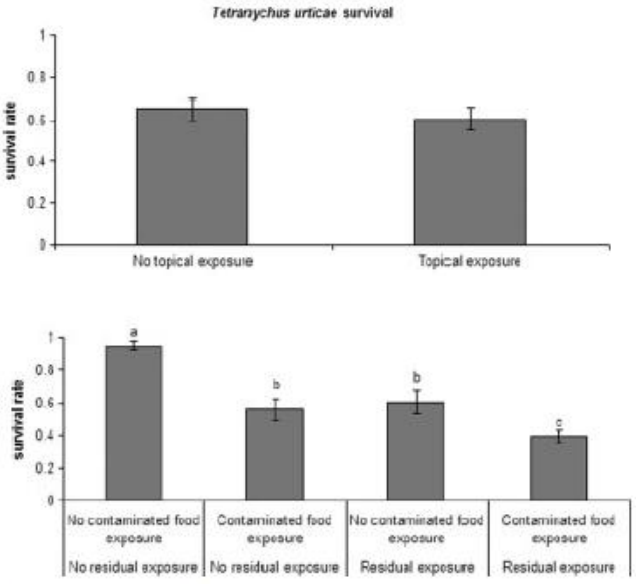

Figure 2. Effect of different routes of exposure on the survival of *Tetranychus urticae* observed at 72 h from the beginning of the experiment. Different letters indicate significant differences in the Wald χ^2 test ($\alpha = 0.05$).

4 DISCUSSION AND CONCLUSIONS

Exposure of *T. urticae* and *P. persimilis* to thiamethoxam resulted in significant negative effects. However, the impact of the insecticide varied among the combinations of different routes of exposure.

For *T. urticae*, high mortality occurred after residual and contaminated food exposures, while no effect on mortality was observed with topical exposure. A synergistic effect on mortality was observed when residual and contaminated food exposures were combined. Fecundity of *T. urticae* was reduced by thiamethoxam with all routes of exposure. Contaminated food exposure was more harmful to *T. urticae* than the other routes, and high effects were seen when this route was combined with the two others. Leaf residue and topical exposures exerted a higher effect on fecundity when combined than either exposure route alone. Previous investigations into the effect of thiamethoxam on *T. urticae* reported low toxicity in terms of mortality when the spider mite was exposed to leaf residues.[30] In the same study, a significant reduction in *T. urticae* fecundity was observed after exposure to leaf residues and to contaminated food, resulting in larger reductions in the latter treatment. The reductions in survival and mortality reported in previous studies were generally lower than

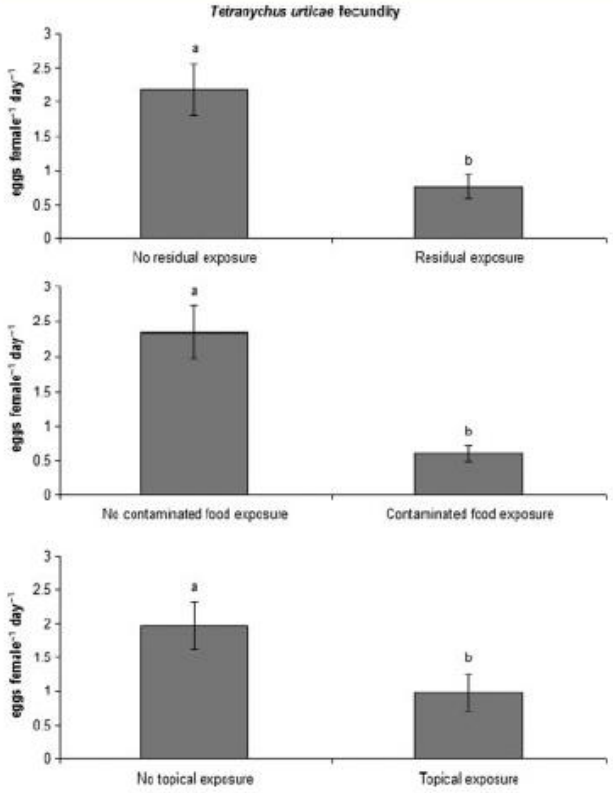

Figure 3. Effect of different routes of exposure on the fecundity of *Tetranychus urticae* observed for 168 h after the beginning of the experiment. Different letters indicate significant differences in the *t*-test of the least-square means ($\alpha = 0.05$).

those shown here. These differences can be related to the *T. urticae* strains used. Indeed, the effect of neonicotinoids on *T. urticae* can be strain dependent and is highest in strains that have low pesticide experience.[31] The *T. urticae* strain used here has likely never been exposed to pesticides in the past, and thus its susceptibility to neonicotinoids is probably higher than for other strains.

The present results show that thiamethoxam reduced *P. persimilis* survival when exposed to leaf residues and contaminated prey. When combined, these two routes of exposure did not induce a greater effect compared with residual exposure alone, but did induce a greater effect compared with contaminated prey alone because, when predatory mites fed on contaminated prey, thiamethoxam residues induced additional mortality. Thiamethoxam application reduced *P. persimilis* fecundity in all routes of exposure. Among the single routes of exposure, relatively high total effects (>70%) were observed for residual and contaminated prey exposures, while, in all routes of exposure combinations, total effects were greater than 90%. The present data confirm previous investigations into thiamethoxam effects on *P. persimilis*, where the use of this insecticide was associated with a toxic effect using residual exposure alone or in combination with topical exposure.[23,32] Using a low concentration of thiamethoxam (i.e. 7.5 ppm), Ahn *et al.*[33] found a low lethal toxicity to *P. persimilis* after residual exposure. Considering other predatory mite species, thiamethoxam showed remarkable effects on *Neoseiulus cucumeris* (Oudemans), *Neoseiulus fallacis* (Garman) and *Galendromus occidentalis* (Nesbitt) fecundity when exposed to fresh residues or subjected to direct application of the insecticide.[19,34,35] In other studies, thiamethoxam slightly reduced survival of *Neoseiulus californicus* McGregor and *Phytoseiulus macropilis* (Banks), but a reduction in the number of spider mite eggs consumed by the latter predator was observed.[36]

