




Susceptibility of the Box tree pyralid *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) to potential biological control agents Neem (NeemAzal[®]-T/S) and entomopathogenic nematodes (Nemastar[®]) assessed in laboratory bioassays and field trials

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Abstract

The Box tree pyralid *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) is one of the accidentally introduced organisms that became invasive and established in Europe within a few years. Thus, eco-friendly preparations are required, which should be accessible, affordable, easy usable and suitable for the application in private and public areas. Therefore, the effects of the plant extract formulation NeemAzal[®]-T/S (active ingredient Azadirachtin A) and the commercial preparation Nemastar[®] (entomopathogenic nematode species *Steinernema carpocapsae*) were investigated on *C. perspectalis* larvae in laboratory bioassays and field trials. When NeemAzal[®]-T/S-treated leaf discs were consumed by larvae in the laboratory, a significant effect on mortality and feeding activity was noted after 14 days of exposure. At this time, 47–62% of the larvae had already died and less than 10% of larvae were still feeding. Application of different *S. carpocapsae* suspensions (10–200 EPN/100 µl, i.e. per larva) demonstrated a high susceptibility of both tested larval instars (2nd: 10–75% and 4th: 45–100% mortality). In field trials neither the application of Nemastar[®] nor the use of NeemAzal[®]-T/S caused mortality rates comparable to those generated by the treatment with plant protection products based on *Bacillus thuringiensis*. In conclusion, there is a higher variability in terms of the effects of the investigated agents compared to the constant effectiveness of *B. thuringiensis*. But their use would be possible if individual feeding damage and proper surveillance will be considered in order to be capable of repeating the application timely and a combination with further measures ought to take place.

Keywords *Buxus* · Biological control · Feeding activity · Larval mortality

Introduction

Organisms are frequently brought into new environments by humans. After importing, the species are capable of reaching different levels of invasion success (introduced, established and pest) as defined by Williamson and Fitter (1996a). Just a small proportion of introduced species have the potential to become established, and only a small amount of these established species will become a serious

pest (Williamson and Fitter 1996b). The Box tree pyralid *Cydalima perspectalis* (Walker 1859) is one of these accidentally introduced, therefore non-native and invasive organisms that have accomplished an establishment in Germany and Europe. Further, it can be classified as pest, because of causing negative economic effects as well. According to the law, trees belong to the owner of the land on which they grow. Their damage or destruction therefore affects the value of the land of which they form a part. Cemeteries and large park facilities with partially several-kilometre-long *Buxus* hedges are strongly affected by a reduction in economic value, depending on feeding damage caused by *C. perspectalis* (Leuthardt 2013). Moreover, *C. perspectalis* continuously spreads (Nacambo et al. 2014) and constantly causes serious damage on ornamental

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plantings and natural *Buxus* stands in Europe (John and Schumacher 2013).

Buxus is a traditionally important evergreen perennial shrub (Oberdorfer et al. 2001) of widespread use. It is planted in large numbers in private and public areas. It is also very popular on cemeteries for adorning graves and enclosing irrigation systems. Most of the plantings are structurally important subdivision elements. Especially in these public urban areas, the application of chemical insecticides often may be necessary, but is disliked or forbidden. Therefore, cemeteries are frequently becoming a source of infection for plants growing nearby, caused by renewed attacks. *C. perspectalis* develops exclusively on plants of the genus *Buxus* and herbivorous; competitors are lacking in Europe (Wan et al. 2014). Only a small amount of sucking organisms utilizes *Buxus* plants as feeding source, for instance the Boxwood psyllid (*Psylla buxi*), the Boxwood spider mite (*Eurytetranychus buxi*) and diverse scales (van Trier and Hermans 2007), but the damage caused is purely aesthetic and not as destructive as by *C. perspectalis* or fungal *Buxus* diseases.

Because of this pest characterisation, efficient pest management is required. Preparations based on *Bacillus thuringiensis* are eco-friendly and specifically effective against lepidopteran larvae. Many strains produce endotoxins as pre-proteins which accumulate in crystalline inclusions in the bacterium. After ingestion, these crystals are dissolved by proteases in the gut of the host insect producing active proteins which interfere with the metabolism of the target insect (Schnepf et al. 1998). Immediate feeding stop occurred, induced by rapid paralysis of mouthparts and gut, followed by gut and haemolymph poisoning, which leads to the death of the insect after a few days (Burgess 1982; Schmutterer and Huber 2005). *B. thuringiensis* is highly effective against *C. perspectalis* and registered. However, two main problems arise: If treatment has to be done, public areas have to be closed for visitors until the spray deposit is completely dry (24 h). Furthermore, as the only available biological control measure, the risk of resistance formation exists with frequent application (Schnepf et al. 1998).

Thus, further eco-friendly preparations are needed which should be accessible, affordable, easy to use and suitable for the application in public areas, e.g. cemeteries, monasteries and public parks as well as house gardens. Since the beginning of biological control in the 1980s, numerous publications and reviews have been written regarding insect control and pointed out many advantages of Neem products and entomopathogenic nematodes (Schmutterer 1990; Kaya and Gaugler 1998; Schmutterer and Huber 2005). These agents are well investigated, commercially available and are used against various pests.

Formulations with Azadirachtin, the most important active ingredient in seeds of the Neem tree (*Azadirachta indica* A. Juss (Meliaceae)), were tested and successfully used against various insect pests. Benelli et al. (2017) reviewed the potential of Neem-based products and pointed out that on the basis of its properties like effective and eco-friendly features, including little non-target effects, multiple mechanisms of action, low cost and easy production, Neem-based products can serve as an advantageous option to build newer and safer arthropod control tools. It serves as insect growth regulator and feeding deterrent to many insects (Isman 2006) and there are anti-ovipositional, fecundity- and fitness-reducing properties as well (Schmutterer 1990).

Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae are mutualistically associated with bacteria of the genus *Xenorhabdus*, serving as vectors. After penetrating a host, this complex causes a fast insect pathogenicity and can only be active against a specific host range (Schmutterer and Huber 2005). In many countries, they are considered as invertebrate beneficials and no registration is necessary.

