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Persistence of imidacloprid, acetamiprid and methomyl in qat leaves

A.J. Al-Rajab[1,*], A.M. Alhababy[2,3] and T. Alfaifi[2]

Summary Qat leaves are chewed on a daily basis by approximately 10 million inhabitants of different countries. This study investigated the persistence of three insecticides most used in qat production, imidacloprid, acetamiprid and methomyl. These chemicals were applied separately on plots of ten qat trees each at the recommended application rates. Samples of qat leaves were collected separately at time 0 (1 h post-treatment) and 1, 3, 7, 12, 19, 26 and 37 days after application. The residues of the investigated pesticides were extracted and then quantified by liquid chromatography (LC-MS/MS). The half-lives of imidacloprid, acetamiprid and methomyl were 12.2, 11.7, and 5.1 days, respectively. Overall, our findings showed that imidacloprid and acetamiprid were more persistent than methomyl in qat leaves. Taking into account the maximum residue limits (MRL) in lettuce, due to lack of MRL in qat leaves, the residue concentrations were below MRL for imidacloprid 7 days after application, and 1 day after application for acetamiprid and methomyl.

*Additional keywords***:** chat, degradation, half-life, insecticide, khat, qat

Introduction

Qat (*Catha edulis*, Forsk) is a perennial shrub, also known by the common names khat or chat, cultivated only in specific regions of a few countries encompassing the Red Sea: Yemen, southwestern Saudi Arabia, Ethiopia, Eritrea, Djibouti, Kenya, and Somalia (Alvi *et al*., 2014; Gebissa, 2010). The fresh qat leaves are chewed for 3–6 hours on a daily basis (usually in the afternoon) by around 10 million inhabitants of these regions; this habit is referred to locally as "takhzeen al-qat" (Hassan *et al*., 2013; Al-Motarreb *et al*., 2010). Moreover, the habit of qat chewing has recently been introduced to other African countries, such as Uganda, Burundi, and Rwanda (Numan, 2012), as well as to the United States, Great Britain, and Western Europe by the Eastern African and Yemeni communities of these countries (Bongard *et al.,* 2015; Al-Motarreb *et al.,* 2010).

The principal active component in qat leaves is cathinone (*(S)*-2-Amino-1-phenyl-1-propanone, $C_9H_{13}NO$), which is known for its mild stimulatory effects; recently, synthetic cathinones have been sold worldwide under the name "bath salts" (Katz *et al.,* 2014; Daba *et al.,* 2011). Qat is moderately used as a traditional medicine by indigenous people of East Africa, but neither the plant itself nor its isolated active ingredients have been widely recognised for their therapeutic use (EMCDDA, 2016). According to the literature, qat-chewing is linked to adverse health effects, such as liver toxicity, an increased risk of cardiovascular events, reproductive problems, psychosis, and periodontal problems (Date *et al.,* 2004; Al-Hebshi and Skaug, 2005).

Qat has been reviewed by the WHO Expert Committee on Drug Dependence (ECDD) on a number of occasions. *Catha edulis* remains outside international control, although cathinone and cathine, which arises from the metabolism of cathinone in the mature plant, have been listed in the 1971 UN Convention under Schedules I and III, respectively, since the early 1980s. Qat is controlled in a number of European countries including Belgium, Denmark, Germany, Greece, France, Ireland, Italy, Latvia, Lithuania, Poland, Slo-

[1] Center for Environmental Research and Studies, Jazan University, Jazan, Saudi Arabia
[2] Department of Biology, Faculty of Science, Jazan University, Jazan, Saudi Arabia
[3] Department of Environment, Faculty of Marine Science and Environment, Hodeidah University, Yemen
* Corresponding author: alrajab@hotmail.com

venia, Finland, Sweden, Norway, Switzerland and recently in U.K. (The Misuse of Drugs Order, 2014; EMCDDA, 2016).

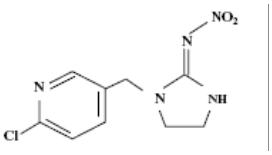
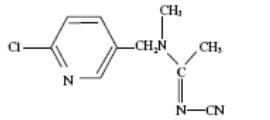
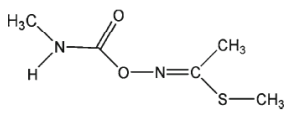
The high demand for qat, combined with the limited amount of cultivated lands, has raised its value in recent years. Its price has increased dramatically to approximately $150–$200/kg, depending on its variety and origin. An individual chews about 100–300 g of fresh leaves daily (Nakajima *et al.,* 2014; Date *et al.,* 2004).

The use of pesticides in qat production is indispensable in protecting the plants from different insects and fungi. Consequently, qat production consumes about 70% of the pesticides used in Yemen; some of these pesticides are banned (e.g. DDT) but continue to be used illegally in the production of qat and other crops (Date *et al*., 2004). Imidacloprid, acetamiprid and methomyl are the most commonly used insecticides in the production of qat; some of their key properties are presented in Table 1. Imidacloprid (*N*-{1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl}nitramide; $C_9H_{10}ClN_5O_2$), the most-used insecticide worldwide, and acetamiprid (*N*-[(6-chloro-3-pyridyl)methyl]-*N*′-cyano-*N*-methyl-acetamidine; $C_{10}H_{11}ClN_4$) are neonicotinoid insecticides that act as insect neurotoxins (Sharma and Singh, 2014). Methomyl (*S*-methyl *N*-(methylcarbamoyloxy) thioacetimidate; $C_5H_{10}N_2O_2S$) is an oxime carbamate insecticide used as a broad-spectrum insecticide since 1968 (Van Scoy *et al.,* 2013), which was banned few years ago but continues to be used illegally by qat farmers.

Therefore, the consumption of qat might be an important source of exposure to pesticides, especially because its leaves are consumed fresh, without any thermal treatments that can reduce pesticide residues (Daba *et al*., 2011). Qat chewing is similar to the smokeless tobacco chewing among workers in tea industry in India (Kausar *et al.,* 2014). Moreover, the failure of some local farmers to respect pesticide labels (dose, application method, and post-harvest interval) might lead to a high risk of qat contamination by pesticides (Date *et al*., 2004). Results obtained by Daba *et al.* (2011) showed high concentrations of the insecticides diazinon (751 µg kg^{-1}) and DDT (1,372 µg kg^{-1}) in qat collected from different farms in Ethiopia. In contrast, Hassan *et al.* (2013) reported the absence of pesticide residues in 120 qat samples collected from Jazan area, Saudi Arabia.

Information on the persistence of pesticides in qat is extremely scarce. The pres-

Table 1. Structure and key properties of imidacloprid, acetamiprid and methomyl (Gupta and Shanker, 2008; Gupta *et al.,* 2008; Tomasevic *et al.,* 2010; Van Scoy *et al.,* 2013).

Compound	M.W.	Solubility (mg L^{-1})	Log K_{ow}	p*Ka*	Structure
Imidacloprid CAS: 3380-34-5 Formula: 5-chloro-2-(2,4-dichlorophenoxy)-phenol	289.5	4.621	4.7	8.1	
Acetamiprid CAS: 101-20-2 Formula: 3,4,4′- trichlorocarbanilide	315.6	0.6479	4.9	n/a	
Methomyl CAS: 16752-77-5 Formula: S-methyl N-(methylcarbamoyloxy)thioacetimidate	162.2	57900	1.24	14	

ent study sought to determine the residue dissipation of the insecticides most used in the production of qat (imidacloprid, acetamiprid and methomyl) to control jassids, thrips and mites. To the best of our knowledge, this is the first work on the residues of these three insecticides in qat.

Materials and Methods

The commercial formula of selected insecticides were purchased from a local market in Jazan, KSA: imidacloprid (Imidor 200 g L^{-1}) (Astrachem, KSA), acetamiprid (Deltaride 20% sp) (Delta, KSA), and methomyl (Lanet 90 WP) (Du Pont, China).

Field trials were conducted at a private farm in the Fyfa Mountains area on the border zone between Yemen and Saudi Arabia (17° 15′ 3.76″ N, 43° 7′ 56.46″ E). Four separated plots of ten trees each, with a buffer zone of 10–15 m between plots were selected for this study. Consequently, the selected trees were labeled for each treatment. Trees were healthy, 10–12 years old, and were not treated with any pesticide for the last 4 years, as declared by the farm's owner. One plot was considered to be a control and treated only with tap water, while other plots were separately treated with the investigated insecticides at the recommended application rates. The applied concentrations of pesticides in the sprayed solutions were 1.25 ml L^{-1}, 0.5 g L^{-1}, and 0.5 g L^{-1} for imidacloprid, acetamiprid, and methomyl, respectively. The total sprayed solution was 10 L for each plot using a hand-operated sprayer (Mythos, Italy). All trees were treated until run-off.

For each treatment, leaves and buds were sampled from the four sides and top of each qat tree; an approximate total of 500 g of green qat leaves and buds were collected in a plastic bag from each plot at time 0 (1 h post-application) and 1, 3, 7, 12, 19, 26, and 37 days after application. At each of the specified times, collected samples (from treated and control plots) were placed in a field cooler at 4 °C and transferred to the laboratory. Accumulated precipitation during the experiment was 53.1 mm; the mean air temperature was 29.7 °C (Fig. 1).

Upon arrival to the laboratory, each sample was chopped separately using a domes-

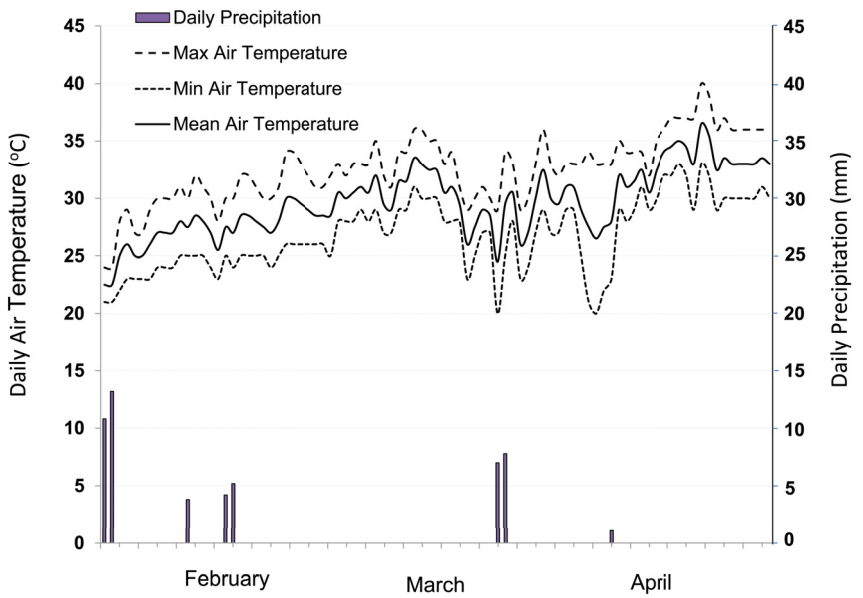


Figure 1. Weather parameters at the research farm of qat during the period of the insectiside residue field experiment.

tic glass blender 1.8 L (Moulinex, France). For each sample, three homogenized subsamples of 150 g were transferred into a 250 mL amber glass bottle with cap and stored at -20 °C until analysis by the end of field experiments. In order to determine the pesticide residues, frozen samples were placed inside a field cooler and shipped frozen to an accredited lab (Australian Laboratory Services, Dammam, Saudi Arabia). The pesticide residues were extracted from a homogenized sample with 1% acetic acid in acetonitrile (QuEChERS procedure) according to the method described by Anastassiades et al. (2003). The extract was analyzed by liquid chromatography with mass detection (LC-MS/MS) as described by Anastassiades et al. (2003) and EC (2007). The calibrated range of the method was 0.01-0.2 µg ml^{-1}, which equates to 0.01–0.2 mg kg^{-1} in the sample (0.02–0.4 mg kg^{-1} in the dry samples). The retention times were 2.68, 3.46 and 3.85 minutes for methomyl, imidacloprid and acetamiprid, respectively (Fig. 2). A recovery test for the extraction method of insecticides was made separately in 3 samples of untreated qat leaves (collected from the control plot trees) spiked with a known amount of each insecticide. A blank of untreated qat leaves was extracted at the same time as control. The recovery rate was acceptable with values of 101.2±3.1%, 98.3±2.7% and 94.6±1.2% of the initial amount for imidacloprid, acetamiprid and methomyl, respectively. None of the investigated pesticides were detected in the samples collected from the control plot.

Data analysis was conducted using Microsoft Excel 2002 (Microsoft Canada, Toronto, ON). Dissipation curves were plotted using SigmaPlot (Version 10, Systat Software Inc., Chicago, IL). The half-life of each insecticide was calculated separately using the equation of first-order rate, as described by Gupta *et al.* (2008).

