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Is microbiological control of the box tree moth feasible? Effectiveness and impact on non-target diurnal Lepidoptera

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HIGHLIGHTS

- *Cydalima perspectalis* is an invasive insect pest affecting *Buxus* spp.
- In European environments, box trees form the protected Habitat 5110.
- *Btk* treatments have been evaluated in field and controlled conditions.
- Potential impact of *Btk* has been assessed on non-target diurnal lepidoptera.
- No major effects were detected on the abundance and richness of non-target species.

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ABSTRACT

Recent outbreaks of the invasive alien species *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) have led to the widespread loss of boxwoods in Europe. Although details on its biology are not fully unraveled, the box tree moth (BTM) can be considered a major pest, severely damaging its primary host, *Buxus sempervirens,* ultimately affecting the associated coenosis. In European environments, box trees form the Habitat 5110, and BTM outbreaks are seriously endangering its long-term survival.

The effectiveness of microbiological treatments with *Bacillus thuringiensis* var. *kurstaki* (*Btk*) on the survival of BTM was evaluated in field and controlled conditions. Given the scant specificity of *Btk* action, an in-depth survey of its potential impact on non-target diurnal Lepidoptera has been conducted by monitoring their communities' changes in treated and control areas.

Btk spraying was highly effective in the field, and five days after treatments larval density reduction was over 90% in all sites and years, with similar mortality trends achieved in controlled conditions.

No significant short-term effects were detected either on non-target diurnal lepidopterans' abundance or species richness.

These results can be combined with the outcomes of spatially explicit models to overcome issues that, up to now, have caused the failure of appropriate strategies to control *C. perspectalis*. Even if no impact has been observed on non-target Lepidoptera, a rigorous plan for the application, detection and surveillance of the potential effects of microbiological treatments needs to be established.

1. Introduction

Defoliating pests, particularly invasive alien species, can seriously threaten ecosystems and economies, damaging local biodiversity extensively (Kenis et al., 2009; Mitchell et al., 2018). Insect defoliators can cause further impacts on forest coenosis than the death of their host plants because of microclimate and nutrient cycle alterations due to leaf loss (e.g., Kenis et al., 2009; Gandhi and Herms, 2010). Following the outbreak of herbivorous pests, many other mechanisms might impact the resident arthropod community by changing predator, parasitoid, competitor dynamics, or food availability (Redman and Scriber, 2000). Therefore, although often occurring at comparatively small spatial scales and rather short time intervals, forest defoliator pests can profoundly alter ecosystem functioning (Crawley, 1983; Suárez-Muñoz

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et al., 2019).

Recently, several European countries suffered the invasion of an Asiatic pest, the box tree moth (BTM), *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) that can utterly defoliate several boxwood species (Kenis et al., 2013; Leuthardt and Baur, 2013). In its native range, the BTM seems also to exploit other plants, such as *Ilex purpurea, Euonymus japonicus,* and *Euonymus alatus*. Still, no shift on secondary species has been recorded in Europe, suggesting the unpalatability of those plant species for BTM (van der Straten and Muus, 2009; Ferracini et al., 2022). Natural forests encompassing *Buxus sempervirens*, *Buxus colchica,* and *Buxus balearica* have faced severe declines over the last century (di Domenico et al., 2012), but, from the first record of the BTM in Germany in 2006, the boxwood loss has increased and extended to entire regions (see Ledru et al., 2022, for boxwood biomass loss in France), following the rapid spread of *C. perspectalis* across Europe, through Anatolia to Iran (Bras et al., 2019). The BTM distribution is limited by the presence of the box trees and by climatic conditions affecting larval development and diapause (Canelles et al., 2021). In European environments, box trees form the Habitat 5110 “Stable xerothermophilous formations with *B. sempervirens* on rock slopes (*Berberidion* p.p.)” according to the 92/43/EEC Habitats Directive. Based on the Natura 2000 European Database, Habitat 5110 is known for 310 Natura 2000 sites (SCIs, SPAs and SACs) distributed across 9 countries: France (146 sites), Spain (109), Italy (33), Belgium (12), Germany (3), Greece (3), Portugal (2), Luxembourg (1), and the UK (1). In Italian environments the box tree enters either as a typical or differential species of several forest and semi-forest associations (Raineri et al., 2017). In NW Italy *B. sempervirens* forms stable shrubs typical of EU Habitat 5110, for which a new Site of Community Importance (SCI) was established in 2019 in Pradleves municipality. However, the conservation status of the Habitat 5110 in the alpine biogeographical region is inadequate (U1), according to the last reporting under Article 17, and the biology of the BTM populations occurring outside the species’ native range has been increasingly investigated in the last few decades (Ferracini et al., 2022). *C. perspectalis* is a multivoltine species, with up to four generations a year in Europe (Nacambo et al., 2014; Ferracini et al., 2022). Eggs are laid in small clusters on the host plants, and overwintering occurs at early larval stages (Poitou et al., 2020; Ferracini et al., 2022). After five to seven instars, caterpillars pupate, and the imagoes emerge in about ten days (Kawazu et al., 2010). Females bear a variable number of eggs according to the generation, showing higher fecundity in the native areas (Ferracini et al., 2022). The adults are estimated to have broad dispersal abilities, up to ten kilometers a year (Canelles et al., 2021).

