Potential for antagonists and direct tools for a control strategy of *Leucoptera scitella* L. in organic apple orchards in Southern Germany

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Abstract

In the last few years, the population of Leucoptera scitella *L. rises and in 2018 this insect became relevant for a considerable part of the organic apple orchards especially in the region of Lake Constance. In consequence, studies were conducted to explore the different tools for a successful control in organic fruit growing. The application of entomopathogenic nematodes (*Steinernema feltiae*) in autumn did not prove successful. NeemAzal-T/S showed an efficacy of about 50 % on the first generation in a field trial. It could be shown in semifield trials that the effect of NeemAzal-T/S is limited to the larvae and that the hatching rate of the larve from the eggs is not compromised by applications before or after egglaying.* Furthermore, there were *studies conducted to identify and monitor population dynamics of* L. scitella *and the role of its antagonists in 14 apple orchards. Parasitation of cocoons and larvae was lower in orchards with higher infestation pressure. Up to now 5 parasitoid species of the larval stage of* L. scitella *were classified, and 8 species of fungi isolated from it.*

Keywords: *Leucoptera scitella*, parasitoid, nematode, entomopathogenic fungi, Neem

Introduction

For several years, *Leucoptera scitella* L. occurred in organic orchards in the Lake Constance area. However, this abundance was focussed on few orchards only. In the dry and hot summer 2018, the infestation with this pest extended to a considerable part of the area under organic cultivation, hence symptoms were observed in many organic orchards in the region. Lately, also some orchards in the Neckar region were concerned.

In autumn 2017, within the scope of a project for development of strategies for insect control in organic fruit growing, in two orchards at Lake Constance area, the applications of entomopathogenic nematodes (EPN) in autumn was tested. The aim was to reduce the number of diapausing codling moth larvae and to check whether these applications could also contribute to a reduction of the infestation potential of *L. scitella*. In this context, the mortality and the occurrence of antagonist on the overwintering pupae and the development of the leafminer population and its antagonists in summer was studied.

In 2018, the potential of the antagonists was examined in 14 orchards with different infestation levels. Furthermore, the potential of NeemAzal-T/S as control agent was tested in field and lab trials.

Material and Methods

For the **trials with EPN** in 2017/2018, to test the efficacy of *Steinernema feltiae* on the overwintering stage of *L. scitella,* two apple orchards (2-4 ha) at Lake of Constance were divided across the rows into two plots. The first one was treated with *S. feltiae* whereas the second served as control. Between the plots a buffer zone of ca. 50 m was not assessed.

EPN applications were carried out at 17.9. resp.19.9.2017 in the evening before a rainy night with temperatures > 8°C. In both orchards, the same concentration (1.5 billion EPN in 2,000 l water /ha) of *S. feltiae* was applied in combination with Trifolio-S-forte 0,1 %. In each plot, winter mortality and impact of antagonists was assessed in 2018. For the assessment of

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summer infestation in each plot, 50 branches were monitored (max 1 branch/ tree) with varying number of leaves. The number of mines was assessed, and the size of the mines was categorized into three groups (big > 5 mm, medium 2- 5 mm and small < 2 mm).

One orchard was treated twice the 4th and 10th July with NeemAzal-T/S and the second remained untreated.

For **monitoring the impact of antagonists in 2018,** 15 apple orchards in the region of Lake Constance were chosen. These orchards were divided into three groups depending on the infestation rate of the leaf miner (heavy, medium and weak). The orchard infestation ratio in 2018 was estimated by farmer's observations and the extension service. In 2019 this was done by calculating the percentage number of the big mines on hundred apple leaves, selected haphazardly, (heavy > 35 %, medium 20-35 %, and weak < 20 % of leaves with big mines).

For the **determination of winter mortality and the impact of antagonists** in the two orchards of the EPN trial in spring 2018 and in 15 orchards with pre-infestation in spring 2019, 500-600 overwintering pupae of *L. scitella* per orchard were collected. They were put into plastic boxes (each 20-25 cocoons) and kept at laboratory conditions under natural light (2018) at 24± 1C° and a photoperiod 16 h (2019) until the adults emerged. The numbers of hatched moths and emerged parasitoids were observed. The numbers of non-hatched moths and parasitoids and of dead larvae and pupae of moths and parasitoids was checked by dissecting the rest of cocoons under microscope (Zeiss Stemi DV 4).