Results and Discussion

The initial concentration of imidacloprid residues in qat leaves was 6.2 mg kg^{-1}. Its degradation then approximated first order (r^2 = 0.95); 37 days after treatment 10.65% of initial residues remained in the qat leaves (Tables 2 and 3). The wash of insecticide with rain was negligible because of the low precipitation after its application (Fig. 1). Itoiz *et al.* (2012) reported similar initial residue concentrations of imidacloprid in lettuce leaves of 5.97 mg kg^{-1}, which then declined to 0.69 mg kg^{-1} 14 days after application. Persistence

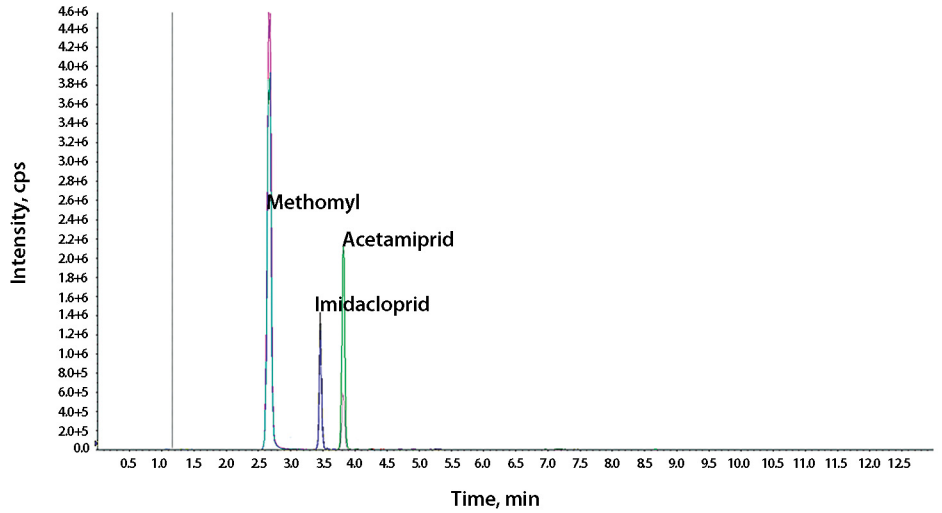


Figure 2. Representative LC-MS/MS chromatogram for standards mixture of imidacloprid, acetamiprid and methomyl.

Table 2. Residues (mg kg^{-1}) of imidacloprid, acetamiprid and methomyl, and the percentage of remaining residues (%) in leaves of qat at different time intervals after application.

Time (days)	Imidacloprid		Acetamiprid		Methomyl	
	Residues	%	Residues	%	Residues	%
0 (1h)	6.2 ± 0.41	100	3.0 ± 0.28	100	12.0 ± 0.63	100
1	5.2 ± 0.33	83.87	2.6 ± 0.30	86.67	2.6 ± 0.43	21.67
3	3.9 ± 0.19	62.90	2.1 ± 0.24	70.00	2.3 ± 0.39	19.17
7	3.1 ± 0.22	50.00	2.0 ± 0.11	66.67	1.1 ± 0.22	9.17
12	1.8 ± 0.12	29.03	1.3 ± 0.08	43.33	0.54 ± 0.18	4.50
19	1.8 ± 0.16	29.03	1.0 ± 0.09	33.33	0.31 ± 0.04	2.58
26	1.1 ± 0.09	17.74	0.89 ± 0.10	29.67	0.17 ± 0.06	1.42
37	0.66 ± 0.07	10.65	0.25 ± 0.05	8.33	0.03 ± 0.01	0.25

Table 3. Dissipation rate parameters for the fit of imidacloprid, acetamiprid and methomyl in qat leaves, to a first order kinetic model.

Pesticide	r^2	K (day^{-1})	C_0 (mg kg^{-1})	Std. error	$t_{1/2}$ (days)
Imidacloprid	0.9542	0.057a	6.2	0.0021	12.2
Acetamiprid	0.9482	0.059a	3.0	0.0017	11.7
Methomyl	0.9290	0.135b	12	0.0009	5.1

r^2: determination coefficient; K: rate constant; C_0: initial concentration of residue; Std. error: Standard error; $t_{1/2}$: half-life; ^{a,b}: significant difference between treatment using a Student T test.

of pesticides in plants, which is longer in the dry season than in the wet season, is influenced by different factors, such as the targeted plants, the physico-chemical properties of the pesticide, and its application methods (Itoiz *et al*., 2012; Gupta *et al.,* 2008; Fujita *et al*., 2014). However, a variety of similar studies have been realized to determine the dissipation of imidacloprid using different application techniques on different plants. The half-life of imidacloprid residues in qat leaves was relatively long at 12.2 days (Table 3), which is consistent with another study showing that the half-lives of imidacloprid in sugarcane leaves were 8.1–9.7 days in two different application doses (Sharma and Singh, 2014). In contrast, imidacloprid was reported to dissipate more rapidly in other plants, such as tea shoots, with a half-life of 1.09–1.25 days (Gupta *et al.,* 2008), 4.4 days in lettuce leaves (Itoiz *et al.,* 2012), and 1.7–2.3 days in chickpea pods and leaves (Chahil *et al.,* 2014).

Until these days, there is no legislation in the qat production countries for the recommended minimum pre-harvest intervals (PHIs) or for the maximum residue limits MRLs of pesticides in qat despite its high consumption by about 10 million people of different countries. Consequently, no value has yet been established with respect to the maximum permissible intake of imidacloprid, acetamiprid and methomyl in qat. Due to the lack of some scientific information on the persistence of pesticides in qat and the absence of MRL values in its leaves in the major guidance documents i.e. Codex (2015) and EU (2005), we used the MRL values already established for lettuce leaves to compare the preharvest intervals of investigated insecticides because both lettuce and qat leaves are chewed fresh and uncooked among the qat consumers and approximately at the same quantity weekly. The MRL for imidacloprid in lettuce is 3.5 mg kg^{-1} and the recommended PHI is 7 days (Global MRL Database, 2015). In our study, the residue concentrations of imidacloprid were below the MRL at 3.1 mg kg^{-1} (Table 2) 7 days after treatment. In contrast, Chahil *et al.* (2014) reported that the residue concentrations of

imidacloprid were below the MRL directly after application (2 h) in chickpea pods and leaves. This difference might be due to the very low initial concentration of imidacloprid (0.29–0.49 mg kg^{-1}) in chickpea pods and leaves. Moreover, imidacloprid residue concentrations ranging from 0.01 to 0.76 mg kg^{-1} were detected in some fruit and vegetable samples collected from the Aegean region in Turkey (Bakirci *et al.,* 2014).

The dissipation kinetics of acetamiprid in qat leaves was similar to that of the other investigated neonicotinoid insecticide, imidacloprid, although the initial concentration of acetamiprid residues in qat leaves was 3.0 mg kg^{-1}, which is 52% less than that of imidacloprid. Therefore, its degradation approximated first order (r^2 = 0.95); 37 days after treatment 8.33% of initial residues remained in the qat leaves (Tables 2 and 3). The initial residue concentrations of acetamiprid obtained in this study (3.0 mg kg^{-1}) were much higher than the concentrations reported in chillies (0.02–0.1 mg kg^{-1}) after treatment at the recommended and double-the-recommended doses (Sanyal *et al*., 2008). A possible explanation for this discrepancy could lie in the fact that qat leaves received a higher amount of pesticide than the chilli peppers due to their larger surface; consequently, the concentrations of the chemical in leaves were found to be higher than those in fruits.

Dissipation of acetamiprid in different plants has been reported in many recent studies; however, the literature contains no information about the dissipation of acetamiprid in qat leaves. In our study, the dissipation rate of acetamiprid in qat was relatively slow (half-life = 11.7 days) compared to that of other plants, such as 1–1.6 days for the mustard plant (Pramanik *et al*., 2006), 1.8–2.3 days for green-tea shoots (Gupta and Shanker, 2008), 2.2–4.8 days in chillies (Sanyal *et al.,* 2008), and 1.9 and 2.5 days in zucchini and zucchini leaves, respectively (Park *et al.,* 2010). Similar to the case of imidacloprid, no MRL value has yet been established for acetamiprid in qat, thus, we used its value in lettuce leaves to compare the preharvest intervals (PHIs). For acetamiprid in lettuce, the MRL = 3 mg kg^{-1}, and the recommended PHI is 7 days (Global MRL Database, 2015). In the present study, the residue concentrations of acetamiprid were below the MRL at 2.6 mg kg^{-1} 1 day after treatment (Table 2). However, a PHI of 1 day after application is recommended for tea shoots to ensure safe consumption (Gupta and Shanker, 2008); the PHI of 1 day could be recommended for acetamiprid in qat leaves. Acetmiprid residues were detected in some fruit and vegetable samples collected from the Aegean region in Turkey, but they ranged from 0.01 to 0.06 mg kg^{-1} and were therefore below the MRL value of 3 mg kg^{-1} (Bakirci *et al.,* 2014).

Despite the relatively high initial residue concentration of methomyl in qat leaves (12 mg kg^{-1}), its dissipation, with a half-life of 5.1 days, was significantly more rapid than that of imidacloprid and acetamiprid (Tables 2 and 3). The decline of methomyl residues was very fast after application: About 78.3% of the residues had dissipated 1 day after treatment; its degradation then approximated first order (r^2 = 0.93), and the residue concentrations at the end of the experimentation, 37 days after treatment, were 0.03 mg kg^{-1}. Similar to the case of imidacloprid and acetamiprid, no value has yet been established with respect to the maximum permissible intake of methomyl in qat. Therefore, we used its value in lettuce leaves to compare the preharvest intervals (PHIs). The MRL value of methomyl residues in lettuce is suggested to be 5 mg kg^{-1} (Global MRL Database 2015). In the present study, the residue concentrations of methomyl, at 2.6 mg kg^{-1}, were below the MRL 1 day after treatment (Table 2); at 5.1 days, its half-life is in agreement with results obtained by Reeve *et al.* (1992), which showed that variable half-lives of methomyl in grape foliage in 36 U.S. fields ranged from 1 to 7.7 days. In contrast, the half-life of methomyl in qat leaves, as demonstrated in our study, is longer than the reported half-lives in other studies of other plants e.g., 0.9-1.34 days for tomatoes (Gambacorta *et al.,* 2005; Malhat *et al*., 2015) and 0.88–0.94 days for okra fruits (Aktar *et al.,* 2008). The half-life of methomyl in plants

increases significantly with the progression of the summer months (Reeve *et al.,* 1992) due to the slow growth of plants and, consequently, less efficiency in the degradation of the pesticides. The initial residual concentration in qat leaves was higher than results reported in tomatoes (1.54 mg kg^{-1}) (Gambacorta *et al.,* 2005) and 5.61–8.42 mg kg^{-1} in okra fruits treated at the recommended and double-the-recommended doses, respectively (Aktar *et al.,* 2008). Methomyl residue concentrations ranging from 0.01 to 1.42 mg kg^{-1} were detected in grape samples collected from the Aegean region in Turkey and in some vegetables (arugula, eggplant, bean, cucumber, leek, mushroom, onion, and pepper); at concentrations ranging from 0.01 to 2.13 mg kg^{-1}; the residues in all samples were below the MRL of 5 mg kg^{-1} (Bakirci *et al.,* 2014). The rapid dissipation of methomyl and its relatively short half-life led to decrease the residues below the MRL value within only 1 day after application.

Conclusions

The present study demonstrates that the neonicotinoid insecticides imidacloprid and acetamiprid have similar dissipation pathways in qat leaves; their persistence was significantly higher than the carbamate insecticide methomyl. Half-life values for imidacloprid, acetamiprid and methomyl, when applied at recommended dosages on qat trees, were 12.2, 11.7 and 5.1 days, respectively. Our results showed that the residues of the investigated pesticides were below the MRL for lettuce 7 days post-application for imidacloprid and 1 day post-application for acetamiprid and methomyl. In view of the increased consumption of qat and the scarce information about the dissipation of pesticides in this plant, more studies are required to assess the risk of human exposure to pesticides by chewing qat leaves.

AJ Al-Rajab designed the experiments, data interpretation, manuscript writing, and submission corresponding. AM Alhababy contributed in experiments design, treatments, samples preparation, data collection and interpretation. T. AlFaifi contributed in pesticides' treatment, sampling, lab preparation and data collection.

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Υπολειμματικότητα imidacloprid, acetamiprid και methomyl σε φύλλα του φυτού *Catha edulis*

A.J. Al-Rajab, A.M. Alhababy και T. Alfaifi

Περίληψη Η μάσηση των φύλλων του φυτού *Catha edulis* αποτελεί συνήθεια σε καθημερινή βάση περίπου 10 εκατομμύριων ανθρώπων σε διάφορες χώρες. Στην παρούσα μελέτη διερευνήθηκε η υπολειμματικότητα των πλέον χρησιμοποιούμενων εντομοκτόνων σκευασμάτων στην καλλιέργεια του φυτού (δραστικές ουσίες imidacloprid, acetamiprid και methomyl). Τα εντομοκτόνα εφαρμόστηκαν ξεχωριστά σε πειραματικά τεμάχια των δέκα φυτών στις συνιστώμενες δόσεις εφαρμογής. Συλλέχθηκαν δείγματα φύλλων 1 ώρα μετά την εφαρμογή και 1, 3, 7, 12, 19, 26 και 37 ημέρες μετά την εφαρμογή. Ο προσδιορισμός των υπολειμμάτων πραγματοποιήθηκε με τη χρήση υγρής χρωματογραφίας σε συνδυασμό με φασματομετρία μάζας τριπλού τετραπόλου (LC -MS/MS). Οι χρόνοι ημίσειας ζωής των imidacloprid, acetamiprid και methomyl ήταν 12.2, 11.7 και 5.1 ημέρες, αντίστοιχα. Συνολικά, τα αποτελέσματα έδειξαν ότι οι ουσίες imidacloprid και acetamiprid παρουσίασαν μεγαλύτερη υπολειμματική διάρκεια σε σύγκριση με το methomyl στα φύλλα του φυτού. Λαμβάνοντας υπόψη τα ανώτατα όρια υπολειμμάτων (MRL) στο μαρούλι, λόγω απουσίας MRL σε φύλλα του ιδίου φυτού, οι συγκεντρώσεις υπολειμμάτων ήταν κάτω από τα MRL για το imidacloprid, 7 ημέρες μετά την εφαρμογή, και 1 ημέρα μετά την εφαρμογή για το acetamiprid και το methomyl.