Overall, species traits, such as high fecundity, fertility, and dispersal, together with an extensive trophic activity of larvae causing severe defoliation to plant death (Kenis et al., 2013), make the BTM a major pest threatening forest ecosystems in Europe. The severity of its impact and the rapidity of its outbreak prompted the authors to list the BTM among the “100 worst alien species for Europe” (Nentwig et al., 2018).

The containment of this pest in natural *Buxus* formations poses a whole series of problems and is a challenge to be pursued. The EU Habitat 5110 provides ecosystem and cultural services, and its loss may have several implications (Mitchell et al., 2018). *Buxus* formations are rich in biodiversity, and several species have been recorded as associated with *Buxus* (Mitchell et al., 2018); hence, any containment other than mechanical removal should be theoretically avoided. On the other hand, the progress of damage caused by BTM larvae jeopardizes this habitat’s medium- to long-term permanence in many geographical areas.

While chemical insecticides are used to eradicate BTMs from ornamental plants in nurseries, integrated pest management strategies have been explored to protect boxwood in the wild. Indeed, BTM control strategies must consider and preserve the complex community of other plants, fungi, or animals including insects hosted by *Buxus* natural formations (Mitchell et al., 2018). Still, sustainable, long-term control treatments are far from being successfully implemented.

Outside its native range, the population dynamics of BTM are not regulated by the moth’s natural predators or parasitoids as it occurs in Asia (Wan et al., 2014). Larvae are generally discarded by most common predators due to their ability to sequester *Buxus* alkaloids. Some predation by arthropods or birds and parasitism by insect wasps were documented (Leuthardt et al., 2013; Mostini, 2018; Bird et al., 2020), but currently no effective control of the pest was ever reported.

The impact of biological control strategies involving parasitoids as *Trichogramma* species (Göttig and Herz, 2016) and entomopathogenic nematodes (Göttig and Herz, 2018) were tested primarily in laboratory conditions but showed low pest parasitism or mortality rates, respectively. Specifically, even if preliminary laboratory tests allowed the isolation of three interesting *Trichogramma* strains, field trials showed that these egg parasitoids were insufficient to control the BTM (Colombel et al., 2022). Further research focused on the predation rate by *Chrysoperla lucasina* (Lacroix, 1912) on BTM eggs and young larvae and by the great spotted cuckoo *Clamator glandarius* (L., 1758) gave more promising results (Espluga and Garcia-Reàdigos, 2020; Colombel et al., 2022).

Pheromone traps are likely to be effective in monitoring, but they proved inadequate for suppressing the moth invasion at larger scales (Santi et al., 2015).