For the **determination of the parasitization rate of larval stage in summer** 50-70 apple leaves were collected with fully developed mines with larvae in the last larval stage (> 5 mm) in summer 2018 regularly in the 2 orchards of the EPN trial and sporadically in 8 other orchards. In summer 2019 the leaves were collected in 15 orchards with different infestation level. The leaves with the mines were placed in small jars and kept under laboratory conditions until the adults (moths, parasitoids) emerged.

The emerged moths, parasitoid adults were counted and the rest of the mines examined under the microscope to determine if dead larvae or parasitoid pupae were inside.

For the **identification of parasitoids** a Zeiss Stemi DV 4 stereomicroscope was used to identify the specimens and take photographs. A scanning electron microscope (SEM) was used to take photographs of the body organs of taxonomic importance such as the head, antennae, thorax, wings and abdomen. The adult parasitoids and the emerged wasps were morphologically identified using the key of Burks (2003).

In a first step, three specimens were selected for DNA extraction by using a Qiagen DNA extraction protocol. Universal primers LCO-1490 and HCO-2198 (Folmer et al., 1994) were used for amplification and sequencing of the DNA barcode (Gariepy et al. 2012).

Fungal strains were isolated from dead larvae of *L. scitella* from the fields studied. The small larval segments were externally sterilized in 100% ethanol for about 1 minute and in sodium hypochlorite solution 2 % for 50 sec. and allowed to air dry for another minute. Sterilized surface segments were put into PDA medium in Petri-dishes and incubated at room temperature in an incubator for ten days. The fungal colonies were observed and the fungal morphology was studied under microscope by observing the colony features (colour, shape, size and hyphae). The DNA Extraction of genomic DNA from the fungi was conducted by using DNeasy Plant Mini Kit. ITS1and ITS4 primers were used to amplify ribosomal internal transcribed spacer (ITS). PCR products were purified using the QIA quick PCR purification kit (Bao et al., 2012).

The sequence for **molecular confirmation** was obtained in all samples compared with public sequences available in the GenBank database using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) confirming the taxonomy of all morphologically identified fungal and parasitoid specimens.

To investigate the **effect of NeemAzal®-T/S** on the infestation of the leafminer, a **field trial** was carried out in 2019 in an organic apple orchard with high infestation in 2018.
The trials were arranged in a randomized complete block design with four replications per treatment (6 rows, 29 trees per row and plot). NeemAzal®-T/S 1,5 l/ha and m tree height was applied two times (25th June and 2nd July 2019) by the farmer.
In the two middle rows of each plot, 25 branches with varying number of leaves were marked (max. one branch/tree). The number of mines on the leaves was assessed before the first application and 10 and 15 days post application to determine the effect of NeemAzal®-T/S on the development of leafminer larvae. Mine size was divided into three categories: small < 2 mm, middle: 3-5 mm and full size > 5 mm).
For a better understanding of the effects of NeemAzal-T/S on the leafminer, **additional laboratory tests** were carried out. In early 2019, overwintering pupae of *L. scitella* were collected and kept at ca. 8 C°. Adults were obtained by transferring the pupae at 24 ± 1C° with a photoperiod of 16 h. All tests were carried out in a semi natural condition by using small potted apple plants (30-35 cm). For mating newly emerged adults males and females were released in a plastic box (20*20*25 cm) covered with nets (0,7 mesh) for 48 h. To obtain the eggs, ten potted plants were exposed in a net cage (frame 80×30× 60 cm) to ovipositing female leaf miner moths. After 24 h, the eggs on the underside of the leaves were counted with the aid of a hand-held magnifier and the females were removed.
In a first trial, it was tested if NeemAzal-T/S or other products containing oils had an effect on leafminer eggs. For this test, four plants containing 24 h old leaf miner moth eggs were treated with NeemAzal®-T/S 0,1 % and with WetCit 0,1% and Prev-AM 0,5 % (both products based on orange oil) using a hand operated sprayer. The five control plants were treated with distilled water. The effect of treatment was assessed by counting the mines on the leaves after 15 days. The efficacy on the eggs was calculated using the formula of Henderson and Tilton comparing the number of eggs before the application with the number of mines. The size of the mines was also recorded.
Since it was also discussed that the application of NeemAzal-T/S before egg laying should have some effect on the hatching of the eggs five plants were treated with NeemAzal-/TS 1 % and 24h later the trees were offered to female leaf miner moths for egg laying together with five control trees. The eggs per leaf and after 15 days the number of mines per leaf were assessed to determine the hatching rate of the eggs.