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SHORT COMMUNICATION

An update on the occurrence of resistance-breaking populations of root-knot nematodes (*Meloidogyne* spp.) on resistant tomato in Greece with six new records from Crete

E.A. Tzortzakakis[1*], M.-C. Vieira dos Santos[2] and I. Conceição[2]

Summary The available published information on the occurrence of resistance-breaking populations of root-knot nematodes (*Meloidogyne* spp.) on resistant tomato in Greece is updated. Within the period 1994-2013, 13 populations (11 *M. javanica* and 2 *M. incognita*) able to reproduce on resistant tomato had been recorded in the regions of Crete, Epirus, Thrace, Peloponissos and Macedonia. In the present study six more resistance-breaking populations, four *M. javanica* and two *M. incognita*, were detected in the period 2013-2014, all originating from greenhouse vegetables in Crete. Four of these populations, two *M. javanica* and two *M. incognita*, originated from the region of Ierapetra. This is the first time that such populations are found in this major area of greenhouse vegetable production of Crete.

Additional keywords: *Meloidogyne javanica*, *M. incognita* , *Mi* gene, pathogenicity, pepper, virulence

Root-knot nematodes (RKN), *Meloidogyne* spp., are among the most economically important nematodes in agriculture with a broad host range (Karssen and Moens, 2006) and a wide distribution in the Mediterranean region (Lamberti, 1981) especially in greenhouses with vegetables. In tomato, there are commercially hybrids with resistance to RKN, which is conferred by the *Mi* gene being effective against *M. arenaria, M. javanica* and *M. incognita* at moderate soil temperatures (Williamson, 1998). However, there are several reports concerning virulent populations of these *Meloidogyne* species, able to reproduce on resistant tomato cultivars, occurring either naturally (Williamson, 1998) or after repeated selection on tomato plants with the *Mi* gene (Castagnone-Sereno *et al*., 1994).

A review on the occurrence of RKN in Greece has been published for the period 1996-2010 (Tzortzakakis *et al*., 2011); no information on the presence of virulent populations on resistant tomato was included. Since 1994, there have been 13 records of resistance-breaking populations of RKN from Greece (Tzortzakakis and Gowen, 1996; Tzortzakakis *et al*., 1999; Tzortzakakis *et al*., 2005; Tzortzakakis and Blok, 2007; Tzortzakakis *et al*., 2008; Tzortzakakis *et al*., 2014).

The aim of the current study was: a) to review the published records on the occurrence of RKN populations able to reproduce on resistant tomato in Greece and b) to evaluate the reproduction of 20 populations of RKN, collected randomly from greenhouses and outdoor crops of Crete during 2013-2014, on a resistant tomato and a susceptible pepper cultivar, and update the existing information.

Materials and Methods

From June 2013 until December 2014, 20 populations of RKN were collected from greenhouses and outdoor crops, from various areas of Crete. All originated from sus-

[1] Department of Viticulture, Vegetable Crops, Floriculture and Plant Protection, Institute of Olive Tree, Subtropical Crops and Viticulture, N.AG.RE.F., Hellenic Agricultural Organization-DEMETER, PO BOX 2228, 71003, Heraklion, Crete, Greece
[2] CFE-Center for Functional Ecology, Department of Life Sciences, University of Coimbra, P 3000-456 Coimbra, Portugal
* Corresponding author: etzortza@her.forthnet.gr

ceptible crops, where no nematode resistant tomato hybrid had been used in the last 4-5 year crop rotation scheme. The nematode populations were established as cultures, by inserting pieces of root galls, in soil around seedlings of the susceptible tomato (*Solanum lycopersicum* L.) cv. ACE grown in pots. The RKN populations originating from pepper were established as cultures on the susceptible pepper (*Capsicum annuum* L.) cv. California Wonder. The plants were watered and fertilized as required; they grew in a controlled environment with 16 h photoperiod and soil temperature 24-26°C, at which the *Mi* gene is effective (Williamson, 1998). Every seven weeks, the plants were uprooted and ten egg masses were transferred to new plants to maintain the nematode populations.

Preliminary tests

Egg masses were used to inoculate resistant tomato plants cv. Silvana (with the *Mi* gene) and the susceptible pepper cv. California Wonder at a rate of 20 egg masses/plant, and were maintained in the conditions described above. Seven weeks after the inoculation, the roots of the resistant tomato and pepper plants were examined. If there were no egg masses, new plants of resistant tomato and pepper were inoculated again using inoculum from the original nematode cultures. In case that there was no reproduction of the nematodes in the second test, the populations were discarded.

From the 20 RKN populations tested, 12 were discarded as they did not reproduce on resistant tomato and pepper. The remaining eight populations, originating exclusively from greenhouse crops, were selected for subsequent studies according to the results of the preliminary tests:

1. Five populations which reproduced on resistant tomato but not on pepper (I1, I2, I3, S and V).
2. One population which reproduced on both resistant tomato and pepper (I4).
3. One population which reproduced on pepper but not on resistant tomato (K).
4. One population which did not reproduce in both, resistant tomato and pepper, was kept as control (I5).

RKN population virulence tests

The populations which reproduced on resistant tomato and pepper were maintained in the same cvs for 4-5 further generations, using each time ten egg masses originating from the tomato cv. Silvana or the pepper cv. California Wonder. All the original populations were simultaneously maintained in the susceptible tomato cv. ACE or in pepper cv. California Wonder.

Females of each population were extracted from the roots and used for identification. Protein extracts were obtained from females and the electrophoretic analysis was carried out using the Mini-Protean Tetra cell system (Bio-Rad) according to Esbenshade and Triantaphyllou (1985ab) and Pais *et al.* (1986), with some modifications. The origins and species of these eight *Meloidogyne* populations are presented in Table 2. In all cases the same species per population was identified in both the susceptible tomato and resistant tomato or pepper, indicating that selection in these plants did not alter the constitution of the initial nematode population.

The reproductive ability of all the original nematode populations, which were maintained either on the susceptible tomato cv. ACE or the susceptible pepper cv. California Wonder for at least four generations, was studied on the resistant tomato cv. Silvana and on the susceptible pepper cv. California Wonder in a pot experiment. Egg masses were left to hatch in extraction dishes and plants of both tomato cvs and pepper grown in 250 ml pots were inoculated with 400 second stage juveniles (J_{2s}) with five replicates per treatment. In each test, the population I5 was used as control to prove the resistance of the tomato cv. Silvana and the non host status of pepper cv. California Wonder, towards the avirulent *M. javanica*. Plants were maintained at 16 h photoperiod and soil temperature 24-26°C. The number of egg masses on roots was assessed seven weeks after inoculation. Egg masses

(c. 5-10) were randomly collected from roots of each plant, transferred into an aqueous solution of sodium hypochoride to release eggs (Hussey and Barker, 1973) and checked under the stereoscope for the presence of eggs. The number of egg masses produced on susceptible tomato, resistant tomato and pepper were compared by ANOVA. The experiment was conducted once.

Results and Discussion

A review of 13 described resistance-breaking populations of RKN from Greece during the period 1994-2013 (Table 1) reveals the following characteristics:

1. From 1994 until 2013, 13 RKN populations, from which 11 *M. javanica* and two *M. incognita*, able to reproduce on resistant tomato, have been recorded in five different regions of Greece: Crete, Epirus, Thrace, Peloponissos and Macedonia.
2. Four populations, three from Crete (*M. javanica* and *M. incognita*) and one from Peloponissos (*M. javanica*), collected from heavily infected roots of nematode resistant tomato, reproduced at high rates on resistant tomatoes in pot tests (Tzortzakakis *et al*., 1999, 2005, 2008).
3. Seven populations, two from Crete (*M. javanica*), four from Epirus (*M. javanica*) and one from Macedonia (*M. incognita*), collected from nematode susceptible crops with no recent history of resistant tomato cultivation in the field sites, reproduced at high rates on resistant tomatoes in pot tests (Tzortzakakis *et al*., 1999, 2005, 2014).
4. Two populations, one from Thrace (*M. arenaria* collected from balm) and one from Crete (*M. incognita* collected from susceptible tomato), were unable to reproduce on resistant tomato in pot tests, when six egg masses were inoculated per plant. However, when resistant tomatoes were inoculated with 30 egg masses per plant, a virulent *M. javanica* was revealed in both cases, composing a minor percentage in the original population which was undetected in the identification process (Tzortzakakis *et al*., 2008).
5. The only two resistance-breaking populations of *M. incognita* found in Greece,

Table 1. Root-knot nematode populations (*Meloidogyne* spp.) from Greece, virulent on resistant tomato hybrids with the *Mi* gene, reported within the period 1994-2013.

Code	Nematode species	Region	Host plant found	References
1HVa and HVb , 4/1 and 4/2[1]	*M. javanic*a	Crete	Resistant tomato	Tzortzakakis and Gowen, 1996; Tzortzakakis *et al*., 1999
16, 17	*M. javanica*	Crete	Susceptible tomato	Tzortzakakis *et al*., 1999
*Mj*P1, *Mj*P2, *Mj*P3, *Mj*P4	*M. javanica*	Epirus	Susceptible tomato, cucumber	Tzortzakakis *et al*., 2005
*Mj*C1	*M. javanica*	Crete	Resistant tomato	Tzortzakakis *et al*., 2005
*Mi*C1	*M. incognita*	Crete	Resistant tomato	Tzortzakakis *et al*., 2005; Tzortzakakis and Blok, 2007
T	*M. javanica*[2]	Thrace	Balm	Tzortzakakis *et al*., 2008
C	*M. javanica*[3]	Crete	Susceptible tomato	Tzortzakakis *et al*., 2008
P	*M. javanica*	Peloponissos	Resistant tomato	Tzortzakakis *et al*., 2008
*Mi*NG	*M. incognita*	Macedonia	Beet	Tzortzakakis *et al*., 2014

[1] The lines 4/1 and 4/2 are the same with lines 1HVa and 1HVb (single egg mass lines from the same nematode population). In the reference Tzortzakakis *et al*., 1999 they were characterized by molecular and biochemical methods.
[2] The original population identified as *M. arenaria*, which was avirulent and the virulent *M. javanica* consisted a minor component selected by resistant tomato.
[3] The original population identified as *M. incognita*, which was avirulent and the virulent *M. javanica* consisted a minor component selected by resistant tomato.

one in Crete and the other in Macedonia, differed in their ability to reproduce on susceptible pepper cultivars (Tzortzakakis and Blok, 2007; Tzortzakakis *et al*., 2014).

In all the above studies referred, small scale surveys had been conducted in two cases: a) in Crete, in a random sampling on 37 greenhouses representative of the main vegetable growing areas, where two resistance-breaking populations of *M. javanica* were found (Tzortzakakis *et al*., 1999) and b) in Preveza Epirus, a random sampling on ten greenhouses, where the presence of four resistance-breaking populations of *M. javanica* was detected (Tzortzakakis *et al*., 2005). The remaining records came from samples which had been sent to the laboratory for identification.

The results of the current study on RKN populations collected in 2013-2014 are presented in Table 2. There was no infection in the resistant tomato cv. Silvana inoculated with the I5 and K populations, which prove that the *Mi* gene was effective under the certain experimental conditions. The population I5, identified as *M. javanica*, did not reproduce on resistant tomato and pepper. The population K, identified as *M. incognita*, reproduced on pepper at lower rate than on susceptible tomato but not on resistant tomato.

Six populations were found to reproduce on resistant tomato at a level which did not differ significantly to that obtained on the susceptible tomato. Furthermore, their ability to reproduce on resistant tomato was stable as they sustained consistent reproduction on resistant tomato for at least four successive generations. The egg masses which were randomly collected from roots of resistant tomato and pepper all contained a sufficient (>100) number of eggs. From the resistance-breaking populations, four were *M. javanica* which did not reproduce on pepper while from the two virulent *M. incognita*, the I4 reproduced on pepper, at lower rate than on tomato, while the I3 did not.

In the 20 tested RKN populations, the resistance-breaking ones were found at a quite high percentage (30%) compared with that of the survey done 18 years earlier (Tzortzakakis *et al*., 1999), in which that percentage was 5% in 37 samples collected from several areas of Crete. The population which was identified as *M. incognita* (I3), able to reproduce on resistant tomato but not on pepper, is similar to another population of *M. incognita* found earlier in another area of Crete (*Mi*C1 in Tzortzakakis and Blok, 2007). However, the *M. incognita* (I4), reproducing on both the resistant tomato and pepper, is reported for the first time in Crete and for

Table 2. Number of egg masses produced by eight populations of root-knot nematodes (*Meloidogyne* spp.) collected from greenhouses of Crete on susceptible tomato, resistant tomato and susceptible pepper.

Code	Origin	Species	No of egg masses				
			Susceptible tomato cv. ACE	Resistant tomato cv. Silvana	Susceptible pepper cv. California Wonder	SED	P value
I1	Tomato	*M. javanica*	56	44	0[1]	7.9	>0.05
I2	Tomato	*M. javanica*	45	38	0	6.6	>0.05
I3	Tomato	*M. incognita*	44	41	0	7.7	>0.05
I4	Pepper	*M. incognita*	41	44	22	6.5	<0.05
I5	Cucumber	*M. javanica*	42	0[1]	0	-	-
S	Tomato	*M. javanica*	38	32	0	5	>0.05
V	Tomato	*M. javanica*	39	36	0	6.7	>0.05
K	Pepper	*M. incognita*	45	0	29	3.8	<0.01

Origins: I1-I5 Ierapetra, S: Skourvoula Messara, V: Vori Messara, K: Kisamos Chania; Average of five replicates per treatment; [1] In case of 0 values the data were excluded from analysis; SED and P values from ANOVA.

the second time in Greece (Tzortzakakis *et al*., 2014). It is important to notice that in Ierapetra which is the most important area of greenhouse vegetable production in Crete, four out of the eight tested RKN populations were resistance-breaking populations. These results are in contrast with the study conducted 18 years ago (Tzortzakakis *et al*., 1999), where no resistance-breaking populations were found in this area.