In the present study, the effect of different routes of exposure to thiamethoxam was partitioned. Results indicated that topical exposure to thiamethoxam had no lethal effects on either mite species, but that sublethal effects were substantial. Residual exposure and contaminated food exposure had effects on both mortality and fecundity. These aspects have implications for the evaluation of pesticide effects on arthropods in laboratory conditions. More comprehensive information on interactions between pesticides and arthropods can be provided by the consideration of multiple routes of exposure in laboratory evaluation.[20,37,38] The present results also indicate that the toxicity of thiamethoxam on both prey and predator increased with the number of routes of exposure involved, and the effects attained by single routes were not constant. Topical exposure resulted in a lower effect on mites compared with the other routes. The differences between topical and residual exposures can be related to the differences in the amount of pesticide that mites take up. The treated area (25–30 mm diameter) of the holding cells is bigger than the body surface of the mites, determining a higher deposit of insecticide solution. Furthermore, insecticide susceptibility is related to the modes of uptake. Foliar deposits are more appropriate for insecticides characterised by tarsal uptake, while deposits on mite bodies should promote integumental absorption. The higher effect of thiamethoxam through residual exposure on survival and fecundity of mites suggests a prominent role of tarsal uptake rather than integument absorption. It should be noted that, in residual toxicity tests, the submerged leaves can take up the compound systemically, determining an increase in routes of exposure. This study showed that the overall effect of thiamethoxam on *T. urticae* was highest through contaminated food exposure compared with other routes. The systemic activity of thiamethoxam on *T. urticae* increased over time, indicating a pattern in the accumulation of this compound or its toxic metabolites in leaves, with an increase in the first 6 days after drench treatment. Other experiments using imidacloprid showed a peak in imidacloprid and its metabolites present in leaves after 8 days from drench application.[30] The present experiments suggest a faster uptake of thiamethoxam, confirming previous investigations where its uptake appeared to peak sooner than that of imidacloprid.[39] The higher effect of dietary exposure compared with residual exposure on *T. urticae* was also seen in previous investigations with neonicotinoids.[30] Moreover, van Leeuwen *et al.*[40] argued that a high effect of systemic uptake of spinosad on *T. urticae* can be caused by an increased exposure (in term of the amount of active ingredient per surface unit) to the insecticide when cells are punctured by cheliceral stylets of mites. The same can also occur for thiamethoxam, where an uptake from roots can lead to higher exposure by ingestion at the foliar level through gut adsorption compared with other routes. Moreover, systemic root uptake of neonicotinoids would reduce photodecomposition compared with foliar spray application, preserving the toxic potential of the insecticide.[39] These aspects can also be involved in the effect of contaminated prey exposure to thiamethoxam on *P. persimilis*, which was higher compared with the other routes. When considering the systemic activity of thiamethoxam, it should be noted that this compound is the neonicotinod precursor of clothianidin.[41–43] The conversion to clothianidin occurs in plant tissues and insects a short time after drench and oral application. The metabolite has a different binding site from its precursor and

Table 3. Mortality, fecundity, egg mortality and coefficient of toxicity (*E*) of thiamethoxam on *Tetranychus urticae* and *Phytoseiulus persimilis* observed in different treatments

Treatments	Routes of exposure	Mortality (72 h)	Fecundity (168 h)	Egg mortality (120 h)	*E*
	Tetranychus urticae				
1	Control	0.04	4.31	0.10	–
2	Contaminated food exposure	0.43	1.23	0.23	78.37
3	Residual exposure	0.35	1.54	0.24	72.38
4	Topical exposure	0.08	2.11	0.16	55.21
5	Residual exp. + contaminated food exposure	0.59	0.22	0.28	96.77
6	Topical exp. + contaminated food exposure	0.44	0.53	0.26	91.14
7	Topical exp. + residual exp.	0.44	0.89	0.27	85.09
8	Topical exp. + residual exp. + contaminated food exposure	0.62	0.16	0.29	97.21
	Phytoseiulus persimilis				
1	Control	0.03	2.45	0.00	–
2	Contaminated prey exposure	0.66	0.78	0.00	74.98
3	Residual exposure	0.73	0.53	0.00	83.50
4	Topical exposure	0.15	1.43	0.00	44.56
5	Residual exp. + contaminated prey exposure	0.78	0.09	0.00	97.38
6	Topical exp. + contaminated prey exposure	0.50	0.27	0.00	90.91
7	Topical exp. + residual exp.	0.73	0.12	0.00	96.17
8	Topical exp. + residual exp. + contaminated prey exposure	0.81	0.00	–	100.00

appears with higher toxic potential than thiamethoxam.[41] Higher concentrations of thiamethoxam than clothianidin were necessary to obtain the same response in terms of wireworm mortality.[44] In seed treatment, clothianidin resulted in significantly greater mortality of ladybug and *Delia antiqua* (Meigen) compared with thiamethoxam.[45,46] The higher toxicity of clothianidin may explain the superior effect obtained with contaminated food exposure. However, to the present authors' knowledge, no data are available on the comparative effects of clothianidin and thiamethoxam on mites, and thus no conclusion can be drawn on these components of the systemic activity of thiamethoxam, and further studies are required to elucidate whether its high systemic effect is determined by its conversion to clothianidin.

The contaminated prey effect on *P. persimilis* may be influenced by *T. urticae* strains. In the present experiment, a *T. urticae* strain considered susceptible to insecticides[21] was used; however, this spider mite species is well known for its ability to develop pesticide resistance.[4] The reduced effect of neonicotinoids has been correlated with resistance to acaricides, and acaricide-resistant strains are expected to be tolerant to neonicotinoids, but mechanisms are still unclear.[28] The conversion of thiamethoxam to clothianidin in the insect body has been studied using susceptible insects,[41,42] and some authors have suggested that resistance mechanisms to thiamethoxam might be related to its activation to clothianidin.[47] Oxidative degradation is considered to be the prominent mechanism in neonicotinoid-resistant insects.[48–50] This process may lead to the production of toxic and non-toxic metabolites,[51] with possible different outcomes on natural enemies feeding on them. Further studies on the metabolism of thiamethoxam and neonicotinoids in mites are required to evaluate the implications for beneficial mites.

The partition of the effect due to each of the routes of exposure in *T. urticae* and its predator can offer an insight into the impact of thiamethoxam on predator–prey interactions, with possible practical implications. The chances for survival of mites increase with reduction in the routes of exposure. In several agricultural systems, compatibility between the biocontrol of *T. urticae* and pesticide

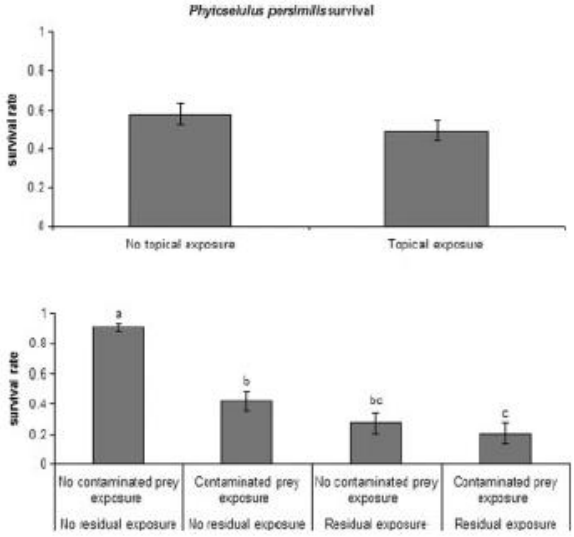

Figure 4. Effect of different routes of exposure on the survival of *Phytoseiulus persimilis* observed at 72 h from the beginning of the experiment. Different letters indicate significant differences in the Wald χ^2 test ($\alpha = 0.05$).