The aim of this study was to assess the potential of other biological control agents as alternative or supplemental method to *B.t.*, which are already commercially available on the market as well as non-target and bystander friendly to the greatest possible extent. Therefore, the biological control agents NeemAzal[®]-T/S and Nemastar[®] were investigated on their potential to affect larvae of *C. perspectalis* using laboratory bioassays and field trials.

Materials and methods

Biological control agents

The plant extract formulation NeemAzal[®]-T/S (NA), containing 1% of the active ingredient Azadirachtin A, oils and surface-active agents, was kindly provided by the company Trifolio-M GmbH (Lahnau, Germany). The commercial preparation Nemastar[®], containing entomopathogenic nematodes (EPN) of the species *Steinernema carpocapsae*, as well as the wetting agent (wa) BREAK-THRU[®] and an adhesive agent (aa) were kindly provided from the company e-nema GmbH (Schwentinental, Germany). The commercially available *Bacillus thuringiensis* (BT) preparations XenTari[®], containing the subspecies *aizawai*, and the product Dipel Es[®], containing the subspecies *kurstaki*, were kindly donated by Cheminova Deutschland GmbH & Co. KG (Stade, Germany).

For conducting the field trials, treatments were made on-site following recommendations by the producers (Table 1). They were randomly assigned in a split plot

Table 1 Treatments considered in field trials on the biological control of *C. perspectalis*

Trial	Product (abbreviation)	Content	Concentration	Application rate
2012	Nemastar [®] (EPN.12.wa)	<i>S. carpocapsae</i>	1.25 million/l	2 l/m ²
		wetting agent (wa)	0.1 ml/l	
	Nemastar [®] (EPN.12.wa+aa)	<i>S. carpocapsae</i>	1.25 million/l	2 l/m ²
		wetting agent (wa)	0.1 ml/l	
		adhesive agent (aa)	1.75 g/l	
	XenTari [®] (BTa.12)	<i>B. thuringiensis aizawai</i>	1 g/l	1 l/m ²
2013	NeemAzal [®] -T/S (NA.13)	1% Azadirachtin A	3 ml/l	0.2 l/m ²
	Nemastar [®] (EPN.13.adj)	<i>S. carpocapsae</i>	1.25 million/l	2 l/m ²
		adjuvant*	2 g/l	
	Dipel ES [®] (BTk.13)	<i>B. thuringiensis kurstaki</i>	1 ml/l	0.2 l/m ²
2014	NeemAzal [®] -T/S (NA.14)	1% Azadirachtin A	5 ml/l	1 l/0.25 m ²
	Nemastar [®] (EPN.14.adj)	<i>S. carpocapsae</i>	2.5 million/l	1 l/0.25 m ²
		adjuvant*	2 g/l	
	Dipel ES [®] (BTk.14)	<i>B. thuringiensis kurstaki</i>	1 ml/l	0.5 l/0.25 m ²

Abbreviations refer to the following figures and tables

*Consists of gel, dispersant and wetting agent

design in four replications at the test area (Fig. 1). The EPN variant incorporated an adjuvant consisting of gel, dispersant and the wetting agent BREAK-THRU[®], which was pre-dissolved in ethanol. It was ensured that EPN did not settle to the bottom by constant movement of the suspension in the laboratory and the sprayer in field. Treatments with water served as control. Applications were conducted using pressure sprayers (APV[®] Akku Roll: 55 l and 7 bar for EPN, filter was removed; Gloria[®] prima 5 (Type 39 TE): 5 l and 3 bar for NA and BT). Plants were

sprayed with defined volumes (Table 1), and it was assured that they were dripping wet.

Plant material

Plants of *Buxus sempervirens* L. var. *arborescens* L. were purchased from a Box tree nursery (Baumschule Atrops, Rheurdt, Germany). A part of the plants were potted and stored in the greenhouse until used for bioassays. Remaining plants had been planted on a testing field for conducting field trials. In addition, cut plant material (twigs

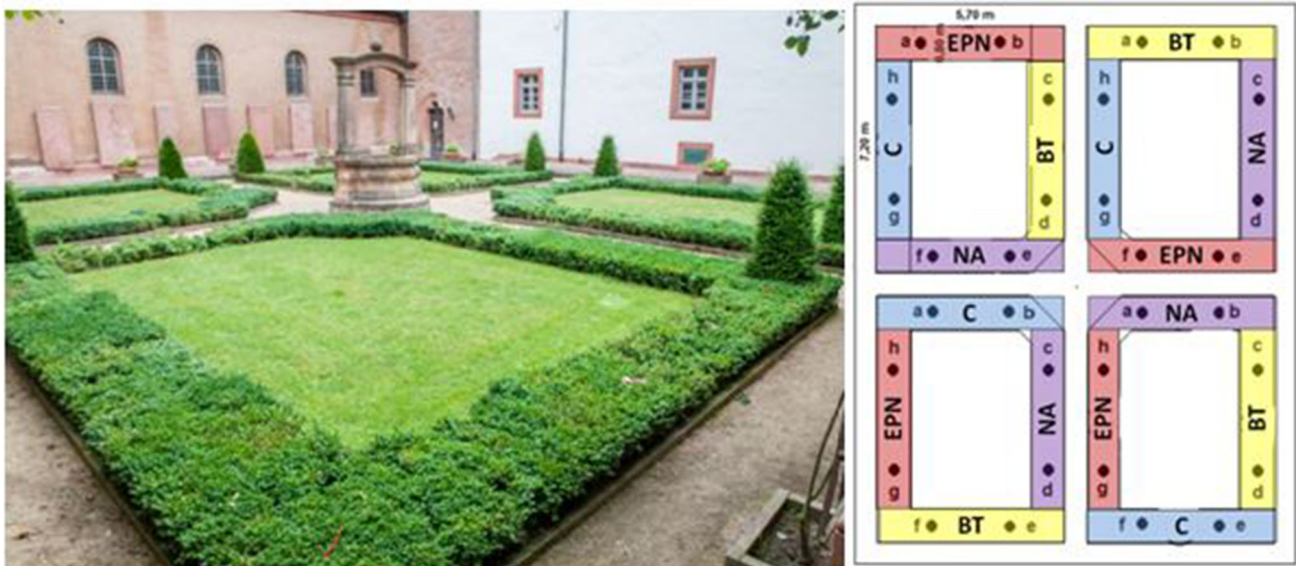


Fig. 1 *Buxus* hedges (left) and experimental design (right) of field trials at the cloister in Seligenstadt conducted in the years 2012 and 2013, including four application sites per variant (coloured) and