Since commercial nurseries provide nematode-free tomato seedlings, a possible explanation for such a 'rise' in the percentage of resistance-breaking populations, could be a 'potential selection' by the increased cultivation of resistant to RKN tomato hybrids. However, in the fields where the pathotypes were found, there was no recent history of cultivation of resistant tomatoes. Furthermore, previous studies in pots indicated that single egg mass lines of *M. javanica* did not have the capacity of adapting to resistant tomatoes (Tzortzakakis *et al*., 1999). Thus it is difficult to provide an explanation for the increase in the percentage of the resistance-breaking RKN populations within the last 18 years period.

In the Mediterranean area, resistance-breaking populations of *M. javanica* and *M. incognita* have been reported in several countries e.g. Cyprus, France, Italy, Morocco, Spain, Tunisia and Turkey, with the majority of them being *M. javanica* (Castagnone-Sereno *et al*., 1994; Devran and Sogut, 2010; Eddaoudi *et al*., 1997; Molinari and Miacola 1997; Ornat *et al*., 2001; Philis and Vakis, 1977; Robertson *et al*., 2006). The results presented herein, constitute additional records on the occurrence of virulent *M. javanica* and *M. incognita* populations in the area of Ierapetra, Crete.

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Επικαιροποίηση της παρουσίας παθοτύπων των νηματωδών του γένους *Meloidogyne* σε ανθεκτική τομάτα στην Ελλάδα με έξι νέες καταγραφές από την Κρήτη

Ε.Α. Τζωρτζακάκης, M.-C. Vieira dos Santos και I. Conceição

Περίληψη Στην παρούσα εργασία γίνεται μία επικαιροποίηση των δημοσιευμένων πληροφοριών που αφορούν στην παρουσία νηματωδών του γένους *Meloidogyne* σε ανθεκτική τομάτα στην Ελλάδα. Την περίοδο 1994-2013, 13 πληθυσμοί (11 *M. javanica* και δύο *M. incognita*) με ικανότητα αναπαραγωγής σε ανθεκτική τομάτα καταγράφηκαν στις περιφέρειες της Κρήτης, Ηπείρου, Θράκης, Πελοποννήσου και Μακεδονίας. Έξι επιπλέον παθότυποι, τέσσερις *M. javanica* και δύο *M. incognita,* επισημάνθηκαν σε θερμοκηπιακές καλλιέργειες κηπευτικών στην Κρήτη, την περίοδο 2013-2014. Τέσσερις από τους παθότυπους, δύο *M. javanica* και δύο *M. incognita,* επισημάνθηκαν για πρώτη φορά στην περιοχή της Ιεράπετρας που αποτελεί τη σημαντικότερη περιοχή καλλιέργειας κηπευτικών σε θερμοκήπια στην Κρήτη.

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Insecticidal effect of *Fusarium subglutinans* on *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae)

O. Demirözer*, A. Uzun, Ş.-E. Arici, İ. Gep and R. Bakay

Summary *Fusarium subglutinans* (Ascomycota: Nectriaceae) is known to have lethal effects on aphid species, while there are limited studies associated with other arthropods. In this study, the effect of different spore concentrations (1x10^4, 1x10^6 and 1x10^8 spores/ml) of *F. subglutinans* 12A, isolated from *Aphis gossypii* in Adana-Karataş (Turkey), was investigated on *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) females and on 2nd instar nymphs (only 1x10^6 spores/ml). The application method was by dipping and observations on mortality of females were conducted 24, 48, 72, 96 hours and 7 and 9 days after application. Mycosis was also observed on dead individulas. Mortality of nymphs was recorded during 8 days after application. Higher average dead females were found in the treatments compared to the control, but there was not significant difference between the tested concentrations (Mycosis rate recorded in 1x10^6 spores/ml was higher than those in 1x10^4 and 1x10^8 spores/ml). The highest and lowest mycosis rates were observed on the 7th and 3rd day, respectively. Average number of dead 2nd instar nymphs recorded in 1x10^6 spores/ml did not differ from control.

Additional keywords: Biological control, entomopathogenic fungi, pest, thrips

Introduction

The Western Flower Thrips (WFT) *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is a serious pest feeding on leaves, fruits and flowers and causing direct and indirect damages on agricultural crops and ornamental plants (Bryan and Smith, 1956; Miliczky and Horton, 2011; Demirözer *et al*., 2012). Due to significant number of thrips vectors for viral pathogens, they are known as destructive pests worldwide.

The WFT spread to the World from North-West of the United States (Kirk and Terry, 2002). The first presence of WFT in Turkey was recorded in vegetable fields of Antalya (Western Mediterranean region) in 1993 (Tunç and Göçmen, 1994) and in a very short time it suppressed the *Frankliniella intonsa* (Trybom) which was the main thrips species in cotton fields in Çukurova region (East Mediterranean) (Atakan *et al*., 1998; Atakan and Özgür, 1998; 2000; Atakan, 2003; Doğanlar and Aydin, 2009).

Süleyman Demirel University, Faculty of Agriculture, Department of Plant Protection 32260 Isparta-Turkey
* Corresponding author: ozandemirozer@sdu.edu.tr

Short generation period, high reproductive capacity and thigmotactic behaviour of *F. occidentalis* are reasons that make it difficult to control. In addition, rapid resistance development ability against insecticides also contributes to the difficulty in the control of *F. occidentalis*. The WFT is known to be resistant to carbamates (bendiocarb, formetanate, methiocarb), organophosphates (diazinon), spinosyn (spinosad) and pyrethroids (acrinathrin, deltamethrin, fenvalerate, permethrin) (Jensen, 2000; Bielza, 2008; Cloyd, 2009).

Besides the difficulties in the suppression of thrips populations, chemical insecticides are known to have side effects on the natural enemies of the WFT (Goettel and Hajek, 2000; Pell *et al*., 2001; Jones *et al*., 2005; Demirözer *et al*., 2012). Since entomopathogens are specific to their hosts and they reproduce they are a desirable alternative in pest control (Charnley and Collins, 2007). Additionally, low risk on the non-target organisims supports safeuse of entomopathogens in control practices (Eilenberg *et al*., 2001; Augustyniuk-Kram & Kram, 2012; Shadid *et al*., 2012).

There are 750 known species of entomopathogenic fungi, which belong to 85 ge-

nus of the Plyla Ascomycota and Zygomycota (Samson *et al*., 1988; McCoy *et al*., 1988; Gillespie and Moorhouse, 1989). A considerable number of these species belong to the genera *Beauveria, Entomophthora, Metarhizium, Neozygites, Nomuraea* and *Lecanicillium* (Desphande, 1999; Shadid *et al*., 2012). Although *Fusarium* spp. (Ascomycota: Nectriaceae) cause diseases in a number of economically important plants, the species *Fusarium subglutinans* isolated from aphids has entomopathogenic action on arthropods (Gerin, 1998; Erkiliç *et al*., 1999; Satar *et al*., 2000).

The aim of this study was to determine the insecticidal effect of three different spore concentrations ($1x10^4$, $1x10^6$ and $1x10^8$ spores/ml) of *F. subglutinans* 12A on adult females of *F. occidentalis.* The mycosis rate was recorded on dead individuals of the thrips. Additionally, the effect of $1x10^6$ spores/ml concentration was investigated on the 2nd instar nymphs of *F. occidentalis*.

Materials and Methods

The study plant was pepper (*Capsicum annuum*). Adult females and 2nd instar nymphs of *F. occidentalis* used in the experiments came from laboratory colonies kept at 25±1°C, 60-70% RH and 16:8 h L:D. The isolate 12A of *F. subglutinans* from *Aphis gossypii* in Adana-Karataş, Turkey was used for making suspensions of the fungus.

In the study, spore concentrations of $1x10^4$, $1x10^6$ and $1x10^8$ spores/ml were prepared by using suspension of *F. subglutinans* 12A; they were cultured on potato dextrose agar (PDA) and incubated at 25°C for 10 days. Spore concentrations were determined by using Thoma counting chamber. In the control, distilled water and Tween 20 (0.1 %) was used.

The experiment was conducted in glass Petri dishes (9 cm diameter) containing pepper leaf discs (5 cm diameter) on filter papers, to feed the thrips. In each treatment 10 individuals, newly emerged adult females or 2nd instar nymphs, were used. Before the treatment, thrips were deprived food for 1 hour. The application method was by dipping for 5 seconds. After treatment the thrips were transferred by using a moisturized fine paint brush to the Petri dishes, which were then covered with parafilm to prevent their possible escape. The experimental design was a complete randomized block with five replications. The Petri dishes were kept in climate-controlled rooms at 25±1°C, 60-70% RH, and 16:8 h D:L.

Observations on mortality were made at 24, 48, 72 and 96 hours, and 7 and 9 days after the dipping. Mycosis observations were performed between the third and ninth day of the study. Counting on the 2nd instar nymphs was initiated 24 hours after the dipping and repeated every 24 hours until the 8th day of the experiment. Re-isolation was made at the end of the counting process on dead individuals.

Statistical Analysis

Square root transformation was applied to the data of dead individuals. Inverse angle transformation was applied to the mycosis data obtained from dead flesh (body) of adults. Data were analysed using repeated measurement analysis of variance in a factorial design (treatment x time). Linear relation between dead individuals and mycosis rates was investigated by calculating the Pearson correlation coefficient. The Mann-Whitney 'U' test was applied to the data obtained from 2nd instar nymphs of *F. occidentalis*, since the data were non-parametric. Significance level was $P< 0.05$.

Results

The mean numbers of dead females of *F. occidentallis* after dipping in solutions of three different spore concentrations of *F. subglutinans* 12A are presented in Table 1. The control had the lowest mean number of dead females, which was significantly different from the other treatments ($P<0.05$). The mean

Table 1. Mean mortality of adult females of *Frankliniella occidentalis* and mycosis rate on dead individuals after treatment with three different spore concentrations of *Fusarium subglutinans* 12A.

Treatments (spores/ml)	Mortality Mean ± s.e.	Mycosis rate Mean ± s.e.
10^4	3.67 ± 0.756 a	0.989 ± 0.230 b
10^6	3.47 ± 0.724 a	1.794 ± 0.351 a
10^8	4.20 ± 0.732 a	1.455 ± 0.262 ab
Control	0.70 ± 0.180 b	

Means with different letter in the same column differ significantly (*P <0.05*); s.e.: standard error

mortality obtained in three spore concentrations did not differ significantly between them (P>0.05).

In the concentrations 10^6 and 10^8 spores/ml, first deaths of adults were observed 24 hours after dipping, whereas this was 48 hours in 10^4 spores/ml (Figure 1). In the control, deaths were observed three days after dipping into the water and the mortality percentage was 16% on the 8th day of the experiment. Mortality percentage was 100% in the three spore concentrations of *F. subglutinans* 12A on the 9th day of the experiment (Figure 1).

The highest mycosis rate was recorded in 10^6 spores/ml (1.794 ± 0.351) and was higher than the average mycosis rates of 10^8 and 10^4 spores/ml concentrations (P<0.05; Table 1). The Pearson correlation coefficient between the death and mycosis rates was 0.68 and it was found significant (P<0.01). In addition, a linear relationship was determined between the death and mycosis rates and mycosis became visible three days after the application (Table 1). The highest mycosis rates were observed on the 7th and 9th day after application and the lowest rate was recorded three days after application in all spore concentrations. The Pearson correlation coefficient between the time and mycosis rate was 0.93 and it was significant (P<0.01).

Deaths of 2nd instar nymphs at 10^6 spores/ml concentration of the fungus were observed starting on the second day of spore applications. The results showed that percentage of mortality was more than 50% on the 8th day of the study (Figure 2). The 'R' value of average dead individuals was 0.412 and it was different from the control (P<0.01).

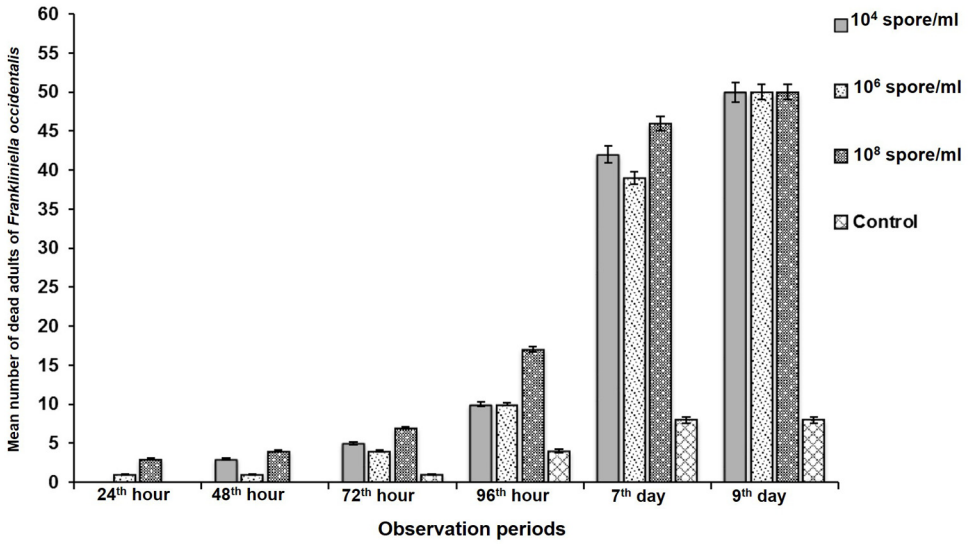

Figure 1. Mean number of dead adult females of *Frankliniella occidentalis* after treatment (dipping) with three different spore concentrations of *Fusarium subglutinans*.