Results

In the trials with EPN the assessment of the summer infestation of the first generation the efficacy (ABBOTT) of the application of *S. feltiae* was for orchard A -7,5 %, 24,4 % and 16,7 % and for orchard B -16,7 %, -0,2 % and -42,5 % at the 1st, 2nd and 3rd assessment date (fig 1).

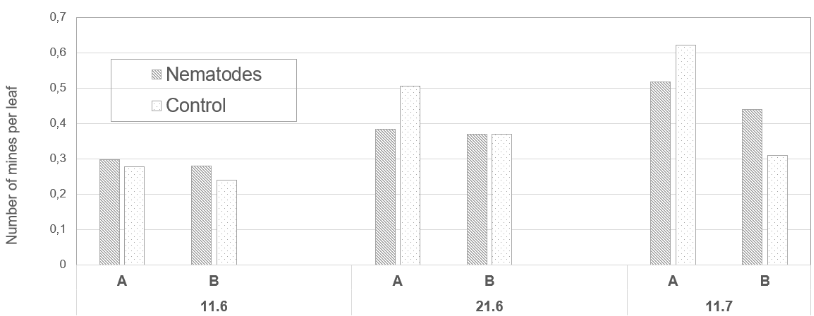

Fig.1: Infestation of *L. scitella* in summer in the trials A and B in the plot treated with *S. feltiae* and in the untreated control plot.

The examination of the collected pupae also did not show a clear effect of the mortality of overwintering stage of *L. scitella*, while in the trial A, the mean percentage mortality increased ca. 5 % compared to the control. Adult parasitoid emergence was not affected by nematode application and the parasitization ratio ranged between 13.1% in trial A -16.6 in trial B (Table 1).

Table 1. Effect of entomopathogenic nematodes on evaluation of the wintering cocoons of the leaf miners collected in spring 2018.

Trials	Treatments	Emerged adult (%)	Not emerged Adult (%)	Dead pupae (%)	Emerged parasitoid (%)	Not emerged parasitoid (%)	Dead larvae (%)
Trial A	Nematodes	21.2	30.5	28.6	6.9	9.7	3.1
	Control	16.5	18.8	29.6	5.7	4.6	24.7
Trial B	Nematodes	4.6	7.6	32.8	2.0	11.1	41.9
	Control	10.0	22.9	33.6	4.7	9.0	19.9

In average, the **parasitization rate of the overwintering leaf miner cocoons** was lower in the orchards with a higher infestation in 2018 compared with the orchards with a medium and a lower infestation. The number of dead larvae and pupae was also lower in the orchards with a higher infestation compared with the others (table 1).

Table 2: Parasitization ratio, dead larvae and pupae of the pupal stage of *L. scitella* in 2019

Orchards	Infestation pressure 2018	Emerged adult (%)	Emerged parasitoid (%)	Not emerged adult (%)	Not emerged parasitoid (%)	Dead pupae (%)	Dead larvae (%)
EB	Weak	18.8	18.8	8.7	31.9	5.2	16.7
EK		15.9	20.4	16.8	38.8	1.0	7.1
FSU		22.0	4.2	20.4	3.8	33.1	16.4
NK		31.7	14.0	3.7	4.5	33.3	12.8
NB		20.2	27.9	13.2	19.0	11.2	8.5
SCHA		11.9	18.5	2.1	7.1	26.5	33.9
SA	Medium	22.9	14.5	33.5	14.9	6.9	7.3
EA		27.9	15.6	28.8	21.1	2.5	4.1
FSH		25.3	10.5	14.7	18.2	16.1	15.1
JA		7.6	12.9	13.3	13.7	34.1	18.5
RO	High	38.2	4.7	23.6	15.2	9.9	8.5
ST		42.9	9.7	28.0	9.7	2.3	7.4
HU		22.1	16.5	4.8	4.5	35.7	16.5
BR		18.8	4.5	25.4	12.9	17.0	21.3
MA		24.2	1.8	16.7	8.0	28.8	20.5

Parasitism of the larval stage of *L. scitella* in summer 2018 varied from 0 to 17 %. In summer 2019 it varied from 0 % to 64 % of parasitized larvae in an orchard where the infestation with the leaf miner was low and strips with flowering plants were present (table 3).