use can be achieved by reducing *P. persimilis* exposure with asynchronous pesticide application and natural enemy release or by maintaining untreated refuges.[52–54] The present results confirm that reduced exposure to pesticides is obtained by limiting one or more of the possible routes of exposure. Topical exposure was more favourable to *P. persimilis* than to *T. urticae*; however, the segregation of this route from the others appears to be difficult in practice. The exposure to thiamethoxam through ingestion of contaminated food was also more favourable to predatory than to herbivore mites. Neonicotinoids are characterised by versatile application methods,[55] and drench application of thiamethoxam proved in this study to be a valuable way to reduce the routes

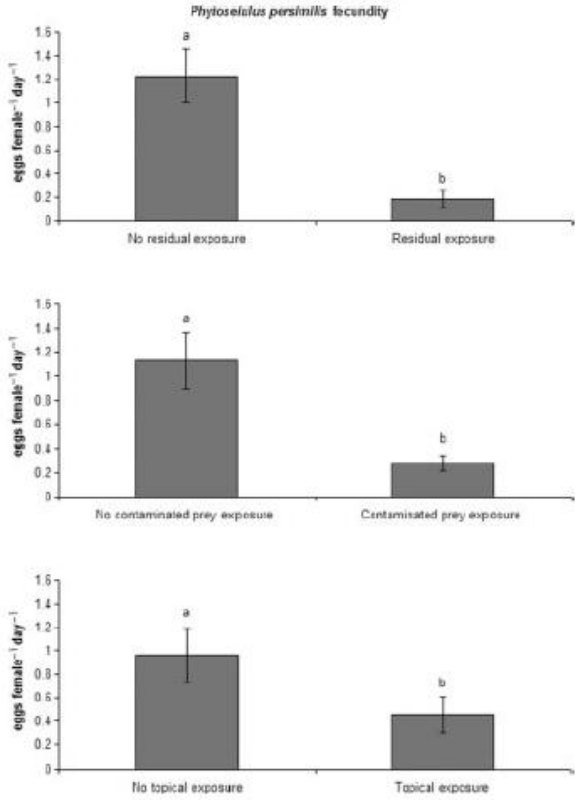

Figure 5. Effect of different routes of exposure on the fecundity of *Phytoseiulus persimilis* observed for 168 h after the beginning of the experiment. Different letters indicate significant differences in the *t*-test of the least-square means ($\alpha = 0.05$).

of exposure in mites. This technique showed promising results for the control of *Bemisia tabaci* Genn. and for preventing tomato yellow leaf curl geminivirus (TYLCV) transmission by whitefly.[56–58] The same application method was effective against thrips and reduced the incidence of tomato spotted wilt virus (TSWV).[59] Drench application of thiamethoxam and its systemic activity provided efficient control against various pests.[10,60–62] According to the results obtained here, drench applications of thiamethoxam are safer for beneficial mites than foliar applications. The present results suggest that drench application is a valuable measure for reducing the negative impact of this insecticide on predatory mites and thus on *P. persimilis*–*T. urticae* interactions.

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Chapter 6

Toxicity of thiamethoxam to *Tetranychus urticae* and *Kampimodromus aberrans* (Acari Tetranychidae, Phytoseiidae) through different routes of exposure

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Introduction

In the last decade, the neonicotinoids have become key components of Integrated Pest Management (IPM) on various crops because of their chemical and biological characteristics, such as excellent uptake and translocation in plant, new mode of action and favourable safety profiles (Maienfisch et al. 2001b, Jeschke and Nauen 2008). Thiamethoxam belongs to the second-generation neonicotinoids and is well known to provide control of a broad range of sucking and chewing insects on various agricultural crops (Maienfisch et al. 2001a, 2001b). This insecticide is characterized by contact, stomach and systemic activity (Elbert et al 2008) and it has been developed both for soil and foliar use as well as seed treatments (Maienfisch et al 2001b).

Thiamethoxam is characterized by systemic and translaminar properties. When applied as a foliar spray it can be adsorbed by foliar surface and distributed throughout the plant by means of the vascular system (i.e., phloem) (Cloyd and Bethke 2010). This results in potential different routes of exposure for natural enemies that can include: direct exposure to pesticide droplets (topical exposure), exposure to pesticide residues (residual exposure), and to contaminated food sources (Longley and Stark 1996). Depending on method and timing of pesticide application, the exposure of a beneficial arthropod to a pesticide may involve different combinations of routes of exposure that can determine variable effects. Moreover, pesticides with systemic translocation represent a special case that can induce the exposure to systemically contaminated food (Cloyd and Bethke 2010).

In European vineyards thiamethoxam is used against leafhoppers species, such as *Scaphoideus titanus* Ball and *Empoasca vitis* (Goethe). In some areas of Northern Italy the extensive use of thiamethoxam appeared to increase the frequency of spider mite problems. The impact of thiamethoxam on prey-predator interactions has been poorly studied. Recently, the effect of thiamethoxam has been studied considering the spider mite *Tetranychus urticae* Koch and the predatory mite *Phytoseiulus persimilis* Athias-Henriot. Mites were exposed to the pesticide via residual, contact or ingestion modes of exposure. Mechanisms of contamination affected

*Paola Tirello contributed to all parts of the experimental work, data analysis and paper writing.

significantly prey-predator system (Pozzebon et al. 2011). In the present study, we investigated the effect of different routes of exposure on the predatory mite *Kampimodromus aberrans* (Oudemans), a key beneficial in European vineyards and fruit orchards (Duso 1989; Girolami et al. 1992; Duso et al. 2009). It plays a fundamental role in preventing spider mite outbreaks in South-European vineyards (Ivancich Gambaro 1973; Girolami 1981; Duso 1989; Kreiter et al. 1991; Papaioannou-Souliotis et al. 1999). We considered three routes that are likely to be involved in *K. aberrans* exposure to thiamethoxam: topical exposure, residual exposure and systemically contaminated prey exposure. Since combinations of multiple routes of exposure can occur, in this study we used a factorial experimental design to study interactions among different routes as well as to partition the effect of single routes (Pozzebon et al. 2011).

Material and methods

Rearing of Kampimodromus aberrans

The strain of *K. aberrans* used in these trials was collected from a vineyard where it showed a low susceptibility to organophosphates (Posenato 1994). Mites were reared in the laboratory at the Department of Environmental Agronomy and Crop Science, University of Padua. Grapevine leaves were used as substrate for predatory mite rearing. They were put on a pad of wet cotton where small pieces of PVC were placed for shelter and oviposition. *Typha latifolia* pollen was provided every two days as food. In experimental trials we used *Tetranychus urticae* Koch as prey for predatory mites. Spider mites were reared on French beans (*Phaseolus vulgaris* L.) at the laboratory at the Department of Environmental Agronomy and Crop Science, University of Padua.

Systemic toxicity assessment on Tetranychus urticae

A preliminary experiment was set up to evaluate the effect of time of treatment in the systemic contamination of *T. urticae* by thiamethoxam. Grapevine leaf petioles were immersed in a thiamethoxam solution (100 ml at 95 mg/L, Actara® 25 WG, Syngenta Crop Protection s.p.a., Milano, Italy) and leaves were used for building test units after 0, 1, 3, and 5 days from immersion. A total of 50 *T. urticae* females (10 replicates) per time of immersion were transferred on treated leaves (5 females per leaf). The effect on survival was evaluated after 72 h from mite transfer while fecundity of surviving females was assessed for additional 168 h.