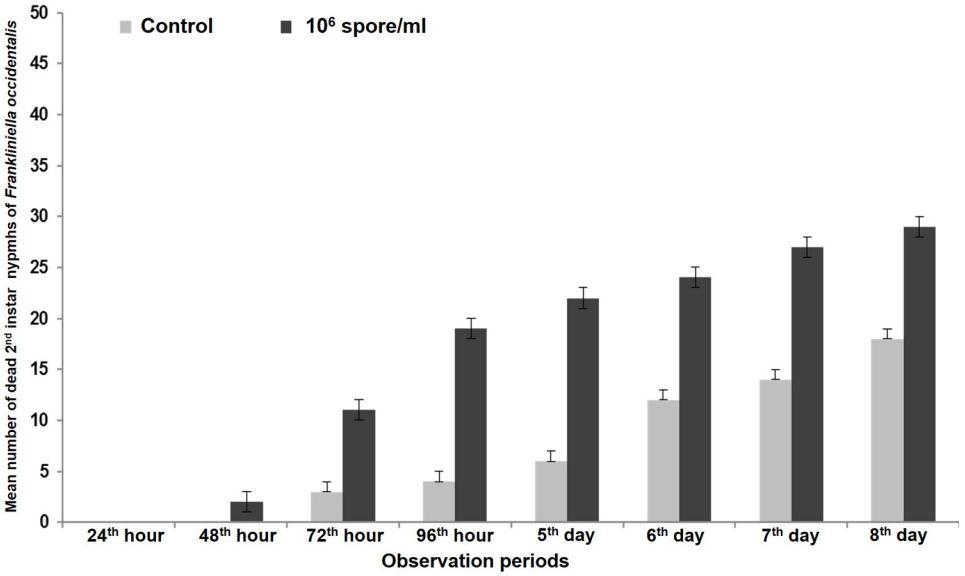

Figure 2. Mean number of 2nd instar nymphs of *Frankliniella occidentalis* after treatment (dipping) with 1x10^6 spores/ml concentration of *Fusarium subglutinans* 12A.

Discussion

Previous studies focused on efficacy of *F. subglutinans* on several aphid species (Erkiliç *et al*., 1999; Satar *et al*., 2000; Satar and Koç, 2004; Arici *et al*., 2012). Satar *et al*. (2000) reported that 1x10^7 spores/ml concentration of *F. subglutinans* caused 16% and 45.5% deaths on *A. gossypii* (Hemiptera: Aphididae) which fed on cotton and eggplant, respectively, and 12.9% on *Myzus persicae* (Hemiptera: Aphididae) which fed on eggplant.

In the present study, 1x10^4-1x10^8 spores/ml concentrations of *F. subglutinans* 12A had similar efficacy (in terms of mortality) against the thrips *F. occidentallis* but the 1x10^6 spores/ml concentration was found appropriate for mycosis. Lethal effect of *F. subglutinans* could be expected to vary on different host plants, pest species and different life stages of pests. The mortality rate of the 10^6 spores/ml concentration of *F. subglutinans* 12A was 58% on the 2nd instar nymphs of the thrips. The concentration 10^6 spores/ml of *F. subglutinans* was the most effective on *A. gossypii* and *A. fabae* in studies by Satar and Koç (2004) and; Arici *et al*. (2012).

Other fungi recorded to have biocidal effect on *F. occidentalis* include *Beauveria bassiana*, *Lecanicillium* (*Verticilium*) *lecanii* (Ascomycota: Cordycipitaceae) and *Metarhizium anisopliae* (Clavicipitaceae) (Jacobson *et al*., 2001; Ludwig and Oetting, 2002; Maniania *et al*., 2002). Whereas recorded mortality rates were 20-70 % for *L. lecanii*, these were 93.5-100% for *M. anisopliae* on different life stages of *F. occidentalis* (Vestergaard *et al*., 1995; Gouli *et al*., 2009). Several efficacy studies of different spore concentrations of *B. bassiana* showed that this fungus caused 67-96% deaths on pre adult stages of *F. occidentalis*. In addition 1x10^7 conidia/ml was the most effective spore concentration in several other studies Gouli *et al*., 2009; Gao *et al*., 2012; Wu *et al*., 2014), However these studies have no data on mycosis rates of the pest.

In conclusion, *F. subglutinans* 12A had a lethal effect on *F. occidentalis* and the fungus was found more effective on adults than the 2nd instar nymphs. Developing hyphae and spores of entomopathogenic fungi provide

infection to other individulas in the pest population (Moutia, 1936; Shahid *et al*., 2012). Present study results suggest that three days are required for mycosis to become visible from the application and seven days later mycosis rate reaches the highest level. Based on the results obtained from this study it is recommended future studies to focus on *F. subglutinans* 12A mode of action on arthropods, infection features and side effects of this fungus on natural enemies.

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Εντομοκτόνος δράση του μύκητα *Fusarium subglutinans* στο θρίπα της Καλιφόρνιας, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae)

O. Demirözer, A. Uzun, Ş.-E. Arici, İ. Gep and R. Bakay

Περίληψη Ο μύκητας *Fusarium subglutinans* (Ascomycota: Nectriaceae) είναι γνωστό ότι έχει θανατηφόρο δράση σε είδη αφίδων, ενώ υπάρχουν περιορισμένες μελέτες που σχετίζονται με άλλα αρθρόποδα. Σε αυτή τη μελέτη, εξετάστηκε η επίδραση διαφορετικών συγκεντρώσεων σπορίων ($1x10^4$, $1x10^6$, και $1x10^8$ σπόρια/ml) του 12A *Fusarium subglutinans*, το οποίο είχε απομονωθεί από την αφίδα *Aphis gossypii* στα Adana-Karataş (Τουρκία), στο θρίπα *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), σε ενήλικα θηλυκά άτομα και σε νύμφες δεύτερης ηλικίας (μόνο $x10^6$ σπόρια/ml). Η μέθοδος εφαρμογής ήταν με εμβάπτιση και παρατηρήσεις σχετικά με τη θνησιμότητα των θηλυκών ατόμων διεξήχθησαν 24, 48, 72, 96 ώρες και 7 και 9 ημέρες μετά την εφαρμογή. Επίσης παρατηρήθηκε το φαινόμενο της μύκωσης σε νεκρά άτομα. Η θνησιμότητα των νυμφών καταγράφηκε κατά τη διάρκεια 8 ημερών από την εφαρμογή. Κατά μέσο όρο, περισσότερα θηλυκά άτομα βρέθηκαν νεκρά στις επεμβάσεις με το μύκητα σε σχέση με το μάρτυρα, αλλά δεν υπήρχε σημαντική διαφορά μεταξύ των συγκεντρώσεων που δοκιμάστηκαν (καταγράφηκε υψηλότερος ρυθμός μύκωσης στη συγκέντρωση $1x10^6$ σπόρια/ml από εκείνους στις συγκεντρώσεις $1x10^4$ και $1x10^8$ σπόρια/ml). Τα υψηλότερα και τα χαμηλότερα ποσοστά μύκωσης παρατηρήθηκαν την $7^η$ και $3^η$ ημέρα, αντίστοιχα. Ο μέσος αριθμός νεκρών νυμφών δεύτερης ηλικίας που καταγράφηκε στη συγκέντρωση $1x10^6$ σπόρια/ml δε διέφερε από το μάρτυρα.

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SHORT COMMUNICATION

Description of the first-stage juveniles of *Xiphinema cretense* and *X. herakliense* - Distribution of *Xiphinema* and *Longidorus* species in olive orchards and grapevines in Crete, Greece

E.A. Tzortzakakis[1*], A. Archidona-Yuste[2], C. Cantalapiedra-Navarrete[2], I.G. Birmpilis[1], E. Nasiou[1], J.E. Palomares-Rius[2] and P. Castillo[2]

Summary The occurrence of nematodes of the family Longidoridae was investigated in soil samples collected from cultivated and wild olives and grapevines in Crete. The first-stage juveniles of *Xiphinema cretense* and *X. herakliense* are described for the first time. The species *X. israeliae*, *X. cretense*, *X. herakliense* and *Longidorus pseudoelongatus*, previously recorded exclusively from olives in Crete, are herein reported in the rhizosphere of grapevines. Also *L. iranicus* is reported for the first time in cultivated olive, while *X. italiae* and *L. closelongatus* are reported for the first time in wild olive in Crete. Data on the occurrence of phytoparasitic nematode species in cultivated olives, wild olives and grapevines are updated with those previously published.

Additional keywords: *Longidorus closelongatus, L. cretensis, L. iranicus*, *L. pseudoelongatus, X. israeliae*

Olive tree and grapevine are the most important crops on the island of Crete, occupying 177,000 and 25,500 hectares, respectively. These represent about 22% for olive trees and 20% for grapevines of the total corresponding cultivated areas in Greece. In addition, wild olive trees are also located in some south coastal areas of the island.

Dagger and needle nematodes of the genera *Xiphinema* and *Longidorus,* respectively, include a number of large plant ectoparasitic nematode species with long life cycles. They cause damage to a wide range of fruit and vegetable crops as well as wild plants by their direct feeding on root cells and transmission of nepoviruses (Decraemer and Robbins, 2007; Taylor and Brown, 1997).

The presence of *Xiphinema* and *Longidorus* nematodes on cultivated and wild olive and grapevines in Crete was investigated by Tzortzakakis *et al*. (2014, 2015). The soil samples were collected from:

a) 101 olive orchards in Heraklion and Lasithi provinces,
b) 22 individual wild olive trees in Heraklion province and
c) 30 vineyards in Heraklion province and assigned to the Nematology Laboratory (affiliation of the 1st author) by farmers for nematode diagnosis.

Five known *Xiphinema* species (viz. *X. index*, *X. israeliae*, *X. italiae*, *X. pachtaicum* and *X. simile*), five known *Longidorus* species (viz. *L. closelongatus*, *L. cretensis, L. iranicus* (synonym of *L. moesicus*), *L. orientalis,* and *L. pseudoelongatus*) and two newly described (for the first time) *Xiphinema* species (viz. *X. cretense* and *X. herakliense*) were found.

The current work presents supplementary data on the occurrence of *Xiphinema* and *Longidorus* nematodes on olive trees and grapevines in Crete based on additional soil samples, which were collected from:

a) the topotype locality (cultivated olive) of

[1] Department of Viticulture, Vegetable Crops, Floriculture and Plant Protection, Institute of Olive Tree, Subtropical Crops and Viticulture, N.AG.RE.F., Hellenic Agricultural Organization-DEMETER, PO BOX 2228, 71003 Heraklion, Crete, Greece
[2] Institute for Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Avenida Menéndez Pidal s/n, 14004 Córdoba, Campus de Excelencia Internacional Agroalimentario, ceiA3, Spain
***** Corresponding author: etzortza@her.forthnet.gr

X. cretense (Tzortzakakis *et al*., 2014) and an olive orchard where *X. herakliense* had been found (Tzortzakakis *et al*., 2015), aiming at the detection of first-stage juveniles (J_1) which could not be detected after repeated samplings at the original description of these species. This life-stage may have a practical significance when distinguishing species closely related (Hunt, 1995),

b) 45 olive orchards in Heraklion, Rethymno and Lasithi provinces and 14 individual wild olive trees in Heraklion province, different to the ones in Tzortzakakis *et al*. (2014, 2015) study and

c) 100 vineyards in Heraklion and Chania provinces and assigned to the Nematology Laboratory (affiliation of the 1st author) by farmers for nematode diagnosis.

Soil sampling (for olive trees), nematode extraction, fixing and identification protocols were carried out as by Tzortzakakis *et al*. (2014, 2015).

Description of J_1s of *Xiphinema cretense* and *Xiphinema herakliense*

In both species, J_1s were positively identified by the position of the replacement odontostyle, which lies mostly within the odontophore, with the anterior tip near the base of the functional odontostyle (Figures 1, 2) (Hunt, 1995).

Xiphinema cretense (J_1)

Measurements (n = 5): L = 1103 ± 24.4 (1083-1128) µm; a = 51.7 ± 1.8 (50.4-53.7); b = 4.3 ± 0.3 (4.0-4.6); c = 22.9 ± 1.3 (22.1-24.4); c′ = 3.2 ± 0.1 (3.1-3.3); odontostyle length = 55.3 ± 0.5 (56.0-57.0) µm; replacement odontostyle length = 73.2 ± 1.2 (73.0-75.0) µm; odontophore length = 41.9 ± 1.0 (41.0-43.0) µm; lip region width = 7.7 ± 0.5 (7.5-8.5) µm; oral aperture-guiding ring distance = 38.7 ± 4.0 (34.0-41.5) µm; tail length = 48.6 ± 2.5 (46.0-51.0) µm; hyaline region at tail tip = 11.5 ± 0.6 (11.0-12.0) µm.

Description: Morphologically similar to adult specimens described by Tzortzakakis *et al*. (2014), apart from developed reproductive system, shorter body length, tail shape and presence of replacement odontostyle (Figure 1A). Anterior part characterized by position of replacement odontostyle just posterior to functional odontostyle, its tip touching or very close to base of functional odontostyle (Figure 1B). Bluntly conoid tail shape well curved dorso-ventrally with a slight dorsal depression at hyaline region level (Figures 1C-E).

Xiphinema herakliense (J_1)

Measurements (n = 1): L = 1183 µm; a = 42.3; b = 5.0; c = 27.5; c′ = 3.0; odontostyle length = 63.0 µm; replacement odontostyle length = 81.5 µm; odontophore length = 39.0 µm; lip region width = 8.0 µm; oral aperture-guiding ring distance = 43.0 µm; tail length = 43.0 µm; hyaline region at tail tip = 20.0 µm.

Description: Only one specimen was found. General morphology agrees closely to adults specimens described by Tzortzakakis *et al*. (2015), except for its developed reproductive system, shorter body length in open C-shape, tail shape and presence of replacement odontostyle (Figure 2A). Anterior part characterized by position of replacement odontostyle into odontophore, just posterior to base of functional odontostyle (Figure 2B). Bluntly conoid tail shape well curved dorso-ventrally with a strong dorsal depression at hyaline region level (Figure 2C).