defined plots to assess the level of infestation before the application (a–h). For full names of variants see Table 1

of 30 cm length) of the large-leaf variant *B. sempervirens* var. *rotundifolia* was purchased and stored in a cooling chamber at 10 °C until cutting leaf discs for bioassays.

***C. perspectalis* larvae**

C. perspectalis have been reared under laboratory conditions since 2010. Adult moths were kept in rearing cages (length 40 cm × width 36 cm × height 50 cm) with diluted sugar (5%) for feeding. Short Box tree twigs (15–20 cm) were regularly introduced into the cages for egg deposition. *C. perspectalis* egg masses (≤ 24 h old) were collected from the offered Box tree twigs. Hatched larvae were fed on watered Box tree twigs in transparent Perspex cylinders (40 cm height × 19.5 cm diameter) with textile covers. The different larval stages used for the laboratory bioassays (2nd, 3rd and 4th) were determined by the average time of moulting until the particular larval stage, initiated from egg masses of equal age. The duration of larval development was adapted from own previous investigations.

Laboratory bioassays

NeemAzal®-T/S (NA) bioassay with 3rd larval instar

The effect of NA was investigated on 3rd instar larvae by feeding them with treated leaf discs. Therefore, the required amount of NA was emulsified with distilled water to prepare different concentrations (0.1, 0.3 and 0.5%). Treatment with water served as control. Leaf discs were cut from *B. sempervirens* var. *rotundifolia* leaves using a cork borer (diameter 1 cm). They were dipped for 30 s into the particular emulsions and air-dried for 30 min. Treated leaf discs were placed in wells (d 3.5 cm) of multiwell plates in which 2 ml water agar (3%) had been filled in order to keep the leaf discs from drying. One larva was each added and 20 larvae were used per concentration. Multiwell plates were incubated at 25 °C, 16-/8-h light/dark condition (L/D), 50% relative humidity (RH). The trial was replicated three times. Larvae were further fed with freshly treated leaf discs two times a week. The vitality and feeding activity were also recorded two times a week. Bioassays were conducted for at least 2 weeks. Larvae were considered to be dead if they did not respond to prodding with forceps.

Entomopathogenic nematodes (EPN) bioassay with 2nd and 4th larval instars and pupae

Laboratory bioassays were conducted to define the susceptibility of young (2nd instar) and further developed (4th instar) *C. perspectalis* larvae to EPN of the species *S. carpocapsae*. A serial dilution of an EPN suspension was prepared to adjust different EPN concentrations (10, 20, 50, 100 and 200 EPN/100 μ l). Therefore, product powder containing infective juveniles (IJs) of EPN was diluted in water (2 g in 1000 ml) and well stirred to prepare a stock suspension containing about 200 EPN/100 μ l. To define the actual EPN concentration and be able to adjust the needed concentrations for the bioassays, a dilution and counting procedure was done following the method reported by Glazer and Lewis (2000). Single *Buxus* leaves were treated with 100 μ l of EPN suspension and air-dried for 15 min. Treated leaves were placed in experimental vials (2 × 2 × 2 cm) in which 1 ml water agar (3%) had been filled in order to keep the leaves from drying. One larva was added per vial, and 15 larvae were included per concentration. Treatment with water served as control. Experimental vials were incubated at 25 °C, 16/8 h L/D, 50% RH. After 3 days, the mortality was determined, food (untreated) was replenished in the case of survived larvae, and vials were incubated for a further 4 days. Thus, the duration of the bioassays was 7 days and the trial was replicated two times per larval stage. Larvae were considered to be dead in the case of a brown coloration of the larvae or if they did not respond to prodding with forceps.

Laboratory bioassays were additionally conducted to define the susceptibility of fresh and matured *C. perspectalis* pupae on EPN of the species *S. carpocapsae*. Tested concentrations (10, 50, 200 EPN/100 μ l) were prepared as mentioned before. Ten fresh and 10 matured pupae were included per concentration. They were individually placed in experimental vials (diameter 3.8 cm) and directly treated with 100 μ l of EPN suspension. Treatment with water served as control. Experimental vials were incubated at 25 °C, 16/8 h L/D, 40% RH. Pupae were observed daily, and hatched moths were counted. After 14 days, all non-developed pupae were dissected to examine them for the presence of nematodes.

Field trials comparing commercial preparations (NA, EPN and BT)

Field trial on already infested *Buxus* hedges at Seligenstadt in 2012

In August 2012, a field trial was conducted on already infested *Buxus* hedges in the cloister of a public convent

garden in Seligenstadt (50°2′38.75″N 8°58′31.25″E; South-Hessia, Germany) to compare the treatments EPN.12.wa, EPN.12.wa+aa with BTa.12 in the field (Table 1). The cloister (Fig. 1) consisted of low planted *Buxus* hedges, symmetrically arranged in four rectangles. Before the treatment, the current level of infestation was assessed. Therefore, eight plots of 0.25 m² (2/replicate) were defined (Fig. 1a–h) and *C. perspectalis* larvae were searched within these plots and counted. Five days after the treatment (DAT5), living and dead larvae were counted on the total area of treated hedges (about 18 m²) for 10 min per replicate to evaluate the mortality directly in field. In addition, treated twigs (5/replicate) were collected at the application day (DAT0). The 5 samples per replicate, thus 20 per treatment, were cut and transferred to single vials. In the laboratory, one *C. perspectalis* larva obtained from own rearing was added to each vial. Vials were incubated in a climate chamber (25 °C, 16/8 h L/D, 50% RH) for a further 14 days, and mortality was determined.