During the years 2018 and 2019, in total more than 600 parasitoid specimens were collected, including different species of the families Eulophidae and Ichneumonidae. More than 90 % of the species belong to the family Eulophidae. Up to now we have classified 5 species that parasitized the larval stage of *L. scitella* as follows: *Pediobius pyrgo* (Walker), *Asecodes sp*., *Eulophus sp*., *Achrysocharoides sp*. and *Ceranisus menes* (Walker). The first three species are known that attack the larvae of *L. scitella* (Cao et al., 2017), but the other two species were recorded for the first time as parasitoids of the leaf miner.

Table 3: Infestation pressure, parasitization ratio, dead larvae and pupae of the larval stage of *L. scitella* in 2019.

Orchard	Infestation pressure of 2018	Infestation rate in 2019 (%)	Emerged Adult (%)	Emerged Parasitoid (%)	Dead larvae (%)	Dead pupae (%)
NK	Weak	18	64.1	5.1	30.8	0.0
NB		16	66.7	13.3	20.0	0.0
SCHA		14	60.0	20.0	12.0	8.0
FSU		19	40.0	0.0	35.0	25.0
EB		10	6.5	67.7	19.4	6.5
EK		17	41.2	0.0	58.8	0.0
EA	medium	25	66.7	7.7	25.6	0.0
FSH		24	69.8	0.0	15.1	15.1
Sa		23	45.3	22.6	9.4	22.6
JA		29	42.3	30.8	26.9	0.0
BR	high	37	88.0	8.0	4.0	0.0
HU		48	45.3	3.1	7.8	43.8
MA		53	50.0	13.3	15.0	21.7
ST		48	42.3	0.0	26.9	30.8
RO		42	63.3	6.7	16.7	13.3

Table 4 shows **the different types of fungi isolated from the larval stage of *L. scitella*.** *Beauveria bassiana*, *Cladosporium cladosporioides* and *Fusarium avenaceum* recorded the highest incidence rate of 42.94 %, 12.88 and 16.56 % respectively, while the other fungal species showed the proportion of 27%. The pathogenicity of the fungal isolates were tested against *Cydia pomonella* (Lepidoptera: Tortricidae) larvae and *Pentatoma rufipes* (Hemiptera: Pentatomidae) nymphs in laboratory assays. They caused different mortality rates in both Codling moth and Stink bug, the mortality rates ranged between15-90% (Alkarrat et al., 2020).

Table 4. Isolated fungi from *L. scitella* larvae

No.	Isolated fungi	Number of Isolates	%
1	*Isaria farinosa*	8	4.91
2	*Cladosporium cladosporioides*	27	16.56
3	*Lecanicillium lecanii*	4	2.45
4	*Fusarium avenaceum*	21	12.88
5	*Beauveria bassiana*	70	42.94
6	*Lecanicillium muscarium*	13	7.98
7	*Clonostachys rosea*	9	5.52
8	*Paecilomyces* sp.	11	6.75

Fig. 2 shows **the effect of NeemAzal-/TS on *L. scitella* development in the field**. Since the egg laying of this leafminer was interrupted in the area of Lake Constance in 2019 by a very cold period that lasted more than three weeks the first application was done when the first mines were already observed. The assessments 7 and 10 days after application showed clearly that the number of fully developed mines and consequently also larvae is considerably reduced. The degree of efficacy (ABBOTT) on the fully developed mines is 57,8 % after 7 days and 42,3 % after 10 days.