So far, because of their effectiveness, treatments with *Bacillus thuringiensis* (*Bt*), specifically using the variety *kurstaki* (*Btk*), are the most widespread strategies to manage BTM population growth, and have been considered in this paper. The killing power of this microbial insecticide resides in the crystalline endotoxin proteins produced during bacterial sporulation, which, once in the alkaline gut, solubilize and devastate the intestine walls (Lambert and Peferoen, 1992). A few minutes after ingestion, the larva stops feeding, but the toxin invades other tissues, causing death from septicemia and paralysis. Although the *Btk* is strongly effective for BTM control, its use is not devoid of disadvantages. The persistence and toxicity of *Btk* are variable depending on humidity and sunlight conditions (Scriber, 2004); thus, repeated treatments are required. Most importantly, *Btk* is not specific to BTM, and it is active against non-target lepidopteran species (Peacock et al., 1998); therefore, to design an effective and efficient pest control using *Btk* in natural areas, it is essential to monitor the potential effects of the treatments on the non-target species. Lepidoptera are the second most numerous order of insects in terms of species, and the majority of insects have been decreasing dramatically in the last decades (Wagner et al., 2021). At EU level many butterflies and moths are threatened with extinction for several reasons (Warren et al., 2021) and 29 butterflies and 6 moths are protected under the Habitats Directive, 60 % of them are present in Italy and Piedmont is the richest part of Italy (Bonelli et al., 2018).

This work aimed to provide insights, which, combined with the outcomes of spatially explicit models (Ledru et al., 2022), can pave the path to overcoming issues that, up to now, have caused the failure of appropriate strategies to control *C. perspectalis*. This three-year work endeavored to evaluate the effectiveness and reliability of microbiological treatments in field and controlled conditions. Ground-based *Btk* applications were performed, and the larval density reduction after spraying was evaluated. Moreover, laboratory tests to assess the BTM survival were performed on different larval instars. The potential impact on the non-target species was evaluated by butterfly monitoring through semi-quantitative transects in the areas subjected to microbiological treatment and in control areas. For non-target species co-occurring with BTM, a method to assess the potential risk of *Btk* exposure was also provided.

2. Materials and Methods

2.1. Study areas

Surveys have been performed for three years (2019–2021) in two NW Italy valleys in the Piedmont region: the Grana valley (Pradleves site; 44°24′44.6″ N, 07°16′16.3″ E; 813 m a.s.l.), and the Vermenagna valley (Vernante site; 44°14′13.1″ N, 07°32′36.3″ E; 857 m a.s.l.). The investigated areas are about 30 km apart.

In Pradleves, *B. sempervirens* forms the Habitat 5110 stable xerothermophilous and calcicolous shrubs with interspersed clearings (SCI, “Comba di Castelmagno” (IT1160065)), while the Vernante site encompasses an alpine meadow regularly mowed and edged by a broadleaf forest where *B. sempervirens* occurs as undergrowth shrubs. Details on vegetation compositions of the two study areas are provided in Table S1a,b.

Since 2019, the *B. sempervirens* habitat occurring in Pradleves and Vernante has been arbitrarily divided into two ha sub-areas; one was used to implement pest control strategies (hereafter called “treated areas”) while the second was used as a control. As chosen within the same habitat type, the two sub-areas were similar in biotic or abiotic features.

2.2. *Insect culture*

A continuous mass-rearing of all BTM development stages was maintained on watered box tree twigs (*B. sempervirens*, 30 cm in length) in cages (1 x 1 x 1 m) having a stainless-steel frame structure supporting a plastic insect-proof net (mesh 0.23 x 0.23 mm). The rearing was started from an initial culture collected from infested boxwood located in the surveyed sites (Ferracini et al., 2022).

2.3. *Control strategies*

The commercially available *Btk* preparation Rapax® was evaluated in field and laboratory conditions to assess BTM larvae mortality. The adhesive agent Lumik Arvensis® was added to reduce the surface tension of water.

Two plots of 1200 m^3 (2 m high x 300 m long x 2 m thick) were selected in each surveyed site (*Btk* treated and control plots). All plants used in the trial were uniform in size and showed a comparable infestation level. In the control plots, no application was performed, and the distance between *Btk* treated and control plots was at least 300 m apart to avoid any drifting effect.