Field trial on already infested *Buxus* hedges at Seligenstadt in 2013

In June 2013, a second field trial was conducted on already infested box hedges in the cloister (Fig. 1) of a public convent garden in Seligenstadt (50°2′38.75″N 8°58′31.25″E; South-Hessia, Germany) to compare treatments NA.13, EPN.13.adj and BTK.13 in the field (Table 1). Before the treatment, the current level of infestation was assessed. Therefore, eight plots of 0.25 m² (2/replicate) were defined (Fig. 1a–h) and *C. perspectalis* larvae were searched within this plots and counted. Seven days after the application, living and dead larvae were counted on the total area of treated hedges for 10 min per replicate to evaluate the mortality directly in field. In addition, treated twigs (5/replicate) were collected three times at DAT0, DAT7 and DAT14. The 5 samples per replicate, thus 20 per treatment, were cut and transferred to single vials. In the laboratory, one *C. perspectalis* larva obtained from own rearing was added to each vial. Vials were incubated in a climate chamber (25 °C, 16/8 h L/D, 50% RH) for a further 14 days, and mortality was determined.

Field trial on intentionally infested *Buxus* plots at Darmstadt in 2014

In August 2014, a field trial was conducted on *Buxus* plants which were planted on an experimental field in Darmstadt (49°51′53″N 8°40′6″E; Hesse, Germany) to compare treatments NA.14, EPN.14.adj and BTK.14. An experimental area was created, consisting of 16 plots in four lines, with one plot comprising four *Buxus* plants

(0.25 m²). Each plot was infected with 10 larvae (4th larval instar) obtained from the laboratory. Within the 4 days before the application, they were allowed to feed and to produce their typically loose webs in the plant. To minimize the impact of solar irradiation on nematodes, the trial was conducted late in the evening (8:00 pm) as recommended by the producers. Four days after the treatment (DAT4), the deployed larvae were collected from the treated plots. Dead larvae were counted, and living larvae were further incubated in the laboratory to determine the cumulative mortality at 3, 7 and 10 days of further incubation (DFI). In addition, samples were collected four times after the treatment (DAT0, 4, 7 and 11) from the plots to assess the persistence of applied variants in the field. Therefore, 40 twigs per variant (10 each plot) were cut, transferred into vials and offered to single *C. perspectalis* larvae obtained from the laboratory. Vials were incubated in a climate chamber (25 °C, 16/8 h L/D, 50% RH) for a further 14 days, and field mortality of these larvae was determined.

Statistical analyses

For statistical analyses, the scientific data analysis software Sigma Plot Version 13.0 was used. The effect of treatment and concentration of NA preparations on feeding and mortality as well as the effect of treatment and concentration of EPN preparations on mortality of young and further developed larvae were assessed via one-way ANOVA ($\alpha = 0.05$). If significant, pairwise multiple comparisons were conducted using Tukey tests. To analyse the effect of EPN concentrations on fresh and matured pupae, proportions were compared using a χ^2 -test. Other results are presented in bar graphs, if possible as mean values with standard deviations.

Results

Laboratory trials

Effect of NeemAzal®-T/S (NA) on 3rd larval instars

Investigation on the effect of NA on *C. perspectalis* demonstrated susceptibility of 3rd instar larvae. After 6 days feeding on treated leaf discs, the amount of vital and still feeding larvae was high and ranged between rates of 60 and 80% of exposed larvae ($n = 20$ /concentration in each trial). No significant impact of treatments on feeding activity could be determined ($F = 2.174$, $df = 3$, $P = 0.169$). Regarding mortality after 6 days, only a few larvae died and no significant impact of treatments was detected ($F = 1.065$, $df = 3$, $P = 0.416$). After 14 days,

feeding activity was significantly reduced to less than 10% in all treatments ($F = 202.872$, $df = 3$, $P < 0.001$). Mortality increased significantly to a maximum of 62% after 14 days of providing leaf discs treated with 0.3% or 0.5% NA ($F = 7.318$, $df = 3$, $P < 0.05$) (Fig. 2).

EPN bioassays with larvae and pupae

Both tested larval instars (2nd and 4th) were highly susceptible to EPN. After 3 days of exposure, mortality was significantly lower for the young larvae ($F = 18.337$, $df = 11$, $P < 0.001$) ranging between 10 and 75% in comparison with further developed larvae where mortality rates ranged between 45 and 100%. The mortality of young larvae was significantly affected after 3 days ($F = 24.279$, $df = 5$, $P < 0.001$) by higher concentrations (100 and 200 EPN/larva, $P < 0.05$), while the 4th instar larvae showed already a significant susceptibility to lower concentrations (20 and 50 EPN/100 μ l ($F = 12.65$, $df = 5$, $P = 0.004$) at the same time. After 7 days of exposure, mortality was significantly affected by all treatments equal to or higher than 10 EPN/larva regarding both tested larval stages (2nd: $F = 129.672$, $df = 5$, $P < 0.001$; 4th: $F = 85.641$, $df = 5$,

$P < 0.001$). A mortality of 100% could be determined after 7 days for all larvae which had been exposed to higher nematode concentrations (50, 100 and 200 EPN/larva) (Table 2).

EPN were much less effective against *C. perspectalis* pupae. Between 70 and 100% of all treated pupae hatched within 14 days. Only a small amount of the pupae did not hatch (16%), including pupae of the untreated control. There was no significant difference in the proportions of dead fresh and matured pupae, and mortality was not related to the tested EPN concentration ($\chi^2 = 1.333$, $df = 2$, $P = 0.513$) (Table 2). Only one matured pupa treated with 200 EPN contained nematodes, indicating a successful penetration into the insect body. No EPN were detected in the remaining dissected pupae.