Distribution of *Xiphinema* and *Longidorus* species in cultivated olive, oleaster and grapevine

In the soil samples of 45 olive orchards, three *Xiphinema* (viz. *X. israeliae*, *X. italiae*, and *X. pachtaicum*) and two *Longidorus* (viz. *L. iranicus,* and *L. pseudoelongatus*) species were found. In three out of the 14 wild olive tree sampling points, two *Xiphinema* (viz. *X. herakliense* and *X. italiae*), and one *Longidorus* species (viz. *L. closelongatus*) were found.

In the grapevine samples, the data on the presence of *X. index*, *X. italiae* and *X. pachtaicum* were not considered in this study, as these are quite common nematode species found on grapevine in Crete. However, in 12 out of the 100 examined grapevine soil

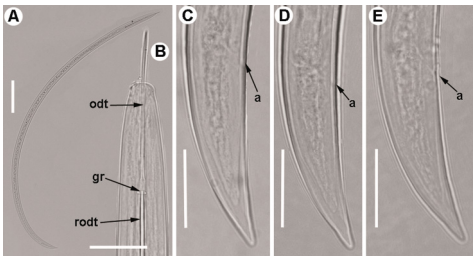

Figure 1. Light micrographs of first-stage juvenile of *Xiphinema cretensis* Tzortzakakis *et al.*, 2014. A) Whole body. B) Anterior region showing odontostyle (odt), replacement odontostyle (rodt), and guiding-ring (gr). C-E) Tail regions showing anus (a). (Scale bars: A = 100 µm; B-E = 20 µm).

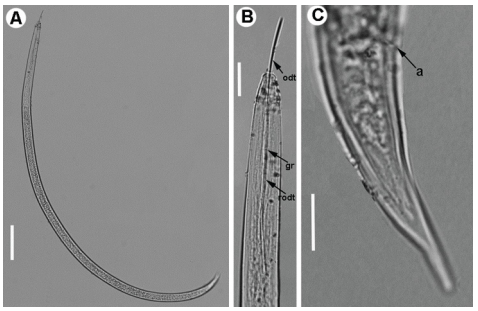

Figure 2. Light micrographs of first-stage juvenile of *Xiphinema herakliense* Tzortzakakis *et al.*, 2015. A) Whole body. B) Anterior region showing odontostyle (odt), replacement odontostyle (rodt), and guiding-ring (gr). C) Tail region showing anus (a). (Scale bars: A = 100 µm; B = 20 µm; C = 10 µm).

Table 1. Longidoridae species found in cultivated olive (OLI), wild olive (OLE) and grapevine (GRA) in Crete.

Nematode species	Plant, locality	Sample code
Longidorus closelongatus Stoyanov 1964	Grapevine, Xamoudochori	GRA36
Longidorus closelongatus	Grapevine, Ag Paraskies	GRA37
Longidorus closelongatus	Grapevine, Akrotiri	GRA39
Longidorus closelongatus	Wild olive, Agiofaraggo	OLE 34
Longidorus cretensis Tzortzakakis *et al*., 2001	Grapevine, P. Elias	GRA34
Longidorus iranicus Sturhan and Barooti, 1983	Olive, Arkalochori	OLI131
Longidorus iranicus	Grapevine, Archanes	GRA33
Longidorus iranicus	Grapevine, Ag. Thomas	GRA35
Longidorus iranicus	Grapevine, Roukani	GRA38
Longidorus iranicus	Grapevine, Lousakies	GRA31
Longidorus pseudoelongatus Altherr, 1976	Olive, Arkalochori	OLI132
Longidorus pseudoelongatus	Olive, Faneromeni	OLI136
Longidorus pseudoelongatus	Grapevine, Gazi	GRA 32
Longidorus pseudoelongatus	Grapevine, P. Elias	GRA40
Xiphinema cretense Tzortzakakis *et al*., 2014	Grapevine, P. Elias	GRA40
Xiphinema herakliense Tzortzakakis *et al*., 2015	Wild olive, Agiofaraggo	OLE 33, 34, 36
Xiphinema herakliense	Grapevine, P. Elias	GRA41
Xiphinema herakliense	Grapevine, P. Elias	GRA42
Xiphinema israeliae Luc *et al*., 1982	Olive, Roufas	OLI14
Xiphinema israeliae	Olive, Neapoli	OLI115
Xiphinema israeliae	Olive, Pyrgiotisa	OLI138
Xiphinema israeliae	Olive, Dermatos	OLI145
Xiphinema israeliae	Olive, Dermatos	OLI146
Xiphinema israeliae	Grapevine, Akrotiri	GRA39
Xiphinema italiae Meyl, 1953	Wild olive, Agiofaraggo	OLE 34
Xiphinema italiae	Olive, Episkopi	OLI 104
Xiphinema pachtaicum (Tulaganov, 1938) Kirjanova 1951	Olive, 12 samples	*

*Sample codes = 101, 102, 103, 105, 107, 110, 123, 129, 130, 137, 139, 140

samples, three *Xiphinema* (viz. *X. cretense, X. herakliense* and *X. israeliae*) and four *Longidorus* species (viz. *L. closelongatus*, *L. cretensis, L. iranicus,* and *L. pseudoelongatus*) were found. Although *L. moesicus* was previously reported on grapevine in Crete (Tzortzakakis *et al*., 2014), after recent studies (Maafi *et al*., 2015), this species has been synonymized with *Longidorus iranicus*. All these data are presented in Table 1, which supplements previously published results (Tzortzakakis *et al*., 2014, 2015) for cultivated and wild olive trees and grapevines from Crete. Thus the updated records, considering also the previous studies, on the percentage of occurrence for the detected nematode species is as follows:

a) In 146 soil samples from olive orchards: *L. closelongatus* 1.4%, *L. cretensis* 0.7%, *L. iranicus* 0.7%, *L. pseudoelongatus* 6.2%, *X. cretense* 2%, *X. herakliense* 0.7%, *X. index* 2% , *X. israeliae* 7.5%, *X. italiae* 7.5% and *X. pachtaicum* 39%,
b) In 36 soil samples from wild olive trees: *L. closelongatus* 2.8%, *X. herakliense* 36.1%, *X. israeliae* 2.8%, *X. italiae* 2.8% and. *X. pachtaicum* 5.6%,
c) In 130 samples from vineyards (excluding *X. index, X. italiae* and *X. pachtaicum*): *L. closelongatus* 5.4%, *L. cretensis* 2.3%, *L. iranicus* 6.2%, *L. orientalis* 0.8%, *L. pseudoelongatus* 1.5%, *X. cretense* 0.8%, *X. herakliense* 1.5%, *X. israeliae* 0.8% and *X. simile* 0.8%.

In conclusion, the data presented herein, indicate some new information for the presence of Longidoridae in Crete. *Xiphinema israeliae*, *X. cretense*, *X. herakliense* and *L. pseudoelongatus* were found on grapevine, while until now they had been found exclusively on olive trees; *L. iranicus* was found on cultivated olive trees, whereas until now it had been found only on grapevines; *X. italiae* and *L. closelongatus* are reported for first time on wild olive trees.

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Περιγραφή της προνύμφης πρώτου σταδίου των νηματωδών *Xiphinema cretense* και *X. herakliense*. Διασπορά των νηματωδών *Xiphinema* και *Longidorus* σε ελιές, αγριελιές και αμπέλια στην Κρήτη

Ε.Α. Τζωρτζακάκης, A. Archidona-Yuste, C. Cantalapiedra-Navarrete, Ι.Γ. Μπιρμπίλης, Ε. Νάσιου, J.E. Palomares-Rius and P. Castillo

Περίληψη Η παρουσία νηματωδών της οικογένειας Longidoridae διερευνήθηκε σε δείγματα εδάφους προερχόμενα από καλλιεργούμενες ελιές, αγριελιές και αμπέλια στην Κρήτη. Έγινε περιγραφή της προνύμφης πρώτου σταδίου των ειδών *Xiphinema cretense* και *X. herakliense*. Τα είδη *X. israeliae*, *X. cretense*, *X. herakliense* και *Longidorus pseudoelongatus*, τα οποία μέχρι τώρα είχαν αναφερθεί μόνο σε ελιά στην Κρήτη, βρέθηκαν στην ριζόσφαιρα αμπελιών. Επιπλέον, το είδος *L. iranicus* αναφέρεται για πρώτη φορά σε καλλιεργούμενη ελιά, ενώ τα είδη *X. italiae* και *L. closelongatus* αναφέρονται για πρώτη φορά σε αγριελιά στην Κρήτη. Η παρουσία φυτοπαρασιτικών ειδών νηματωδών σε καλλιεργούμενη ελιά, αγριελιά και αμπέλι συνοψίζεται λαμβάνοντας υπόψη προηγούμενες δημοσιεύσεις.

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Bioecology of *Nephopterygia austeritella* (Lep.: Pyralidae), a potential biological control agent of *Prosopis farcta* (Fabaceae) in central Iran

A. Mohammadi-Khoramabadi[1]*, H. Alipanah[2], S. Belokobylskij[3] and M.R. Nematollahi[4]

Summary *Prosopis farcta* (Fabaceae) is a native and common perennial weed plant in Iran. In search of environmental-friendly control methods against *P. farcta,* we discovered the seed feeder moth *Nephopterygia austeritella* (Lepidoptera; Pyralidae) in central Iran and studied its bioecology for the first time from 2008 through 2009. Infestation pattern, larval feeding behaviour, developmental period, seasonal occurrence and the adverse impact of the moth on the reproductive organs of *P. farcta* were investigated. Diagnostic morphological characters of the fifth larval instar of *N. austeritella* are provided. Two gregarious ectoparasitoids were reared and identified as *Apanteles subcamilla* and *Phanerotoma leucobasis* (Hymenoptera: Braconidae). Mortality rates of the larvae were 3.03 and 13.44% in 2008 and 2009, respectively. Larvae destroyed 29.6-38.4% of the pods of their host plants. The potential of *N. austeritella* as an efficient biological control agent in IPM programs against *P. farcta* is discussed.

Additional keywords: mesquite, impact, parasitoid, pest, seed, weed.

Introduction

Syrian mesquite, *Prosopis farcta* (Banks & Solander) (Fabaceae), is a perennial, thorny, xerophilous and salt-tolerant shrub which is widely spread from India to Algeria between latitudes *ca* 10° (in Yemen) and 50° in Kazakhstan (Bazzaz, 1973; Bisby *et al.,* 2011). *Prosopis farcta* is an economically multifaceted plant. It has been regarded as a useful plant for fixation of nitrogen and the production of nutrient-rich pods and foliage, especially in saline and arid environments and serves as a source of fodder in many countries (Said *et al*., 2002; Dogan *et al.,* 2004; Omidi *et al*., 2012). However, special biological attributes of *P. farcta* have increased the competitive and prevalent properties of this weed in orchards (e.g. olive and temperate-zone fruits) and fields (e.g. sesame and vegetables) (Johnson, 1983; Pasiecznik *et al.,* 2004; Sertkaya *et al.,* 2005; Qasem, 2007) such as its deep rhizobia-symbiont root system (Bazzaz, 1973; Canadell *et al.,* 1996; Atomov and Aktoklu, 2007; Fterich *et al.,* 2011) that propagates through both long-lived seeds and rhizome buds (Qasem, 2007). These features allow *P. farcta* to produce enormous and durable dense stands and quickly become a dominant weed in agroecosystems. *Prosopis farcta* is also a host of witches' broom disease which is the most destructive disease of alfalfa in Iran (Esmailzadeh-Hosseini *et al.,* 2011).

In order to control *P. farcta* and other species of the same genus in agricultural ecosystems, efforts were made to preserve the beneficial attributes of these plants while limitting their dispersal and competition with agricultural crops. To date, soil solarisation, mechanical methods and chemical control (Qasem, 2007) have failed to effectively control *P. farcta*. By contrast, biological agents can be used against *P. farcta* in an integrated management program (Johnson, 1983; Mc Kay and Gandolfo, 2007; Qasem,

[1] Department of Plant Production, College of Agriculture and Natural Resources of Darab, Shiraz University, Darab, Iran
[2] Department of Insect taxonomy, Iranian Research Institute of Plant Protection, Tehran, Iran
[3] Zoological Institute of Russian Academy of Sciences, St. Petersburg, Russia
[4] Department of Plant Protection, Isfahan Research Center for Agriculture and Natural Resources, P.O.Box 81785-199, Isfahan, Iran
* Corresponding author: Mohamadk@Shirazu.ac.ir

2007). Among the insects associated with *P. farcta*, the seed beetle *Caryedon angeri* Semenov (Coleoptera: Chrysomelidae) is reported as the most harmful agent attacking the reproductive organs of *P. farcta* in the Middle East (Johnson, 1983; Sertkaya *et al*., 2005). *Caryedon angeri* usually infests *Acacia* spp. and *P. farcta*, but there are concerns about its tendency to attack non-target species, such as groundnut (*Arachis hypogaea* L. (Fabaceae) (Bagheri-Zenous, 1992; Anton and Delobel, 2004). It is argued that *C. angeri* is not a good candidate for the biological control of the weed (Sertkaya *et al.,* 2005). Parasitoid braconid wasps attacking lepidopteran species of the families Lycaenidae, Geometridae and Gelechiidae that feed on *P. farcta* were recorded by Halperin (1986).

Recent observations on *P. farcta* shrubs in southern Iran revealed that the pyralid moth *Nephopterygia austeritella* Amsel (Lepidoptera: Pyralidae, Phycitinae) can feed on the pods of *P. farcta* (Alipanah *et al.,* 2012). *Nephopterygia austeritella* has been recorded from Sudan (Amsel, 1965) to the Canary Islands (Spain), Egypt (Asselbergs, 2009) and Iran (Alipanah *et al.,* 2012). This work was intended to study the bioecology of *N. austeritella* under natural conditions in central Iran and its negative impact on the reproductive organs of *P. farcta*.