A logistic regression model was used to evaluate the relationship between the time of immersion in the thiamethoxam solution and the survival or fecundity of *T. urticae*. We considered the number of living mites as response variable with binomially distributed errors (Agresti 2002). The analyses were performed using the GENMOD procedure of SAS (SAS Institute Inc. 1999). The effect of

time was evaluated with a Likelihood–ratio G test ($\alpha = 0.05$). Using the LSMEAN statement we performed a Wald χ^2 test on the pairwise comparison of different times of immersion ($\alpha = 0.05$) (SAS Institute Inc. 1999). Data on fecundity were analyzed using a Restricted Maximum Likelihood (REML) analysis of variance using the PROC MIXED of SAS (SAS Institute Inc. 1999). We evaluated the variation in *T. urticae* fecundity over the time with F-test ($\alpha = 0.05$) and we applied a pairwise t-test ($\alpha = 0.05$) on the differences between least squares means of fecundity observed at each time of immersion (Littell et al. 2002). Data on fecundity were checked for normality and homoscedasticity prior to the analyses, and were log (x+1) transformed to meet REML assumption.

Effect of different routes of exposure on K. aberrans

We evaluated the effect of the different routes of exposure to thiamethoxam on *K. aberrans* survival, fecundity, and egg hatching by a three-factorial design experiment. We considered the exposure to the insecticide through the three routes of exposure (topical, residual and systemically contaminated prey) as experimental factors (Pozzebon et al. 2011). The experimental design comprised eight treatments obtained by all routes of exposure combinations (Table 1). Micro-immersion bioassays (Dennehy et al. 1993; Castagnoli et al. 2005; Duso et al. 2008) were used for insecticide treatments in topical exposure. Mites were drawn into a small pipette tip, after which the insecticide solution was drawn up, immersing the mites for 30 s. The mites were ejected from the pipette, dried on filter paper and transferred into the leaf sections. In residual exposure, we used foliar residues. Grape leaves were dipped in the insecticide solution for 30 s before adding them to the wet cotton as substrate for mite rearing. To obtain the systemic contamination of prey, *T. urticae* was maintained for 24 hours on leaf sections treated for two days as above described.

Table 1. Description of treatments used in factorial experiments with *K. aberrans* to examine the influence of different routes of exposure to thiamethoxam. The symbol (+) means routes of exposure involved while the symbol (-) indicate route of exposures excluded in each treatments.

Treatments	Topical exposure	Residual exposure	Contaminated prey
1	-	-	-
2	-	-	+
3	-	+	-
4	+	-	-
5	-	+	+
6	+	-	+
7	+	+	-
8	+	+	+

Toxicological trials were performed by using grape leaf sections (about 6 cm^2) put on a wet cotton pad ; cotton barriers were created along leaf section perimeter to avoid predatory mite escape. Two *K. aberrans* mated females (about 10 d old) were gently transferred to each leaf section. Motile forms of *T. urticae* were provided as food only at the begin of treatment. Experimental units were kept into a climatic chamber at 25 ±2° C, 70±10% relative humidity and 16L:8D photoperiod.

Female mortality was assessed 72 h after treatments and fecundity was recorded for four additional days. Then the surviving females and young stages were removed and the eggs were monitored until they hatched out completely in the control. The escaping rate was calculated as the number of mites that were found on wet cotton at the end of trials over the initial number of mites. In all experiments a solution of 95 mg/L of thiamethoxam (Actara® 25 WG, Syngenta Crop Protection s.p.a., Milano, Italy) was used. Experiments were performed using at least 40 *K. aberrans* females per treatment.

Factorial logistic regression models were used to evaluate the relationship between the different routes of exposure to thiamethoxam and the survival and hatching rates of *K. aberrans* observed during the experiments (Pozzebon et al. 2011). The analyses were performed using the GENMOD procedure of SAS (SAS institute Inc. 1999). The effect of each experimental factor was evaluated with a Likelihood–ratio G test ($\alpha = 0.05$). We were also interested to study interactions among the different routes of exposure. Using the LSMEAN statement we performed a Wald test to partitioning the effect of a single route of exposure when combined with the others (SAS Institute Inc. 1999). Data on fecundity were analyzed using a REML analysis of variance. The effect of each experimental factors and their interactions was evaluated with an F-test, while significant interactions were investigated with t-tests to the differences between least squares means of

treatments with a Bonferroni adjustment (α = 0.05) (Littell R.C. 2002). To meet REML assumptions, data on fecundity were log (x+1) transformed prior to the analyses.
Total effects (E) of thiamethoxam observed in each route of exposure and their combinations were calculated using the Overmeer and Van Zon formula (1982):

E (%) = 100 % – (100 % - M)*R

where M is the percentage of mortality (Abbott W.S. 1925); R is the ratio between the average number of hatched eggs produced by treated females and the average number of hatched eggs produced by females in the control group.

Results

Systemic toxicity assessment on Tetranychus urticae

The survival of *T. urticae* confined on leaves systemically treated with thiamethoxam, decreased along time after treatment (G = 87.10; $p < 0.001$). No differences were observed between 0 days (d) and 1 d ($\chi^2 = 0.07$; $p = 0.78$). Survival of spider mites was higher after 0 d compared to 3 d ($\chi^2 = 14.27$; $p < 0.001$) and 5 d ($\chi^2 = 50.06$; $p < 0.001$). A reduction in *T. urticae* survival was also found after 3 d ($\chi^2 = 11.62$; $p < 0.001$) and 5 d ($\chi^2 = 37.81$; $p < 0.001$) compared to 1 d and after 4 d compared to 3 d ($\chi^2 = 21.95$; $p < 0.001$).

The effect of thiamethoxam on *T. urticae* fecundity depended on time from drench application ($F_{3, 54} = 3.84$; $p = 0.014$; Figure 1). Fecundity was higher at 0 d compared to 1 d ($t_{54} = 2.40$; $p = 0.019$), 3 d ($t_{54} = 2.73$; $p = 0.008$) and 5 d ($t_{54} = 2.89$; $p = 0.006$). No differences were observed after 1 d compared to 3 d ($t_{54} = 0.04$; $p = 0.967$), and 5 d ($t_{54} = 0.45$; $p = 0.656$), and between 3 d and 5 d ($t_{54} = 0.45$; $p = 0.654$).