Field trials

Field trial on naturally infested Buxus hedges at Seligenstadt in 2012

The number of *C. perspectalis* found in the *Buxus* hedges before the application of treatments EPN.12.wa,

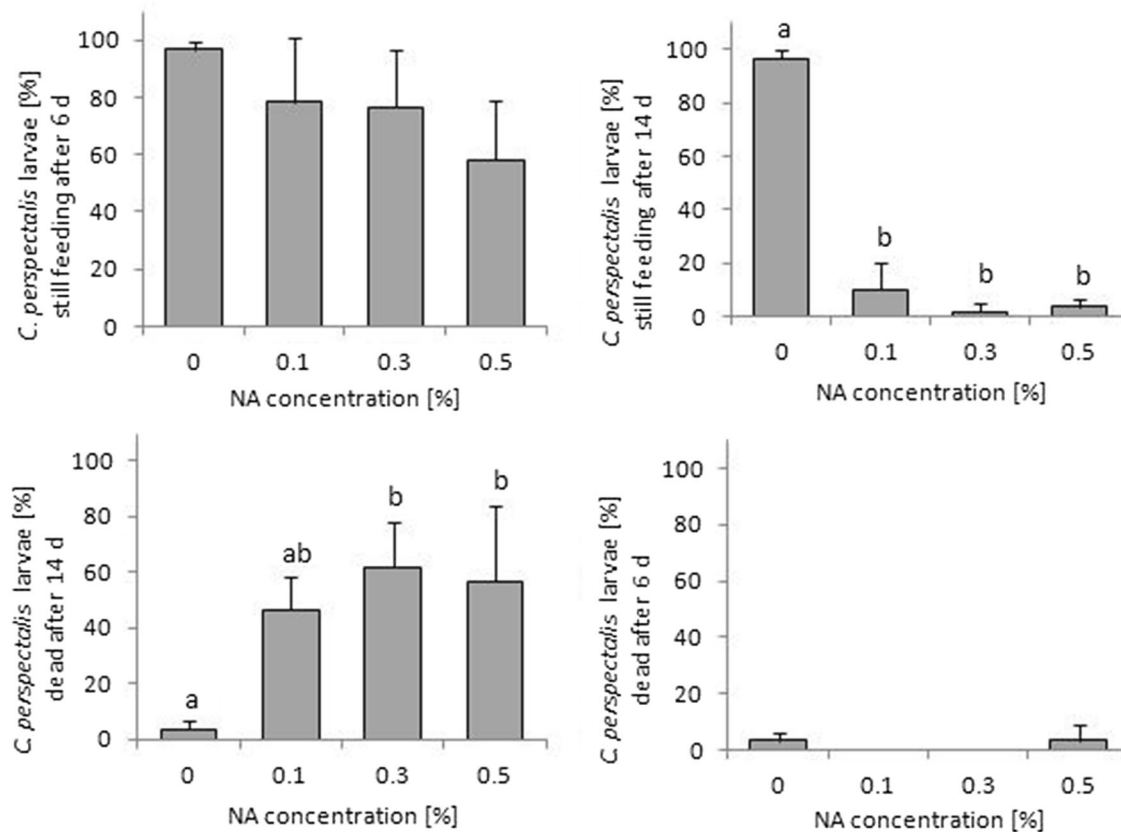


Fig. 2 Mean proportion [%] of *C. perspectalis* 3rd instar larvae ($n = 20$ /concentration) still feeding or dead after 6 and 14 days of providing *Buxus* leaf discs treated with different NeemAzal®-T/S

(NA) concentrations ($n = 3$ trials). Different letters indicate significant differences (ANOVA, Tukey Test, $\alpha = 0.05$)

Table 2 Mortality [%] (mean \pm SD) of young *C. perspectalis* larvae (2nd instar, $n = 2 \times 15$) and further developed larvae (4th instar, $n = 2 \times 15$) after 3 and 7 days exposed to *Buxus* leaves treated with different nematode concentrations [EPN/larva] (ANOVA, TukeyTest, $\alpha = 0.05$) as well as fresh (*f*, $n = 10$) and matured (*m*, $n = 10$) pupae once directly treated with different EPN concentrations [EPN/pupa] (χ^2 test, $\alpha = 0.05$)

[EPN/larva] [EPN/pupa]	2nd instar		4th instar		Pupae	
	3 days	7 days	3 days	7 days	<i>f</i>	<i>m</i>
0	0 \pm 0 a	3 \pm 4.7 a	0 \pm 0 a	0 \pm 0 a	10	10 a
10	10 \pm 14.1 a	70 \pm 4.7 b	47 \pm 28.3 a	77 \pm 14.1 b	10	30 a
20	10 \pm 4.7 a	87 \pm 9.4 bc	70 \pm 14.1 b	97 \pm 4.7 b		
50	33 \pm 0 a	100 \pm 0 c	87 \pm 18.9 b	100 \pm 0 b	20	20 a
100	70 \pm 14.1 b	100 \pm 0 c	97 \pm 4.7 b	100 \pm 0 b		
200	73 \pm 9.4 b	100 \pm 0 c	100 \pm 0 b	100 \pm 0 b	20	20 a

Different letters indicate significant differences between the impacts of concentrations

EPN.12.wa+aa and BTa.12 was 191 individuals in total on 8 m² (32 \times 0.25 m² plots), i.e. 24 *C. perspectalis*/m² (including larvae and a small amount of pupae). 5 days after the application, the estimated number of *C. perspectalis* was 421 including 102 dead larvae on the total area of treated hedges (18 m²), i.e. 23 *C. perspectalis*/m², including 6 dead larvae/m². The mean number of dead larvae was high in the BTa.12 treatment where only a few vital larvae were found. In contrast, in the EPN treatments the number of vital larvae was high and only a few dead larvae were found (Fig. 3).