In the treated plots, ground-based applications of *Btk* were performed, and plants were treated with defined volumes sprayed at high pressure (1 l Rapax®, and 0.4 l adhesive agent Lumik Arvensis® diluted in 400 l of water), directing the spray into the canopy to ensure reaching the inner part of the plants. Treatments were performed by the Forest Fire Volunteers Corps of Piedmont with equipment consisting of a 400-liter tank in food steel, with an internal combustion engine allowing to pressurize the outgoing liquid solution. Two treatments were performed in mid-July and early September (the time of intervention was chosen in relation to the presence of L1/L2 instar larvae, based on field visual inspections and according to previous investigations carried out in the same area; see Ferracini et al., 2022). The trials were conducted late in the evening, as recommended by the producers (after 6:00 pm, when the area to be treated was entirely in the shade), to minimize the impact of solar irradiation. In case of adverse weather, the treatment was postponed.

2.4. *Mortality tests*

2.4.1. *Field experiments*

A field trial was conducted on highly infested natural *Buxus* formations. Before microbiological applications, the plants were selected based on the BTM density and signs of infestation (feeding damage, frass, typical silky webs). Larval population density was estimated in each site before spraying by counting the number of larvae on ten randomly sampled branches (about 10 cm in length) per tree from ten consecutive box tree plants (100 branches/plot/sampling date). The percentage of mortality was calculated according to Schneider-Orelli’s formula (Pünterner, 1981).

The larval population density was estimated two and five days after spraying, using the same method described above.

2.4.2. *Laboratory experiments*

Five age-cohorts for each larval instar (L1, L2, L3, L4, L5 instar) were selected by following Nacambo et al. (2014). The effect of *Btk* was investigated on BTM larvae by feeding them with treated box tree leaves. *Btk* and the adhesive agent Lumik Arvensis® were emulsified with sterile distilled water (the concentration was the same as used in the field experiments), and treatment with sterile distilled water alone served as a control. A leaf dip bioassay method was followed, as described by Ranjbari et al. (2011). Leaves were first washed with distilled water containing 0.1 % Triton X-100 thoroughly and dried. The foliage of the experimental saplings was dipped into the bacterial suspension and sterile distilled water by tilting and bending the plants so that the whole foliage was in the suspension. After treatment for 10 s, the saplings were left to dry under room conditions for 1 h, according to Er et al. (2007). Five healthy larvae of the same cohort were put inside a Petri dish (Ø 15 cm) on treated box tree leaves, and the trial was replicated four times. Leaf areas were roughly equivalent among replications. The same method was used with sterile distilled water, as a control. Accordingly, 20 larvae for each instar were tested with the bacterial suspension and 20 larvae with the sterile distilled water, for a total of 200 larvae tested.

Petri dishes were kept at 24 ± 1 °C, 55 ± 5 % RH, with a 16:8 L:D photoperiod. The vitality and feeding activities were recorded every 6 h. Larvae were considered dead if they were still and did not feed.

2.5. *Impact of control strategies on non-target diurnal Lepidoptera*

2.5.1. *Butterfly communities*

The butterfly community composition and its changes over time were assessed in treated and control sub-areas to evaluate the effect of *Btk* treatment on the non-target species.

The census of non-target butterflies was performed by 300 m long linear transects (Pollard and Yates, 1993) walked in 45 min. Two transects were traced per study site; one crossed the treated part and the other the control sub-areas. Surveys were conducted approximately every ten days only if the wind was absent or scarce, on sunny days, from 10:30 am to 3:30 pm. The sampling period lasted from mid-May until mid-September for a maximum of 11 monitoring events. This census can be considered exhaustive, covering the entire period of activity of butterflies and allowing the detection of both the early and late species. All the individuals were gathered by an entomological net, but only a few samples were killed and brought to the laboratory for further identification, while most species were immediately identified in the field. In addition to their occurrence and abundance, butterfly species were characterized according to their functional traits and ecological requirements (Balletto and Kudrna, 1985) in terms of voltinism (univoltine, bivoltine, multivoltine) and host plant range (i.e., stenophagy: one plant species – monophagous; one plant genus - strictly oligophagous; one plant family – oligophagous).