Materials and methods

Infestation by the herbivore

The infestation of *P. farcta* by *N. austeritella* was studied in an abandoned orchard of approximately 10 hectares in Yazd County (31°89´ N, 54°36´ E, 1230 m a.s.l.), Yazd province, Iran. A map showing the study site is illustrated in Figure 1. Sampling was performed according to the *P. farcta* phenology, from May (early spring coinciding with leaf formation) to November (late autumn during leaf fall) at 10-15 day intervals, during 2008 and 2009. A random sample of 100 pods was made from branches of *P. farcta*. The sampled pods were then transferred to the laboratory where they were dissected with a sharp knife. The number of infested pods, larvae and externally parasitized larvae (as in Figure 2B-F) were recorded. The phenology of the host plant was recorded in each sampling date.

Infestation pattern

Infestation pattern of pods of *P. farcta* by *N. austeritella* was examined in a sample taken on 27 June 2009 from Yazd County area coinciding with the late emergence of adults (Figure 3). Fifty stems of *P. farcta* (one stem in each bush) were randomly selected and all pods (114), the number of infested pods and larva(e) within each pod (Figure 2B-C) were recorded. Infestation pattern of pods was calculated based on single or multiple larvae in each pod.

Description of larval instars

Larval instars were documented by determining the distance between the external extreme of the ocelli as the breadth of the head capsule of the larvae (Freitas, 1993). The body length of the larvae was measured from the anterior edge of the anteclypeus to the posterior edge of the anal plate. These data were used for determining the larval instars of *N. austeritella* using Dyar's rule. All measurements were made using a calibrated ocular micrometer of an Olympus stereomicroscope on 5-10 larvae collected from the study area of Yazd County in each sampling date. The larvae are described here for the first time. The fifth larval instar was described and illustrated in detail to distinguish it from other species of the family Pyralidae. Their mouthparts were dissected following the methods of Godfrey (1972), and the setal nomenclature follows that of Hasenfuss and Kristensen (2003).

Impact of *N. austeritella* on pods of *P. farcta*

Natural impact of *N. austeritella* on *P. farcta* pods was evaluated in March 2008 and 2009 (late winter) coinciding with the end of annual growing period of the plant at three areas in central Iran: Abarkouh coun-

Figure 1. Map of sampling areas of *Nephopterygia austeritella* in central Iran.

ty, Yazd province (31°12´ N, 53°28´ E, 1510 m a.s.l.), Darab county, Fars province (28°47´ N, 54°33´ E, 1100 m a.s.l.) and Yazd county, Yazd province, Iran (Figure 1). For this purpose, several bushes of *P. farcta* were randomly selected and all pods of a single branch were selected to provide a sample of 100 pods. There were six replicates (totally 600 pods) in each area. The pods were then dissected in the laboratory and the rate of damaged pods was calculated.

Natural enemies of *N. austeritella*

We inspected the infested pods of *P. farcta* for larval parasitoids of *N. austeritella* in the study area of Yazd County. Anaesthetized or with observable parasitoid larvae were transferred to the laboratory and kept in ventilated plastic rearing boxes. Adult parasitoids were collected and identified by the third author (Tobias *et al.,* 1986; Van Achterberg, 1990).

Results

General biology

The first adults of *N. austeritella* emerged in late May (Figures 2A and 3) and their appearance lasted until mid-June. This period was synchronized with the first flowering period of *P. farcta*, which began from early May, in the studied areas, when the formation of green fruits occurs. The females laid their eggs singly on the surface of the young green pods of the host plant (Figure 2B).

The first larval instar ate the egg chorion immediately after hatching and it then

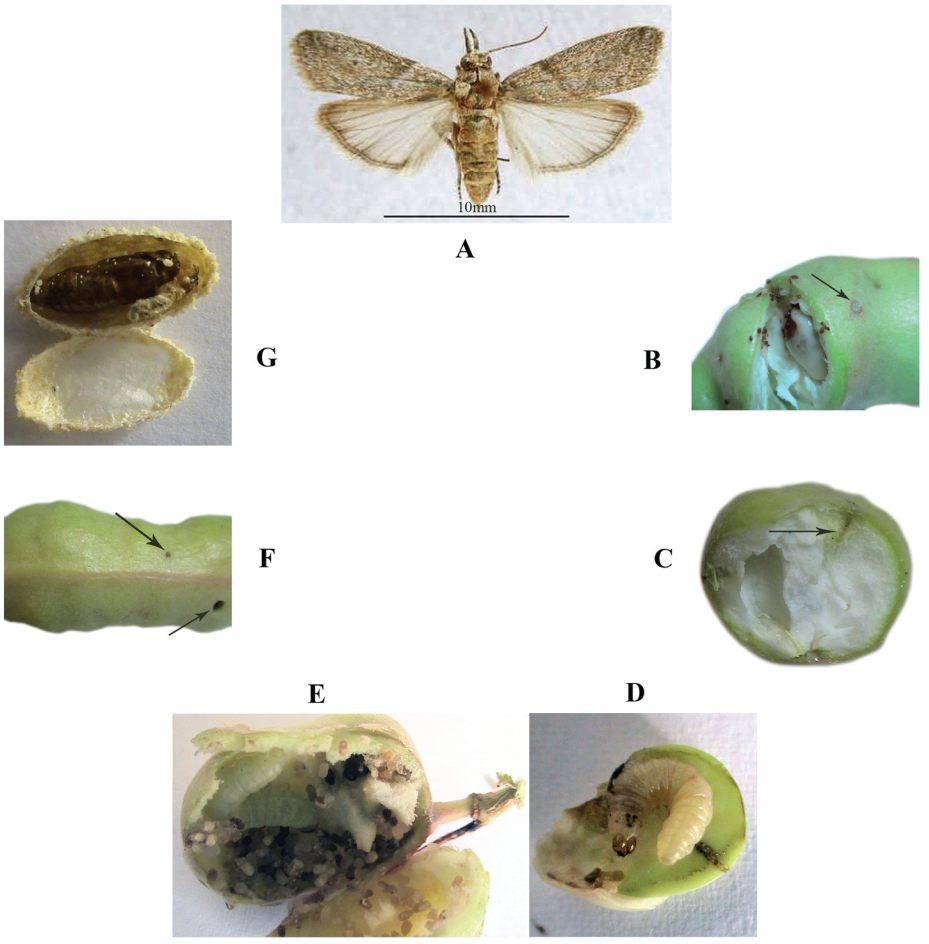

Figure 2. Life cycle of *Nephopterygia austeritella*. The adult female (A) lays its eggs on the surface of a green pod (B), the first instar larva moves to the seed in pod (C), the second instar larva feeds within the seed (D), the larva in the third and following instars completely destroys the mesocarp and seeds of the ripening pod (E), the larva makes a hole to exit from the destroyed pod (the above and below arrow indicates the position of egg and the exiting hole on the pod, respectively) (F), The last instar larva pupates within an oval silken cocoon (G).

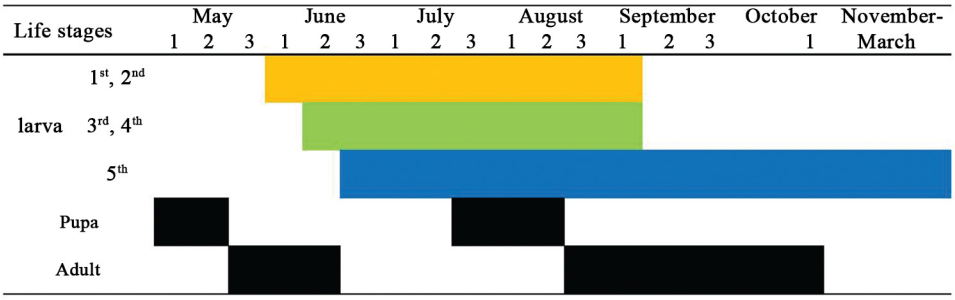

Figure 3. Phenology of *Nephopterygia austeritella* on the Syrian mesquite, *Prosopis farcta*, in central Iran.

penetrated into the pod just beneath the egg and entered the seed (Figure 2C) by chewing a tunnel through the cotyledon. The first and second instars fed on the seed (Figure 2D-E) and destroyed it completely. These two instars were observed from early June to early September (Figure 3). Third larval instar appeared in mid-June, when they left the remains of the seed to feed on the other seeds including mesocarp (internal tissues of the pod), leaving the outer shell intact (Figure 2E). To complete the larval stage, the larva had to exit the pod by making a hole (Figure 2F). After entering another pod, the larva sealed the entrance with silk fibres. Each pod usually contains one larva, although up to two larvae were rarely seen in the same pod. Movement of the larvae from one pod to another was facilitated by silk fibres. Pupation began from late June, when the last larval instar abandoned the damaged pod and descended on the surface of the soil (Figure 3). In the rearing boxes, the last larval instar spun silken cocoons 10-13 mm in length (Figure 2G), at different heights of the boxes. Adult moths emerged (at 30 ± 2° C, 20 ± 5% R. H.) about two weeks later. Emergence period was long and lasted from mid-August to mid-September in 2008 and from mid-August to early October in 2009 (Figure 3). This was the second emergence period, which indicates the second generation of *N. austeritella*.

Our sampling in late autumn and winter showed that no larvae existed in the pods of *P. farcta* at that time. The pods had very hard outer shells and could be hardly broken by larvae. Therefore, it may be that *N. austeritella* overwinters as a full-grown fifth larval instar within a cocoon outside the pod of *P. farcta*.

Infestation pattern

Counting the number of larvae (first or second instars) within a sample taken on 27 June 2009 showed that 65.91, 20.45, 11.36 and 2.27% of pods had one to four larvae, respectively.

Description of the immature stages of *N. austeritella*

Based on the measurement of the head capsule, the insect has five larval instars (Table 1) as follows:

First larval instar

Pale yellow to creamy white, with light brownish head and prothoracic plate light orange posteriorly.

Second and third instars

Nearly in the same colour as the first one.

Fourth larval instar

Head, thoracic plate, body, thoracic legs, prolegs and anal plate of the same colour and pattern as in the fifth larval instar.

Fifth larval instar in detail

Colour: Head light brownish, with pale mottled pattern, a very short coronal suture and an ellipse of six ocelli. Ocellar area dark brown, forming dark lateral patch encompassing ocelli 1 to 5; lower part of gena close to antennal region with a dark brown patch; anteclypeal region, frontal and adfrontal sclerites pale brown; labrum dark brown, notch edged with dark brown to black; mandible light brown and edged with brown distally (Figure 4E); spinneret and labial palpi light brown; antennal segments cream (Figure 4F); body creamy yellow, integument gran-

Table 1. Measurement of head capsule width and body length of larvae of *Nephopterygia austeritella* in each instar.

Larval instar	Nr of examined larvae	Head capsule width (mm± SD)	Maximum body length (mm)
First	5	0.18 ± 0.02	1.30
Second	5	0.71 ± 0.02	7.04
Third	5	1.06 ± 0.06	10.63
Fourth	6	1.19 ± 0.01	12.75
Fifth	10	1.62 ± 0.09	14.88

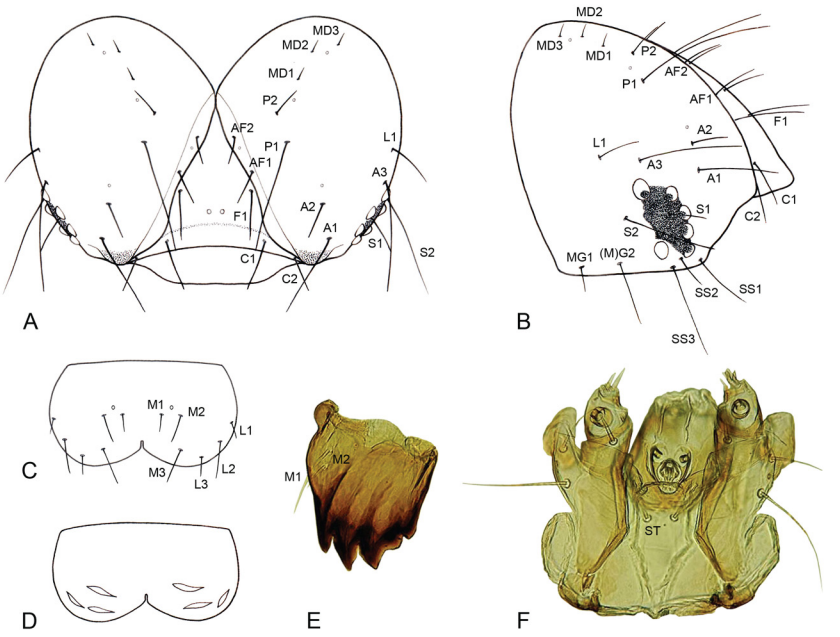

Figure 4. Head structure of the fifth larval instar of *Nephopterygia austeritella*. A) frontal view, B) lateral view, C) labrum, D) epipharynx, E) right mandible, F) maxillo-labium. Sensillium designations: A, anterior; AF, adfrontal; C, clypeal; F, frontal; L, lateral; M (in C and E designation for labral and mandibular setae); MD, microdorsal; MG, microgenal; P, parietal; S, stemmatal (= ocular, O); SS, substemmal (= subocular, SO); ST, stipular (in maxillo-labium).

ulose under low magnification; prothoracic plate very slightly darker than the ground colour, with a postero-median notch and a pattern of pale brown markings; thoracic legs yellowish cream with brownish claw (Figure 5B); anal plate creamy yellow; crochets brown; peritremes of spiracles dark.

Chaetotaxy: Head (Figure 4A–F): Frontal sclerite extended to almost four-fifths of head length, the latter slightly more than its breadth at base; adfrontals slightly tapered medially; ocellus 5 slightly extended out of the circumscribed ocellar semicircle; seta P1 almost 4 x as long as seta P2; distance between setae AF1 and AF2 nearly equal to distance between setae P1 and P2; the length of seta A2 slightly less than the length of seta A3; seta A3 almost 4 x as long as seta A1; seta S2 more than 3 x as long as seta S1.