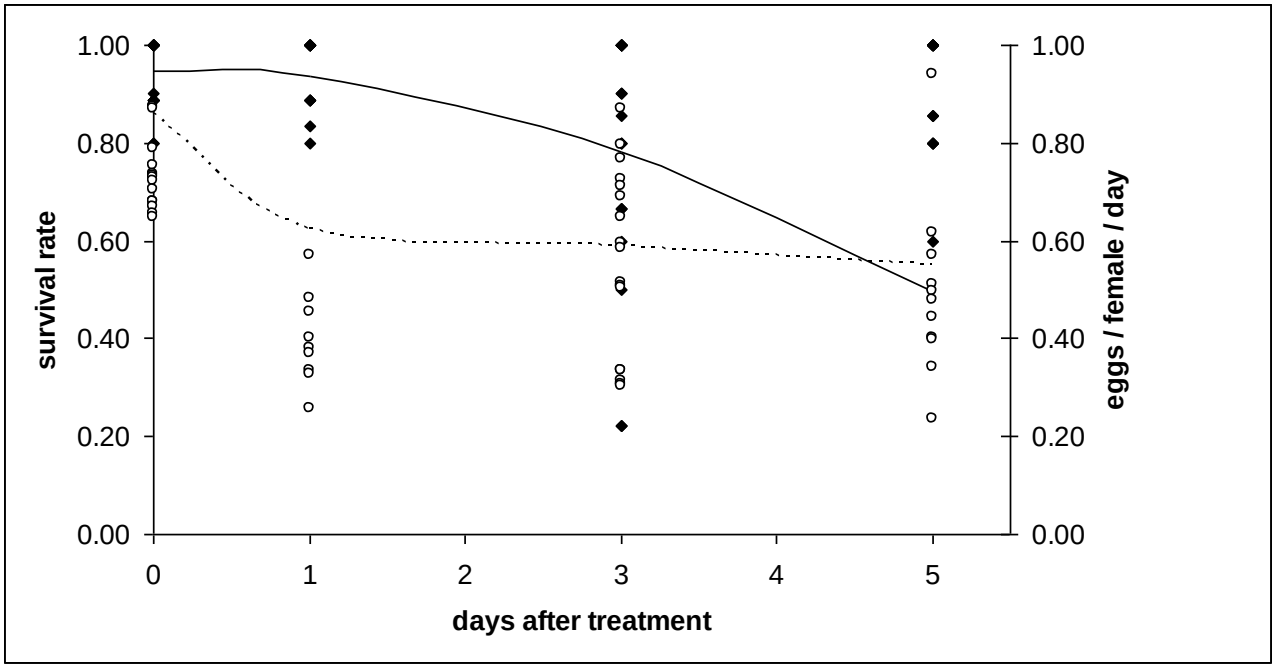

Figure 1**.** Survival at 72 h and fecundity at 168 h of *Tetranychus urticae* females placed on grapevine leaves whose petioles were immersed for 0, 1, 3 or 5 days in the thiamethoxam solution. Observed values are denoted by circles while lines indicate fitted values (solid lines for survival, dashed lines for fecundity).

Table 2. Results of factorial logistic regression of survival and fecundity, and REML (Restricted Maximum Likelihood) factorial analysis of variance of fecundity expressed by *K. aberrans* during experiments.

	Survival		Fecundity		
Sourse of variation	Chi Square	P	F	df	P
contaminated prey exposure	0.1	0.9044	25.40	1,255	< 0.0001
residual exposure	0.38	0.5353	4.67	1,255	0.031
topical exposure	0.1	0.7478	1.06	1,255	0.304
contaminated prey exp. * residual exp.	0.34	0.5594	4.56	1,255	0.033
contaminated prey exp. * topical exp.	0.04	0.8352	0.55	1,255	0.461
residual exp. * topical exp.	3.03	0.0817	0.18	1,255	0.679
contaminated prey exp. * residual exp. * topical exp.	0.63	0.4273	0.08	1,255	0.781

Effect of different routes of exposure on Kampimodromus aberrans

Predatory mite survival was not affected by different exposure to thiamethoxam, while a reduction in fecundity was observed where predatory mites where exposed to thiamethoxam through contaminated prey and fresh residues (Table 2, Figure 1). We found a significant effect of the interaction "contaminated prey exposure × residual exposure" (Table 2). The fecundity observed in treatments where contaminated prey and residual exposures were combined was similar to contaminated prey exposure, but lower compared to residual exposure treatments. Egg hatching rates reached 100% in all treatments.

Among single routes of exposure, a higher overall effect was observed for contaminated prey exposure than for topical and residual treatments. Exposure to multiple routes resulted slightly harmful in all combinations (Table 3), however total effect was highest where contaminated prey exposure was involved.

Table 3. Mortality, fecundity and coefficient of toxicity (E) of thiamethoxam on *Kampimodromus aberrans* observed in different treatments. Egg hatching was not included because it reached 100% in all treatments.

Treatments	Routes of exposure	Mortality (72 hours)	Fecundity (168 hours)	E
1	control	0.01	0.22	-
2	contaminated prey exposure	0.04	0.10	55.05
3	residual exposure	0.03	0.16	27.33
4	topical exposure	0.00	0.20	9.46
5	contaminated prey exp. + residual exp.	0.00	0.10	55.24
6	contaminated prey exp. + topical exp.	0.09	0.10	55.51
7	residual exp. + topical exp.	0.03	0.13	43.94
8	contaminated prey exp. + residual exp. + topical exp.	0.09	0.09	57.95