2.6. *Host plant communities*

The vegetation survey was carried out with the Braun-Blanquet (1964) method within a total of 16 squares of 5 m x 5 m (25 m^2). The plant diversity was explored in four plots, randomly scattered along each transect (n = 4) used to assess the butterfly community. The percentage of coverage is transformed using the Braun-Blanquet scale (density higher than 75 % –5; between 50 % and 75 % – 4; between 25 % and 50 % – 3; between 5 % and 25 % – 2; between 1 % and 5 % – 1; sporadic presence- +). The surveys were carried out at the end of May with a monthly check until August to account for late flowering plants.

2.7. Risk assessment

Given the broad spectrum of Lepidoptera larvae on which the toxicity of *Btk* is considered effective, the risk each butterfly species observed in the treated areas would face was estimated. The risk categories were assigned based on a combination of species' functional traits (following Balletto and Kudrna, 1985) and the presence/abundance of their host plants (Table 1).

The following features were considered pivotal to determining the risk index (RIx) and the entity of impact (I) due to the *Btk* spraying (Table 1):

1) PHENOLOGY (based on Balletto and Kudrna, 1985)

A. *The presence of larval stages*. If active (=feeding) larvae are absent in July and/or September according to the species' life cycle, then the RIx drops to zero. The length of the periods in which treatments with *Btk* can be implemented has been considered extended to the whole month to cope with the precautionary principle;

B. *Voltinism*. A score of 3.75 was assigned to univoltine species that face the highest risk of local extinction if the *Btk* treatment kills the single larval cohort of the year. Bivoltine species (two generations per year) received a score of 1.75. The lowest score (0.75) was ascribed to multivoltine species that cope with a lower risk.

2) HOST PLANTS

The presence and abundance of the host plant(s) were assessed in the areas where *Btk* treatments were performed and in control zones (see Methods: Host plant community for details).

C. If no host plant is present in treated areas, the risk is considered null because the butterfly species just flies through avoiding the site for reproduction; thus, larvae have no chance to feed on *Btk*-treated plants;

D. The higher the host plant density in the treated zones compared to the control area, the higher the risk for the butterfly species. Thus, a score of 0.25 was assigned when the host plant density is lower in the untreated areas, 0.5 when the host plant density is equal in treated and untreated zones, and 1.25 in the case that the host plant density is higher in the treated areas.

3) RISK CATEGORIES

The Risk Index (RIx) is given by the formula $AC (B + D)$ and is used to classify butterfly species according to the degree of the potential negative impact they face in areas treated with *Btk*. Impact categories (I) are defined as follows: Null ($RIx = 0$); Low ($1 \le RIx \le 2$); Moderate ($2 < RIx \le 4$); High ($RIx > 4$). The scores assigned to voltinism and host plant density are weighted to reach a final index (RIx) that varies between 0 and 5.

2.8. Statistical analyses

For the analysis of parametric data, homogeneity of variance was verified using Levene's test. A one-way Analysis of Variance (ANOVA), followed by Tukey post hoc test at 0.05 level of significance, was performed to test for differences in larval infestation (i.e., average number of larvae counted on 100 branches) before treatment, and two and five days after *Btk* application. Differences in species richness, abundance, and Shannon index of non-target butterfly communities were also tested by ANOVA, followed by Tukey post hoc test for pairwise comparisons.

The efficacy of *Btk* treatments was evaluated by considering larval density reduction compared to initial density. The larval reduction due to *Btk* treatments was corrected for natural mortality using the Schneider–Orelli formula (Pünterner, 1981).