The application of EPN.12 and BT.12 caused significant differences in field mortality among the treatments 5 days after the application in 2012 (ANOVA, $F = 81.245$, $df = 3$, $P < 0.001$). BTa.12 (96%) significantly differed from EPN.12.wa (11%), EPN.12.wa+aa (16%) and the control ($P < 0.001$). These rates were supported in parallel by the mortality rates achieved in the laboratory, after treated twigs were collected at DAT0 and offered to larvae from the laboratory: EPN.12.wa (55%), EPN.12.wa+aa (80%) and BTa.12 (95%). BT was the most effective agent (Table 3).

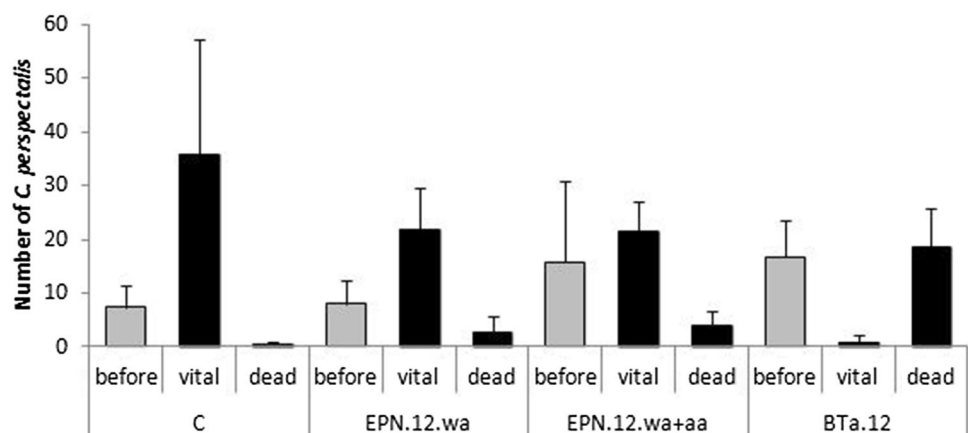
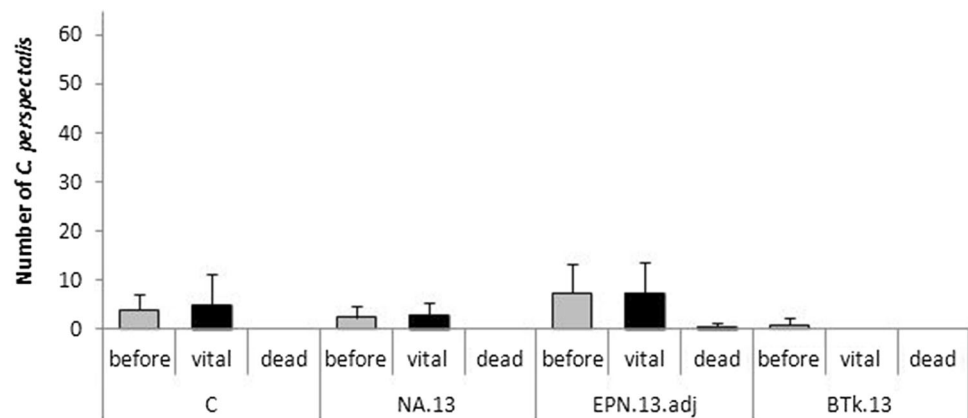
Fig. 3 Mean number of *C. perspectalis* found at already infested *Buxus* hedges ($n = 4$ sites/treatment) before (grey bars, all vital) and 5 days after (black bars) the application of different variants at the cloister at Seligenstadt in 2012 (see Fig. 1). For full names of tested biological control agents see Table 1

Table 3 Larval mortality [%] of *C. perspectalis* in field as well as after exposure of treated twigs collected at different days after treatment (DAT) and further incubated in the laboratory in trials performed in the years 2012 and 2013

	2012				2013			
	C	EPN.12.wa	EPN.12.wa+aa	BTa.12	C	NA.13	EPN.13.adj	BTk.13
Field	1	11	16	96	0	0	7	0
DAT0	0	55	80	95	0	30	55	75
DAT7	–	–	–	–	0	0	0	15
DAT14	–	–	–	–	10	0	0	10

For full names of tested biological control agents see Table 1

Fig. 4 Mean number of *C. perspectalis* found at already infested *Buxus* hedges ($n = 4$ sites/treatment) before (grey bars, all vital) and 7 days after (black bars) the application of different variants at the cloister at Seligenstadt in 2013 (see Fig. 1). For full names of tested biological control agents see Table 1



effective agent, and dead larvae were found in 2013 also on DAT7 and DAT14 (10–15%) (Table 3).

Field trial on intentionally infested *Buxus* plots at Darmstadt in 2014

Not all deposited larvae were retrieved from *Buxus* plots 4 days after the treatment (Fig. 5, numbers in parentheses). At this day (DAT4) 66% of retrieved larvae, which were exposed to BTK.14, and 30% of larvae exposed to EPN.14.adj were dead. In contrast, all collected larvae from C and NA.14 were alive. Mortality rates of the field collected larvae further increased with the days of further

incubation (DFI3, 7 and 10) of the surviving larvae in the laboratory. Thus, the mortality of BTK.14-treated larvae rose to 100% at DFI3. Mortality of EPN.14.adj-treated larvae reached the maximum of 63% at DFI3. In addition larvae treated with NA.14 showed an increased mortality, which reached 54% at DFI10 (Fig. 5). These results suggest that larvae already have been affected in field.