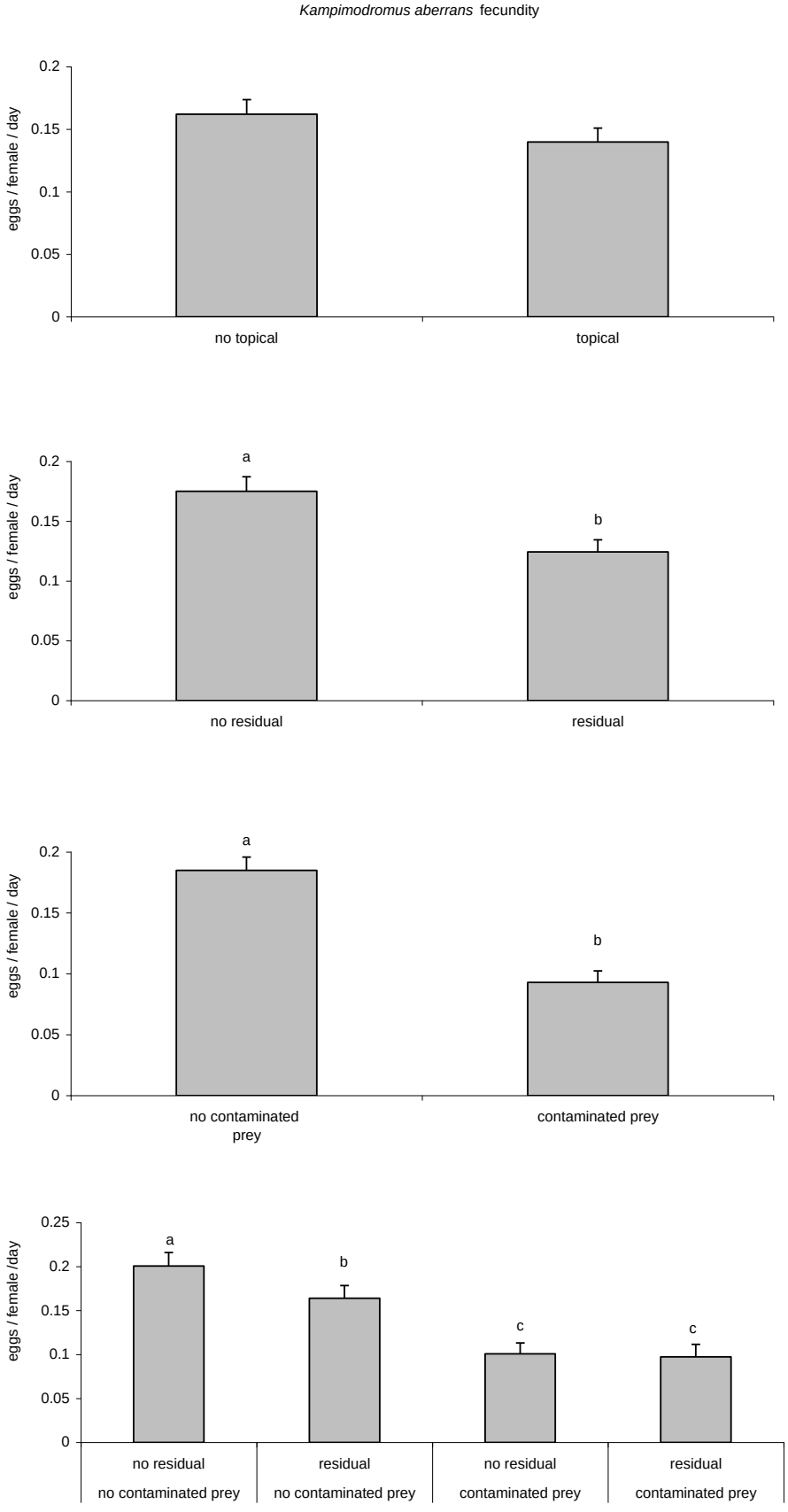

Figure 1. Effect of different routes of exposure on the fecundity of *Kampimodromus aberrans*. Different letters indicate significant differences at t-test to the least square means ($\alpha = 0.05$).

Discussion

Thiamethoxam did not affect the survival of *K. aberrans* females. However, sub-lethal effects emerged after insecticide application. These effects varied among different routes of exposure and their combinations. *Kampimodromus aberrans* fecundity was not affected by topical application of thiamethoxam but this occurred with residual and contaminated prey exposures. When combined, these two routes of exposures did not induce a greater effect compared to contaminated prey exposure alone, but greater than residual alone.

Thiamethoxam can be moderately toxic to *K. aberrans*. Among single routes of exposure, total effects (E = 55%) were higher for contaminated prey exposures than for residual exposure (E < 30%). Route of exposure combinations determined an increase in toxicity, and in particular where contaminated prey exposure was involved total effects exceeded always 55%. Regarding other predatory mite species, thiamethoxam proved to be moderately to highly toxic to *P. persimilis* (Lee et al. 2002; Duso et al. 2008; Pozzebon et al. 2011). A reduction in fecundity after the use of thiamethoxam was reported for *Neoseiulus cucumeris* (Oudemans), *Neoseiulus fallacis* (Garman) and *Galendromus occidentalis* (Nesbitt) (Kim et al. 2005; Villanueva and Walgenbach 2005; Bostanian et al. 2009). In other studies thiamethoxam affected survival of *Neoseiulus californicus* (Mc Gregor) and *Phytoseiulus macropilis* (Bancks) and caused a decrease in prey consumption of the latter (Poletti et al. 2007). Field experiments conducted on *Amblyseius andersoni* (Chant) and *G. occidentalis* showed that repeated applications of neonicotinoids during the season, thiamethoxam included, induced spider mite outbreaks probably due to their effects on predatory mites (Baldessari et al. 2010; Beers et al. 2005).

Systemically contaminated prey exposure increased risks for predatory mites. However, residual exposure can also induce systemic contamination since the immersed leaves can adsorb the pesticide. These results are consistent with those of studies conducted on *P. persimilis* (Pozzebon et al. 2011). Predatory mites exposed to contaminated prey can find on them a relatively high concentration of thiamethoxam and biomagnification effects increased toxicity. Moreover, thiamethoxam is promptly converted to clothianidin in insects and plants, a molecule characterized by a different binding site and apparently a higher toxic potential than its precursor (Nauen et al. 2003).

Our results confirm those obtained for other predatory mites and stress on the effects of contaminated prey exposure as major component of toxicity. This phenomenon, could by implied in spider mites outbreaks associated with thiamethoxam use. Ad hoc experiments will be addressed to this topic.

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Chapter 7

Interactions between *K. aberrans* – pollen – insecticides: laboratory experiments

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Introduction

Generalist predatory mites play a fundamental role in controlling spider mites and eriophyoids in European orchards (Helle and Sabelis 1985). Detailed studies have been conducted on the biology and the behavior of some species, i.e. *Typhlodomus pyri* Scheuten, *Amblyseius andersoni* (Chant), and *Euseius finlandicus* (Oudemans) (e.g., Chant 1959; Dosse 1961; Sabelis 1981; Dicke et al. 1990; Wei and Walde 1997; Schausberger 1998; Broufas and Koveos 2000). *Kampimodromus aberrans* (Oudemans) in another generalist predatory mite inhabiting perennial crops (Schausberger 1998; Kasap 2005; Broufas et al. 2007). Its role has been widely investigated in vineyards (Duso 1989; Tixier et al. 2002; Duso et al. 2009) much less in apple orchards (Duso et al. 2009). Pollen is an important food source for generalist predatory mites that exhibit higher demographic parameters when fed with pollen compared to spider mites (McMurtry 1992; McMurtry and Croft 1997). Studies conducted in vineyards showed that leaves are excellent pollen traps, and when analyzed over the growing season show definite trends in pollen fluctuations (Eichhorn and Hoos 1990; Duso et al. 1997). Populations of generalist predatory mites (e.g. *K. aberrans*) increase when windborne pollen is largely available on grape (Engel and Onhesorge 1994b; Duso et al. 1997) and apple leaves (Girolami et al. 2000). Windborne pollen is an exterminated resource that probably can be managed in conservation biological control tactics.

Predatory mite populations can be seriously affected by pesticide use with obvious consequences for phytophagous mite outbreaks. It is expected that environmental conditions favorable to predatory mites will accelerate the process of resilience. The availability of primary or alternative food could favor the response by predatory mite populations to detrimental pesticides. In this framework, windborne pollen could play a major role.

Grass pollen is a resource largely available for predatory mite in orchards (Girolami et al. 2000; Addison et al. 2002) and in vineyards (Engel and Ohnesorge 1994b; Duso et al. 1997, 2002).