All analyses were performed using SPSS version 23.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Field experiments

Before *Btk* treatments, no significant difference in the larval population density was recorded among all sites. In both treated and control plots, the mean number of BTM larvae/tree was 51, 66, and 47 in 2019, 2020, and 2021, respectively, and did not differ significantly (2019: $F = 0.049$, $df = 1$, $P = 0.825$; 2020: $F = 1.466$, $df = 1$, $P = 0.227$; 2021: $F = 0.934$, $df = 1$, $P = 0.335$). The average (mean ± standard error) BTM larval densities occurred in 2019, 2020, and 2021 in untreated and *Btk*-treated plots before insecticide applications are reported in Table 2.

Btk treatments were always applied when the BTM larval population was in L1 and L2 instars. The application of *Btk* caused a clear larval density reduction (Fig. 1). In Pradleves, considering all experimental years, the reduction attested at around 41 % and 92 %, two and five days after *Btk* treatment, respectively. In Vernante, the reduction had similar values and reached 44 % and 94 %, two and five days after *Btk* treatment, respectively. In the control plots, no larval mortality was ever recorded.

3.2. Laboratory experiments

The *Btk* treatments demonstrated a high susceptibility of young instar larvae. After one day of feeding on treated box tree leaves, about 80 % of the L1 larvae died, while the same mortality for L2 instar larvae was reached after 36 h. Conversely, L3 and L4 instar larvae reached 80 % mortality 66 and 72 h after the treatment. After 96 h, all the larvae had died regardless of their age. All the larvae in the control Petri dishes, sprayed only with sterile deionized water, remained alive for the entire duration of the trial. The mortality of the different instar larvae is shown in Fig. 2.

3.3. Impact of Btk treatments on non-target diurnal Lepidoptera

3.3.1. Butterfly communities

Excluding species occurring once over three years, the non-target butterfly communities encompassed the same species in *Btk*-treated and the control areas, accounting for a total of 45 species in Pradleves and 49 in Vernante. No impoverishment of the butterfly communities was detected when comparing the areas treated with *Btk* and the control patches in the two sites. In Pradleves, both the species richness and the individuals' abundance are similar in control and treated areas, while in Vernante a slightly, although not significant, higher average abundance and species richness was found in the treated areas than in the controls (Fig. 3A; Pradleves: $\text{Abundance}_{\text{mean}Btk} = 216 \pm 42$; $\text{Abundance}_{\text{meanControl}} = 231 \pm 45$; $F = 0.182$, $df = 1$, $P = 0.691$; Vernante: $\text{Abundance}_{\text{mean}Btk} = 331 \pm 116$; $\text{Abundance}_{\text{meanControl}} = 239 \pm 87$; $F = 1.212$, $df = 1$, $P = 0.333$; Fig. 3B; Pradleves: $S_{\text{mean}Btk} = 33 \pm 8$; $S_{\text{meanControl}} = 34 \pm 9$; $F = 0.039$, $df = 1$, $P = 0.853$; Vernante: $S_{\text{mean}Btk} = 40 \pm 5$; $S_{\text{meanControl}} = 33 \pm 6$; $F = 2.630$, $df = 1$, $P = 0.180$). Similarities in the overall abundance and species richness contributed to explaining the absence of difference in the Shannon index (H'index) calculated on the butterfly communities occurring in treated and control areas (Fig. 3C; Pradleves: $H'_{\text{mean}Btk} = 4.30 \pm 0.59$; $H'_{\text{meanControl}} = 4.37 \pm 0.36$; $F = 0.036$, $df = 1$, $P = 0.860$; Vernante: $H'_{\text{mean}Btk} = 4.50 \pm 0.30$; $H'_{\text{meanControl}} = 4.29 \pm 0.30$; $F = 0.067$, $df = 1$, $P = 0.437$).