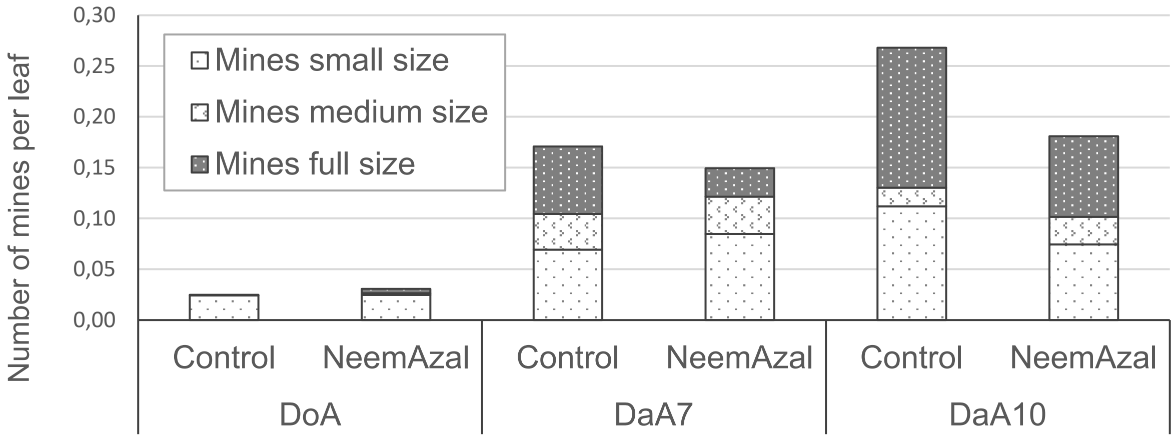

Fig. 2: Effect of NeemAzal on the number and the size of the mines in the field trial

The laboratory trials showed an efficacy (Henderson and Tilton) **of the application on the eggs on egg hatching** of 16,6 % for NeemAzal-T/S, 10,1 % for Wetcit and 28,7 % for Prev-AM.

In the trial with **application of NeemAzal-T/S before egglaying** in the control 19,9 % of the eggs did not hatch whether in the NeemAzal-T/S treatment 9,0 % did not hatch. However, only 12,9 % of the mines developed normally whether in the control 90,7 % of the mines developed with fully grown larvae.

Discussion

The application of *S. feltiae* in autumn did not show an effect on the mortality of the overwintering adults or an effect on the infestation pressure in the following year. Since it is known that *L. scitella* overwinters as pupa and it is improbable that the pupae can be attacked successfully by the nematodes, the time interval for a successful attack of the larvae in the cocoons will be very small and not contemporaneous for all individuals of a population. Thus, nematodes seem to be not very adapted to serve as a valuable tool in the control strategy of *L. scitella*.

Since the parasitation rate is higher in the orchards with lower infestation, parasitoids may play an important role in population control. In practice, it is often observed that high populations break down naturally after several years. Currently, the parasitoid fauna is studied more intensively to find out if it would be an interesting option to bring parasitized material from orchards with low infestation into orchards with fresh outbreaks to accelerate the parasitation. For this, the species and the biology of the main parasitoids in the region have to be clear. Currently, the identification of more species is running. With these data, an information board for the identification of the parasitoids of this species will be set on to allow the extension service an easy identification.

A considerable number of entomopathogenic fungi was isolated from dead larvae indicating that these antagonists are also important as already observed by Pitta (1990).

Also interesting is the observation that there were a lot of adults unable to hatch from the cocoons. In 2018 this was considered to be due to the storage conditions of the cocoons but in 2019 this phenomenon was also observed in the field. The reasons for this have to be studied more intensively to find out if it can contribute to develop valuable tools for the control of the overwintering stage.

NeemAzal-T/S is a tool for the direct control. Since there was some uncertainty about the effects these were studied more in detail to improve the recommendations for practice.

With the laboratory results, an effect on egg hatching of applications before and after egg laying can be excluded. However, the effect on the development of the larvae could be demonstrated very clear. The field studies show this effect even if the infestation pressure in the orchard was not so high as expected. NeemAzal-T/S therefore is a tool that can reduce both damage (since the mines remain small) of the first generation and the infestation pressure for the second generation (since the larvae of first generation donot develop). This confirms the findings of Steinle & Zebitz (2015) and Zwahlen & Hunkeler (2017). Further studies are needed to explore the situation where there is a real need for a second application and if the amount per ha can be eventually reduced. Since NeemAzal-T/S is not expected to be harmful on adult parasitoids and only higher larval stages of the the larvae in the mines are attacked by parasitoids (Mey, 1993) the side effects of NeemAzal-T/S on these antagonists should be low. Since *L. scitella* is an insect that causes losses only at high infestation rates (the level for a significant reduction of yield is given by Baufeld & Freier (1991) as 0.5 – 2.5 mines per leaf in autumn) there is a good chance to develop a combination strategy of direct and indirect tools to prevent outbreaks and to maintain the population on a tolerable level.

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