Tillage or a high mowing frequency could reduce the possibility to produce pollen by grasses growing in apples orchards resulting in lower pollen availability for predatory mites (Madinelli et al. 2002; Baldessari et al. 2005). Recently, relationships between ground management, pesticides

*Paola Tirello contributed to all parts of the experimental work, data analysis and paper writing.

and predatory mites have been explored in apple orchards (Baldessari et al. 2005). In two contiguous apple orchards two different grass managements were compared. In the first orchard mowing was applied only in mid June while in the second grasses were mowed every week from late May to September to minimize grass flowering. In both orchards pesticides were applied simultaneously according to a randomized block design. Spinosad was detrimental for predatory mites in both orchards while chlorpyriphos exerted negative effects in the frequently mowed orchard only. Reducing mowing frequency resulted in a higher pollen flow and this situation appeared to favor the recovery of predatory mites in chlorpyriphos treated plots. Unfortunately it was not possible to study the interaction between grass management and pesticides in the same orchard.

In this paper we explored the role of pollen in mediating the effects of pesticides on *K. aberrans* through laboratory experiments. Predatory mites were exposed to pesticide fresh residues. Pollen was provided at two doses and two frequencies of application in order to assess the effect of pollen amount and fresh pollen availability on the impact of pesticides on predatory mites.

Materials and methods

Predatory mites strains

This study was performed on a *K. aberrans* strain collected from a commercial apple orchard (SM) (San Michele all'Adige, Trento province, Trentino-Alto Adige region) where an OPs resistant strain (chapter 4 of this thesis) had been introduced in 1997. In this orchards OPs (i.e., fenitrothion, chlorpyriphos) were still used in the last decade.

Predatory mites were mass-reared on artificial arenas using an apple leaf placed on a wet cotton layer as a substrate. A cotton barrier was created on the edges of leaves to prevent mite escape. *Kampimodromus aberrans* females were collected from leaves and transferred onto rearing arenas using a fine brush. Small pieces of PVC were placed on each arena as oviposition sites. *Typha latifolia* pollen was used as food source and was replenished every two days.

Laboratory trial

We studied the role of pollen in alleviating the impact of pesticides on *K. aberrans* using a factorial design, where experimental factors were: insecticides application (control, chlorpyriphos, etofenprox, indoxacarb and spinosad); pollen amount (low: 0.03 mg/cm^2; high: 0.33 mg/cm^2) and pollen application frequency (low: once at the start of the experiment; high: every 48 h after the first 72 h). Apple leaf sections were immersed in the pesticide solution for 30 s. When pesticide residues dried out leaf sections were used to build experimental units similar to those above described. Two

coeval *K. aberrans* females were transferred onto each unit. For each pesticide formulation we used the highest field recommended dose (Table 1).

Female survival and escaping were recorded at 72 and 168 h after treatments, and fecundity was recorded for 168 h. Data on survival and escaping were analyzed with a factorial logistic regression ($p = 0.05$), while data on fecundity were analyzed with a REML model ($p = 0.05$).

Table 1 – Characteristics of pesticides considered in laboratory trials.

Trade name	Active ingredient (a.i.)	Dose	Chemical class	Mode of action
Dursban ®	Chlorpyriphos	525 mg a.i L^{-1}	Organophosphates	Acetylcholine esterase inhibitors
Trebon ® star	Etofenprox	158 mg a.i. L^{-1}	Pyrethroids	Sodium channel modulators
Steward ®	Indoxacarb	49.5 mg a.i L^{-1}	Oxadiazines	Voltage-dependent sodium channel blockers
Laser ®	Spinosad	96 mg a.i. L^{-1}	Spynosines	Nicotinic acetylcholine receptor allosteric activators

Results

Laboratory studies evidenced a significant effect of insecticide applications on *K. aberrans* survival observed at 72 h from treatments ($X^2 = 414.95$; df = 4; $p < 0.001$). Survival was higher in the control and indoxacarb than on other treatments. Survival was higher on chlorpyriphos than on spinosad and intermediate between the latter for etofenprox (Figure 1). Survival was not affected by different pollen amounts ($X^2 = 0.34$; df = 1; $p = 0.562$). Insecticides affected escape rate assessed at 72 h ($X^2 = 27.35$; df = 4; $p < 0.001$) that resulted higher in spinosad and etofenprox than on other treatments. Pollen amount did not affect escape rate after 72 h ($X^2 = 0.92$; df = 1; $p = 0.337$).

Insecticides influenced survival rate calculated at 168 h ($X^2 = 36.98$; df = 4; $p < 0.001$). Predatory mites did not survive on spinosad, and a significant mortality was induced by etofenprox. No effects of pollen amount and pollen application frequency were observed on survival assessed at 168 h ($X^2 = 0.14$; df = 1; $p = 0.71$; $X^2 = 0.09$; df = 1; $p = 0.763$; respectively). At 168 h, insecticide application affected escaping ($X^2 = 9.75$; df = 4; $p = 0.049$), resulting higher where spinosad was applied compared to control and chlorpyriphos treatments. No effects of pollen amount or pollen application frequency were observed on escaping at 168 h ($X^2 = 0.56$; df = 1; $p = 0.453$; $X^2 = 1.54$; df = 1; $p = 0.215$; respectively).