In addition, the persistence in field differed between the variants. When exposing larvae to twigs collected at DAT0, 93% of EPN.14.adj-treated larvae and 78% of BTK.14-treated larvae died. BTK.14 was the most persistent variant and caused high mortality rates (> 90%) of larvae exposed to twigs collected at DAT4 and 11. The

Fig. 5 Cumulative mortality [%] of retrieved *C. perspectalis* larvae, collected in 2014 from *Buxus* plots in field ($n = 4$) 4 days after treatment (DAT). Mortality was assessed after 3, 7 and 10 days of further incubation (DFI). Numbers in parentheses show number of larvae (retrieved/deployed). For full names of tested biological control agents see Table 1

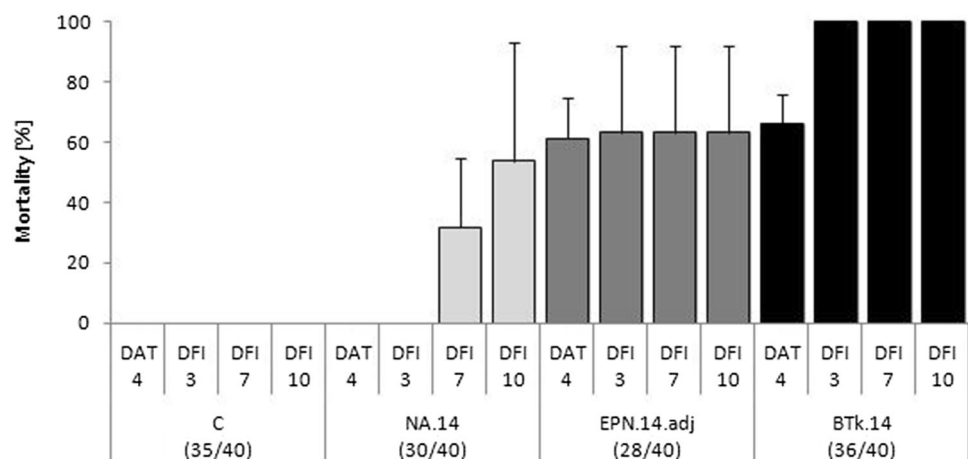
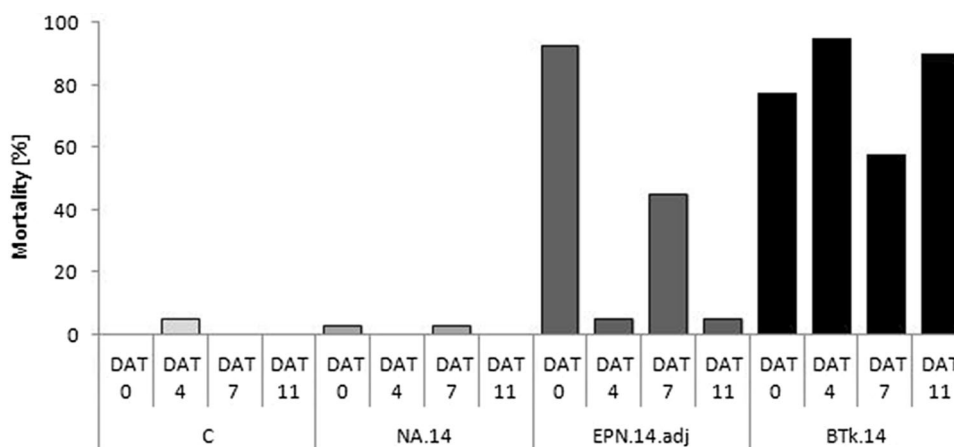


Fig. 6 Persistence of potential biological control agents in the field. Mortality [%] of *C. perspectalis* larvae ($n = 40$ /treatment) fed with treated Box twigs collected in 2014 from *Buxus* plots in field at 0, 4, 7 and 11 days after treatment (DAT) after application of different variants in the field. For full names of tested biological control agents see Table 1



persistence of EPN.14.adj fluctuated and rapidly decreased in field, but after 7 days larvae were infected by nematodes again as conferred by dissection. In contrast, larvae exposed to twigs treated with water or NA.14 were not affected (Fig. 6).

Discussion

This is the first report on the susceptibility of European *C. perspectalis* populations to the biological control agents Nemastar® (EPN) and NeemAzal®-T/S (NA). The results demonstrate clearly the potential of both tested agents to control *C. perspectalis*, but also the challenges of application on *Buxus* plants in the field.

The vulnerability of *C. perspectalis* larvae (3rd instar) to NA was assessed by the potential impact on feeding activity and survival of larvae after repeated ingestion of treated leaf discs. In a preliminary trial, the impact on young (2nd instar) and further developed (4th instar) larvae was tested based on uniquely ingestion of single treated *Buxus* leaves. The responding time was highly extended, and larvae were starving many weeks until all of them died (up to seven weeks). Only minor differences occurred in the reaction of the tested larval stages (Table 4). In the present study, the method of investigation was improved to a more realistic situation. Third instar larvae had been fed continuously with freshly treated leaf discs. There was only little variation in the response on the different tested NA concentrations (1–5 ml/l). None of the tested concentrations led to mortality earlier than 6 days after first ingestion of treated leaf discs. This kind of time delay is well known regarding investigations on the impact of NA to several pests. Most insect pests continue to feed on the treated plants for some time, but as a rule, the amount of food ingested by them is considerably reduced, due to the influence of the “secondary” anti-feedant effect (Schmutterer 1990). For example Efil et al. (2005) as well noted a

Table 4 Duration [days] until > 50% *C. perspectalis* larvae (mean \pm SD) of different larval stages (2nd and 4th) stopped feeding and died after unique ingestion of one *Buxus* leaf treated with different concentrations of NeemAzal-T/S. Results of a previous trial not described in this manuscript

	0.1% NeemAzal- T/S	0.3% NeemAzal- T/S	0.5% NeemAzal- T/S	1.0% NeemAzal- T/S
Feeding stop				
2nd larval instar	17.5 \pm 0.7	12.0 \pm 2.8	12.0 \pm 2.8	10.5 \pm 0.7
4th larval instar	15.0 \pm 4.2	12.0	12.0	12.0
Mortality				
2nd larval instar	29.5 \pm 6.4	21.0 \pm 5.7	17.5 \pm 0.7	17.5 \pm 0.7
4th larval instar	30.0 \pm 4.2	27.0	22.0 \pm 1.4	18.0

duration of 6 days until larval mortality occurred, when leaf discs had been treated with concentrations of 0.5–2.5 ml/l before offering them to larvae of the Beet armyworm *Spodoptera exiqua* Hübner (Lepidoptera: Noctuidae). Application rates used in our study complied with the usual applications of 1–7 ml/l NA for bioassays and recommended rates of 250 ml/100 l water against lepidopteran pests in the field (Dammini Premachandra et al. 2005; Erler et al. 2010). It can be concluded that *C. perspectalis* larvae are susceptible to ingested NA at common concentrations (preferably 3–5 ml/l), causing feeding stop and larval mortality within two weeks in laboratory bioassays.