Mouthparts: Labrum deeply notched medially (Figure 4C, D); mandible with three distinct dents along the cutting margin and two small blunt dents at the base (Figure 4E).

Thorax: (Figure 5A, C): Prothoracic plate and pre-spiracular plate separate; prothoracic plate with mottled pattern; setae XD1, XD2 and SD1 nearly equidistant from one another; seta SD1 1.2 x as long as seta XD1 and 3 x that of seta XD2; seta D2 4–5 x as long as seta D1. Seta L1 almost 3.5–4.0 x as long as seta L2; seta SV1 nearly 5 x as long as seta SV2; spiracle oval, slightly longer than the length of A1 spiracle; mesothorax and metathorax (Figure 5A): seta D2 nearly 3.5 x as long as seta D1; seta SD1 almost 5 x as long as seta SD2; setae L1, L2 and L3 equal in length and each on a separate pinaculum.

Abdomen (Figure 5A–F): Anal plate almond-shaped and more convex posteriorly; seta SD1 slightly longer than seta D2, more than 1.5 x as long as seta D1, and 3 or more times as long as seta D3. Ventral prolegs on A3 to

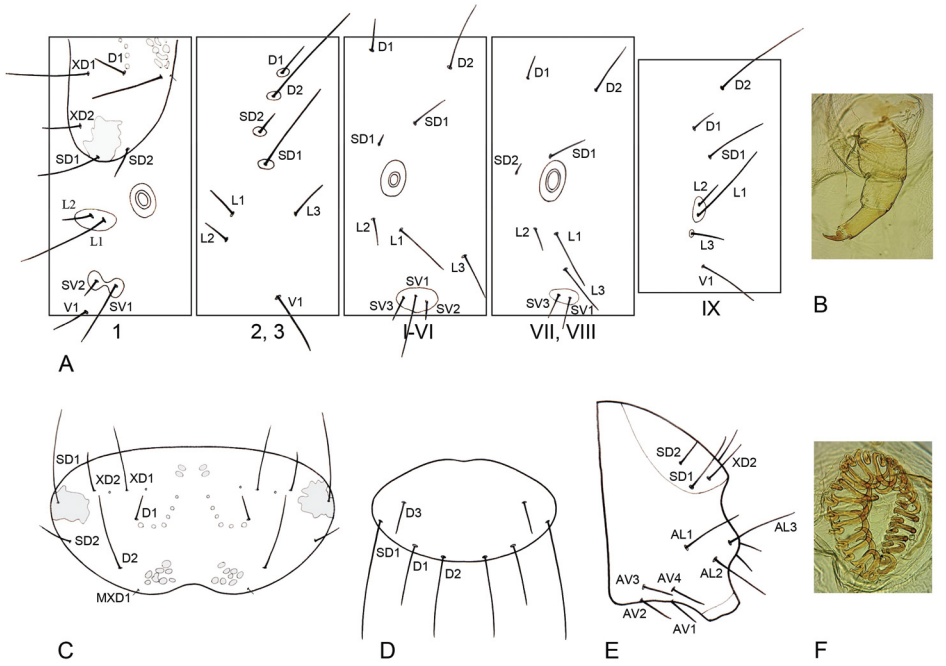

Figure 5. Diagrammatic segmental maps of the setae and sclerotizations of thorax and abdomen in the fifth larval instar of *Nephopterygia austeritella*. A) thoracic (1–3) and abdominal segments (I–IX), B) thoracic leg, C) prothoracic plate, D) anal plate, E) last (X) abdominal segment, F) crochet. Sensillium designations: A, prefix for some anal segments; dorsal, D; lateral, L; supernumary icrodorsal, MXD; subdorsal, SD; subventral, SV; ventral, V; supernumary dorsal, XD.

A6 and A10, crochets uniserial, biordinal, arranged in a complete circle (Figure 5F). In segments A1 to A8: Setae D1 and D2 nearly equidistant; seta D2 more than 2 x as long as seta D1; seta SD2 very short compared to SD1; L group trisetose (each in a unique pinaculum). SV group in A1 to A6 trisetose, while in A7 and A8 bisetose (all originate from a common pinaculum). Segment A9: seta D1 dorsal to seta D2; L group trisetose, L1 and L2 very close to each other on a common pinaculum, and L3 on a separate pinaculum; L2 more than 3.5 x as long as L1; seta L3 nearly equal in length to seta L1.

Pupa

Maximum length 6.8 mm (n=8); brown, integument almost smooth.

Impact of *N. austeritella* on *P. farcta*

Nephopterygia austeritella larvae consumed 29.6-38.4% of *P. farcta* pods in the studied areas during 2008-2009 (Table 2). All the seeds along the mesocarp of the pods were completely destroyed (Figure 2E).

Natural enemies of *N. austeritella*

From a total of 218 larvae of *N. austeritella* which were collected and reared over two years, 5 parasitized and 14 dead larvae

Table 2. Impact of *Nephoterygia austeritella* on pods of the Syrian mesquite, *P. farcta,* in central Iran.

Locality	Year	Damaged pods (%)*
Yazd	2008	32.3
	2009	36.3
Abarkouh	2008	29.8
	2009	38.4
Darab	2008	29.6
	2009	35.3

* The total number of sampled pods in all cases was 600.

Table 3. Natural enemies of *Nephopterygia austeritella* larvae in Yazd, Iran.

Year	Nr of collected larvae	Nr of parasitized larvae	Nr of dead larvae	Mortality (%)	Natural enemy
2008	99	3	------	3.03	*Apanteles subcamilla* *Phanerotoma leucobasis*
		2	------	1.68	*Phanerotoma leucobasis*
2009	119	------	13	10.92	unknown
		------	1	0.84	spider
Total	218	5	14	8.71	

were found (Table 3). The highest mortality (10.92%) of the larvae was recorded in 2009 due to an unknown factor (internal parasitoid or entomopathogen). Three and two larvae were found to be parasitized in 2008 and 2009, respectively. Two braconid parasitoids, *Apanteles subcamilla* Tobias and *Phanerotoma leucobasis* Kriechbaumer were found to parasitize the larvae of *N. austeritella* (Table 3). An unidentified spider was observed guarding its egg mass near the anaesthetized larva of *N. austeritella*.

Both parasitoid wasps were gregarious larval ectoparasitoids of *N. austeritella*. Each host larva was attacked by at most six parasitoid larvae in the pods of *P. farcta*. The last larval instar of the parasitoids underwent its pupal stage inside a white cocoon near its host body and the adult wasp emerged in September. These species were responsible for about 1.68-3.03% of mortality of *N. austeritella* larvae (Table 3).

Discussion

The present study has revealed for the first time some basic bioecological aspects of *N. austeritella* in its native habitat as a fruit feeding agent of *P. farcta*. This information will be valuable for identifying biological control agents from the genus *Prosopis* (Johnson, 1983; Mc Kay and Gandolfo, 2007). The life cycle of *N. austeritella* synchronized well with the period of fruit formation of *P. farcta*. The larvae consumed all the seeds and mesocarp of ripening pods of *P. farcta* (Figure 2E). Larval feeding resulted in a destruction of 29.6-38.4% of the pods of the plant leaving no viable seeds. Considering that each *P. farcta* pod consists of 1-9 seeds, *N. austeritella* has a larger impact on decreasing the long-lived seed bank of the plant in nature compared with the bruchid beetle, *Caryedon angeri* which consumes a fraction of the seeds of *P. farcta* (less than 50%) within a pod (Johnson, 1983; Sertkaya *et al.,* 2005).

Nephopterygia austeritella was the only pyralid moth consuming the ripening pods of *P. farcta*. Pyralids are ecologically important herbivores attacking noxious weeds (McFadyen, 1998; Blossy, 2007; Roe *et al.,* 2015). Seven species of the family Pyralidae have been universally reported targeting reproductive organs of *P. alba*, *P. glandulosa*, *P. juliflora* and *P. velutina* (Beccaloni *et al.,* 2003), six of which occur only in the New World.

As host specificity is one of the most important advantages of a biological control agent (Sheppard *et al.,* 2005; Bourchier *et al.,* 2006; Blossy, 2007), *N. austeritella* which has been yet only reported on *P. farcta*, can be considered as a promising candidate for biological control of the species of the genus *Prosopis*. Its closely related genus *Nephopterix* has a restricted host range to the species of *Euphorbia* (Cristofaro *et al.,* 1998). In North America, larvae of *Nephopterix divisella* Duponchel complete their life cycle on seven species, all in the genus *Euphorbia*. Cristofaro *et al*. (1998) considered *N. divisella* as a natural agent against two *Euphorbia* species namely, *E. milii* Desmoulins and *E. trigona* Haworth. Five species of the genus *Prosopis* are recorded from Iran, where

P. farcta, *P. koelziana* Burkart and *P. cineraria* (Linnaeus) are native and the remaining two species *P. glandulosa* Torrey and *P. juliflora* (Swartz) are introduced (Mozaffarian, 2006; Zare *et al.,* 2011). *Prosopis juliflora* is a common weed in the south of Iran (Nadjafi-Tireh-Shabankareh and Jalili, 2009). Future studies will reveal the host range and specificity of *N. austeritella* in Iran.

The Braconid parasitoids, *A. subcamilla* (Microgaterinae) and *Ph. leucobasis* (Cheloninae), attack larvae of lepidopteran species (Tobias *et al.,* 1986). *Apanteles subcamilla* has been only reported from Azerbaijan without any host record but *Ph. leucobasis* is known from Egypt, Ethiopia, Kenya, Madagascar, Nigeria, S.W. Africa, Saudi Arabia, Socotra island (Yemen), Somalia, Tanzania, Togo and Iran (Ameri *et al.,* 2012; Gadallah and Ghahari, 2013) with a wide range of hosts in the families Cosmopterygidae, Pyralidae and Gelechidae (Van Achterberg, 1990; Sobhani *et al.,* 2012; Yu *et al.,* 2012). Based on the literature, associations of these parasitoid species with *N. austeritella* and *P. farcta* are new.

Egg and pupal parasitoids of *N. austeritella* were not detected in the study, although they may be attacked by hymenopterous parasitoids (Triplehorns and Johnson, 2005). Thus, an intensive or longer period of field surveying is required to improve our knowledge of natural enemies of *N. austeritella* (Blossy, 2007). Parasitoids and other factors were responsible for the mortality of *N. austeritella* individuals during 2008 and 2009 (Table 3). Mortality factors in biological control agents of weeds have been regarded as a threat (Zalucki and Van Klinken, 2006; Zachariades *et al.,* 2011). The rate of larval parasitism of *Caryedon angeri*, a bruchid seed-beetle of *P. farcta*, by *Rhaconotus major* (Hym.: Braconidae) in Turkey varied from 62.3-100% (Sertkaya *et al.,* 2005). Parasitism of larvae and pupae of *Melipotis indomita* Walker (Lep.: Noctuidae), a biological control agent of *P. glandulosa*, was considered as an important mortality factor affecting *M. indomita* population (Cuda *et al*., 1990). The low rate of larval parasitism of *N. austeritella* may be due to the fact that moth larvae are concealed in the pods of *P. farcta* and remain less vulnerable to the attack by parasitoids (Hill and Hulley, 1995; Van Klinken and Burwell, 2005).

N. austeritella could be used as a potential biological control agent in an integrated pest management program against *P. farcta*. Further studies would be necessary to evaluate different aspects of the biology, demography, behavior, host specificity of *N. austeritella* and its possible impact on native plants and other species of the genus *Prosopis* in Iran and neighboring countries (McFadyen, 1998; Bourchier *et al.,* 2006; Blossy, 2007).

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Βιο-οικολογία του *Nephopterygia austeritella* (Lep.: Pyralidae), ενός εν δυνάμει παράγοντα βιολογικής αντιμετώπισης του ζιζανίου *Prosopis farcta* (Fabaceae) στο κεντρικό Ιράν

A. Mohammadi-Khoramabadi, H. Alipanah, S. Belokobylskij and M.R. Nematollahi

Περίληψη Το *Prosopis farcta* (Fabaceae) είναι ιθαγενές και κοινό πολυετές ζιζάνιο στο Ιράν. Σε αναζήτηση φιλικών προς το περιβάλλον μεθόδων αντιμετώπισης του *P. farcta*, εντοπίστηκε στο κεντρικό Ιράν το νυκτόβιο λεπιδόπτερο *Nephopterygia austeritella* (Lepidoptera: Pyralidae), το οποίο τρέφεται με τους σπόρους του ζιζανίου, και μελετήθηκε η βιο-οικολογία του για πρώτη φορά από το 2008 έως το 2009. Επίσης μελετήθηκαν η χωρική εξάπλωση-κατανομή της προσβολής, η διατροφική συμπεριφορά των προνυμφών, η περίοδος ανάπτυξης, η εποχιακή εμφάνιση και οι αρνητικές επιδράσεις του εντόμου στα αναπαραγωγικά όργανα του ζιζανίου. Παρουσιάζονται οι διαγνωστικοί μορφολογικοί χαρακτήρες της πέμπτης ηλικίας προνυμφών του εντόμου. Προσδιορίζονται δύο αγελαία εκτοπαρασιτοειδή του εντόμου, ως *Apanteles subcamilla* και *Phanerotoma leucobasis* (Hymenoptera: Braconidae). Τα ποσοστά θνησιμότητας των προνυμφών ήταν 3,03 και 13,44% το 2008 και το 2009, αντίστοιχα. Οι προνύμφες κατέστρεψαν το 29,6 έως 38,4% των λοβών των φυτών-ξενιστών τους. Γίνεται συζήτηση για το *N. austeritella* ως ένα δυνητικά αποτελεσματικό παράγοντα βιολογικής αντιμετώπισης σε προγράμματα Ολοκληρωμένης Αντιμετώπισης του *P. farcta*.

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