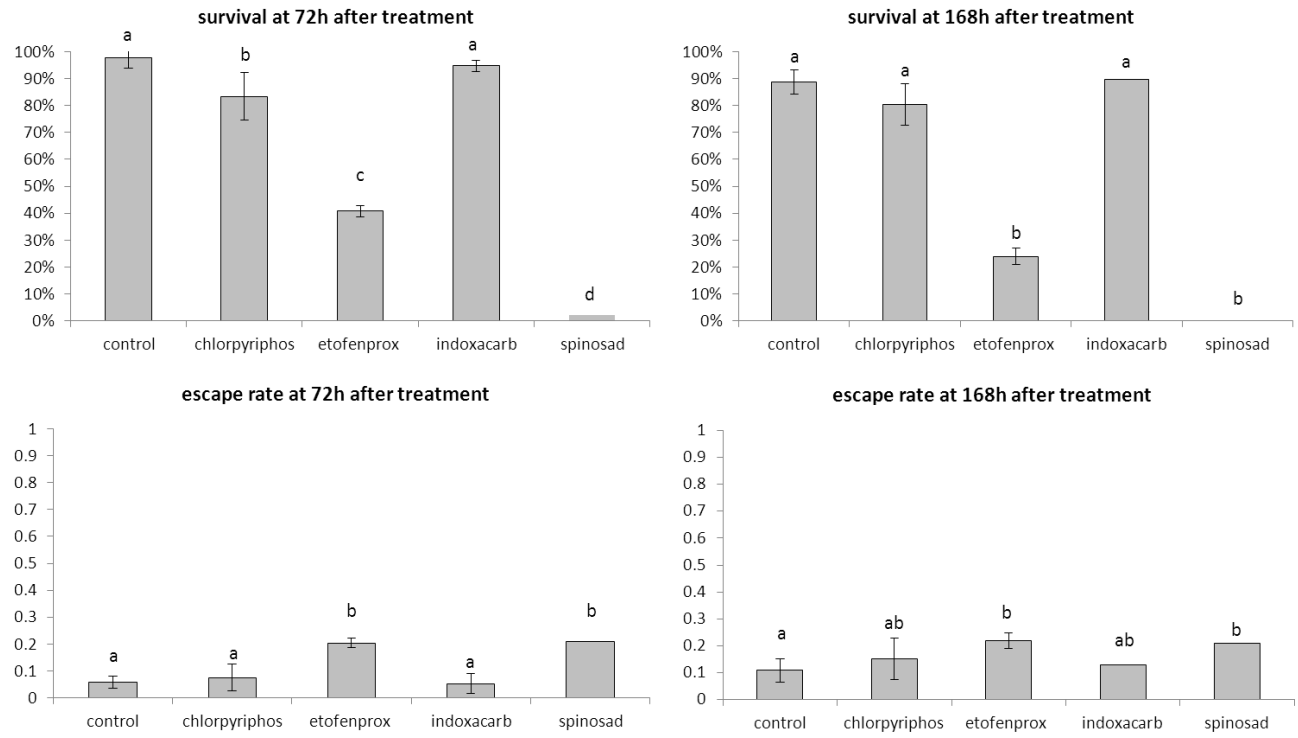

Figure 1- Effects of insecticide applications on survival and escape rate on *K. aberrans*. Different letters indicate significant differences (p = 0.05) at factorial logistic regression.

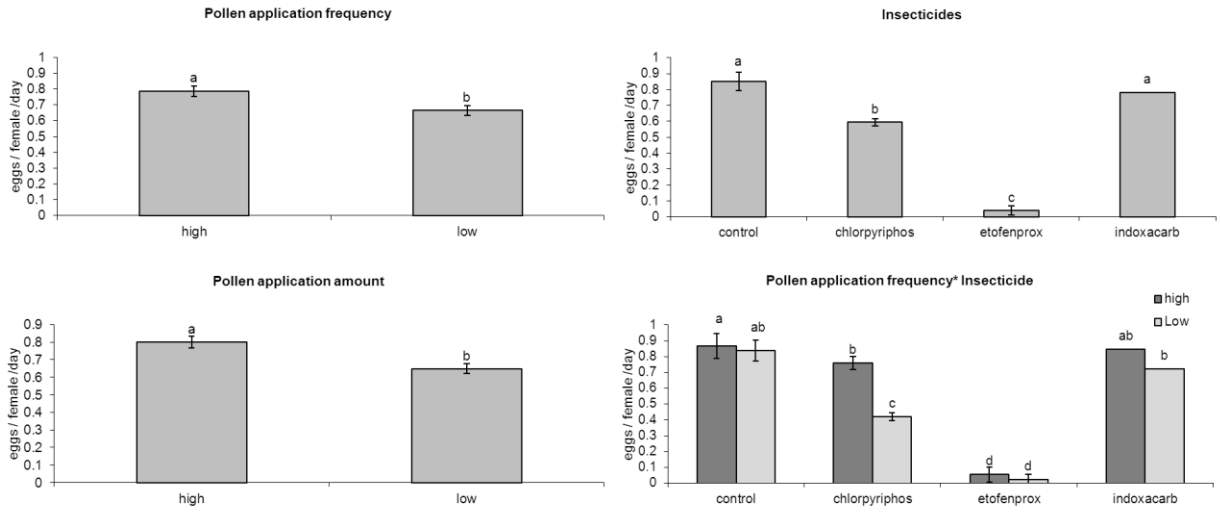

Figure 2- Effects of insecticide applications on *K. aberrans* fecundity. Different letters indicate significant differences (p = 0.05) at REML (Restricted Maximum Likelihood) factorial analysis of variance of fecundity.

Fecundity of *K. aberrans* was influenced by insecticide application (F = 41.41; df = 3. 217; p < 0.001). No eggs were laid where spinosad was applied, and a higher fecundity was observed in the control and indoxacarb than on chlorpyriphos and etofenprox (Figure 2). Predatory mite fecundity increased by augmenting pollen amount (F = 5.44; df = 1, 217; p = 0.021) and pollen application

frequency (F = 7.89; df = 1, 217; p = 0.005). A significant interaction "insecticide application*pollen application frequency" was observed (F = 4.26; df = 3, 217; p = 0.006). Pollen application frequency reduced the negative effect of chlorpyriphos but not that of etofenprox (Figure 2). With high pollen application frequency predatory mites fecundity treated with indoxacarb and chlorpyriphos was similar (Figure 2).

Discussion

Pesticides effects on *K. aberrans* populations may depend on environmental conditions, included food availability. In previous field experiments chlorpyriphos affected predatory mites in orchard plots frequently mowed but not in plots rarely mowed. The detrimental effects of this pesticide were less pronounced where grass management induced a high pollen flow (Baldessari et al. 2005). The results obtained in the factorial experiment conducted on pollen and pesticides suggest some mechanisms involved in the phenomenon reported in field experiment. Insecticide applications affected significantly survival and fecundity of *K. aberrans*. Spinosad was found with highest toxicity on *K. aberrans* determining high mortality and no oviposition. Moreover mite escaping increased when spinosad was applied suggesting that this compound may be repellent to predatory mites. Etofenprox caused effects similar to spinosad, with high mortality and less fecundity. Chlorpyriphos determined a reduction in survival and fecundity but was less toxic than spinosad and etofenprox. Indoxacarb was selective toward *K. aberrans*, no significant effects on mortality and fecundity were found. Pollen application did not affect survival and escaping of predatory mites but increased their fecundity. Pollen application frequency had positive effect on predatory mites exposed to chlorpyriphos residues and in particular fresh pollen availability seemed to reduce the effect of this insecticide. A first factor possibly involved in this phenomenon is pollen contamination by insecticides. Pollen adsorption of organic compounds such as chlorpyriphos is related to the bioconcentration capability in the vegetal biomass (Paterson et al. 1991; Villa et al. 2000). This is likely to increase with time of contact with insecticide residues. Predatory mites feeding on contaminated pollen are exposed to insecticides through ingestion. In our experiment this determined an increase of exposure to insecticides, with more pronounced sub-lethal effects. Indeed, detrimental effects of an insecticide can increase when multiple routes of exposure are considered (Banken and Stark 1991; Galvan et al. 2006; Pozzebon et al. 2011). A second factor can be related to food conversion efficiency into egg biomass by predatory mites that can be disrupted by insecticides. Pollen nutritional quality degrades over the time and mites exposed to chlorpyriphos manifested a reduced fecundity. This suggests a reduction in the food conversion efficiency into egg biomass by predatory mites. They probably take an advantage from fresh pollen

with more available nutritional load per biomass unit, indeed the effect of pollen application frequency was independent from pollen amount provided. A third factor can be related to the feeding capacity of mites. Insecticides may repress the capacity of mites to detect, pierce and ingest old pollen grains. Fresh pollen can be more suitable to be eaten than treated pollen or the latter may induce repellence. Further studies are required to investigate mechanisms that determine the response of mites exposed to chlorpyriphos to fresh pollen availability.

The present study shed light on some aspects influencing predatory mites population dynamics in perennial crops systems. Habitat management that enhances alternative food availability can favor predatory mites persistence and resilience after pesticide use.

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