The presented results suggest that also EPN affected *C. perspectalis* larvae. In preliminary investigations by the authors, three EPN species were examined on their impact on *C. perspectalis* larvae. The species *Heterorhabditis bacteriophora* showed the lowest effect on larval mortality (8–15%) in comparison with *S. feltiae* (46–100%) and *S.*

carpocapsae (85–100%) at concentrations of 25–200 EPN/larva (Göttig 2012). Shannag and Capinera (1995) compared five EPN species against the Melonworm moth *Diphanthia hyalinata* Linnaeus (Crambidae), a close relative to *C. perspectalis*, and determined highest infectiousness of *S. carpocapsae* as well. Furthermore, *D. hyalinata* was significantly less susceptible at first instar larval stage and pupae than older larvae, which was also evident in this study. In contrast, Choo et al. (1991) reported that there was no difference in infection on Japanese *C. perspectalis* larvae by *H. bacteriophora* and *S. carpocapsae* achieving 100% mortality in all treatments ranging between 10 and 80 EPN/larva. Our investigation on the infectivity of different *S. carpocapsae* concentrations (10–200 EPN) on *C. perspectalis* demonstrated a high susceptibility of both tested larval instars (2nd instar: 10–75% and 4th instar: 45–100%). Infection of further developed larvae was faster in comparison with young larvae, regarding the pathogenicity after 3 days. It is known that insect size as well as nematode size can influence the potential of EPN species to invade potential host species, because they are most frequently penetrated via body orifices (Bastidas et al. 2014). Further developed larvae have bigger orifices or probably fed more of the leaves or had more contact to the surface. It was found that pupae were less vulnerable to EPN, even if adult nematodes were found in one of 13 dead and dissected pupae in our study. This could be observed in the case of treated winter cocoons of *C. perspectalis* larvae (usually 3rd instar larvae), too (data not shown). In contrast, infection of cocooned codling moth larvae (*Cydia pomonella* L., *Lepidoptera: Tortricidae*) was high and mortality ranged between 30 and 100% after application of *S. carpocapsae* (0.5–5.0 million/tree) in the field (Unruh and Lacey 2001).

Although both biological control agents can affect *C. perspectalis* in the laboratory, limited success was achieved in field applications. In the presented field trials on naturally infested *Buxus* hedges (year 2012 and 2013), it was not possible to detect an exact difference in the occurring amount of *C. perspectalis* before and after the treatment. In general, the method of feeding treated leaf samples to laboratory reared larvae proved to be reliable in order to quantify effects even in field trials. In addition, field studies conducted on *Buxus* hedges intentionally infested with a defined number of larvae (year 2014) facilitated the quantitative recording of the effect. Thus, the method was suitable to assess field mortality.

In this study, the particular ranking of the effectiveness of NA, EPN and BT was comparable in each experiment. The application of NA resulted in a low response of treated larvae, and larval mortality constantly took a long time. This extensive duration until the larvae stopped feeding and died could be problematic for rapid efficiency in acute

damage cases. But NA may be considered for applications on small larvae, as already mentioned for other targets, if and when a temporary feeding damage does not endanger the plants. The application would be of great advantage if other sucking pests are present on the *Buxus* plant or being part of applications combining more than one control agent. Impacts were proved on over 400 insect species, especially on lepidopteran species (> 138) (Schmutterer and Huber 2005).

Using EPN in field produced mortality rates of about 16–65% at a range of 2.5 million EPN/m², but high fluctuations were observed in persistence tests. Mortality rose up to 80%, if field-treated twigs collected at the application day were fed to larvae in the laboratory. Field mortality was in the same range as results presented by Shannag and Capinera (1995), where infection rates of 52–55% were produced in field applications of 5 billion EPN per hectare (0.5 million/m²) on *D. hyalinata* on crookneck squash foliage. As invertebrate macroorganisms, the application of EPN requires no registration as plant protection product and in many countries there are no user restrictions. The rate of mortality in field and nematode success to penetrate host species is strongly related to weather conditions, the application technique and easy accessibility of the host on the plant foliage, in contrast to soil applications. Factors like the UV irradiation and temperature, as well as the relative humidity, play a decisive role in foliar application and the persistence of EPN in field (Glazer 1992; Smits 1996; Arthurs et al. 2004). Many advantages also apply to EPN. They are partially able to actively seek their host; they are almost harmless to non-target organisms, are easily mass produced, have a broad host range, and have the ability to kill their hosts rapidly (Shannag and Capinera 1995; Kaya and Gaugler 1998).

Because larvae of *C. perspectalis* are highly susceptible to BTK and BTa, the use of plant protection products based on *Bacillus thuringiensis* is currently the only alternative to chemical insecticides and available effective biological control methods. However, the requirements for proper application are high and pesticide resistance is becoming an increasingly important factor in the selection of suitable control agents, even for using BT against other lepidopteran species (Heckel et al. 2007; Kang et al. 2014). Resistance rapidly can change the available insecticides arsenal. Therefore, plant-derived natural products like NA as well as beneficials like EPN are considered to be valuable candidates to prevent resistance on common pesticides.

In conclusion, there is a higher variability in terms of the effects of the investigated agents Nemastar[®] and NeemAzal[®]-T/S compared to the constant effectiveness of Dipel ES[®] in the field. But their use would be possible if feeding damage and proper surveillance of the result will

be considered in order to be capable of repeating the application timely. More frequent and timely applied treatments may improve efficacy and a combination with further measures ought to take place.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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