Because the H' index considers both the species richness and their abundance, its variation was compared among the three sampling years (Fig. 3D). The H' index of the non-target butterflies in treated and control patches follows similar trends, showing a peak in 2020. However, the H' index variation in the treated areas is only nearly significant ($F = 7.834$, $df = 2$, $P = 0.064$), with a significant difference only between the data collected in 2019 and 2020 (Tukey, $P < 0.001$). In the control areas, the H' index varies among the years ($F = 35.479$, $df = 2$, $P = 0.008$), showing significant pairwise differences between 2019 and

Table 1

Risk assessment. A. Presence of larval stages (1 = active larvae present; 0 = larvae absent). B. Number of generations (0.75 = Multivoltine, more than two generations a year; 1.75 = Bivoltine, two generations a year; 3.75 = Univoltine, one generation a year). C. Presence of host plant (1 = at least one host plant present in the treated site; 0 = host plant absent). D. Relative abundance of the host plant in the treated compared to the control areas measured with Braun-Blanquet see 2.4.2 for more details ("<" Lower abundance of the host plant in the treated than control areas = 0.25; "=" Equal abundance of the host plant and control areas = 0.5; ">" Higher abundance of the host plant in the treated than control areas = 1.25). Risk Index (RIx) is given by AC*(B + D). Impact categories (I) are defined as: Null (RIx = 0); Low ($1 \leq \text{RIx} \leq 2$); Moderate ($2 < \text{RIx} \leq 4$); High (RIx > 4).

Butterfly Phenology		**Host plants**		**Risk Categories**	
A. Presence of larval stages during treatment with *Btk*	B. Number of generations	C. Host plants present in the area treated with *Btk*	D. Abundance of the host plant in the treated vs. control areas	Index RIx=AC(B+D)	Impact (I)
0	.	0	.	0	Null
0	.	1	.	0	Null
1	.	0	.	0	Null
1	Multivoltine (0.75)	1	< (0.25)	1	Low
1	Multivoltine (0.75)	1	= (0.5)	1.25	Low
1	Multivoltine (0.75)	1	> (1.25)	2	Low
1	Bivoltine (1.75)	1	< (0.25)	2	Low
1	Bivoltine (1.75)	1	= (0.5)	2.25	Moderate
1	Bivoltine (1.75)	1	> (1.25)	3	Moderate
1	Univoltine (3.75)	1	< (0.25)	4	Moderate
1	Univoltine (3.75)	1	= (0.5)	4.25	High
1	Univoltine (3.75)	1	> (1.25)	5	High

Table 2
Average (mean ± standard error) number of *Cydalima perspectalis* larvae recorded in 2019, 2020, and 2021 in *Btk* treated and control plots before insecticide applications. Data refers to both Pradleves and Vernante sites. Analysis of Variance (ANOVA) statistics and p-value are reported.

Year	*Btk* treated plot	Control plot	F	P
2019	5.32 ± 0.225	5.25 ± 0.223	0.049	0.825
2020	4.58 ± 0.171	4.30 ± 10.155	1.466	0.227
2021	4.26 ± 0.186	4.41 ± 0.179	0.934	0.335

2020, as well as 2019 and 2021 communities.

As expected, the abundance of butterflies varies during the season and peaks from mid-July to early August in both the sites, Pradleves and Vernante (III-IV sampling events; Fig. 4). The butterfly abundance showed a similar trend in treated and control areas. Differences between the total number of individuals recorded in the treated and control patches in single monitoring are significant only in the VIII event in Vernante ($\text{Abundance}_{\text{mean}Btk} = 29 \pm 5$; $\text{Abundance}_{\text{meanControl}} = 15 \pm 1$; F = 24.615, df = 1, P = 0.008), where a higher number of individuals was found where the *Btk* was sprayed, and in Pradleves ($\text{Abundance}_{\text{mean}Btk} = 8 \pm 2$; $\text{Abundance}_{\text{meanControl}} = 12 \pm 2$; F = 10.286, df = 1, P = 0.033), where a higher number of specimens was observed in the control area.

3.4. Risk assessment

Coverages of butterfly host plants occurring in the two sites within the two experimental zones (*Btk*-treated and control) are reported in Table S1a,b.

Following the procedure described in the method section, each butterfly species received a score between 0 and 5, namely the risk index (RIx), which is translated into impact category (I), ranging from null to high (see Table S2a,b for details). Overall, among the butterfly species occurring in all three years, 27 out of 56 (accounting for 48.2 %) in Vernante and 21 out of 56 (accounting for 37.5 %) in Pradleves

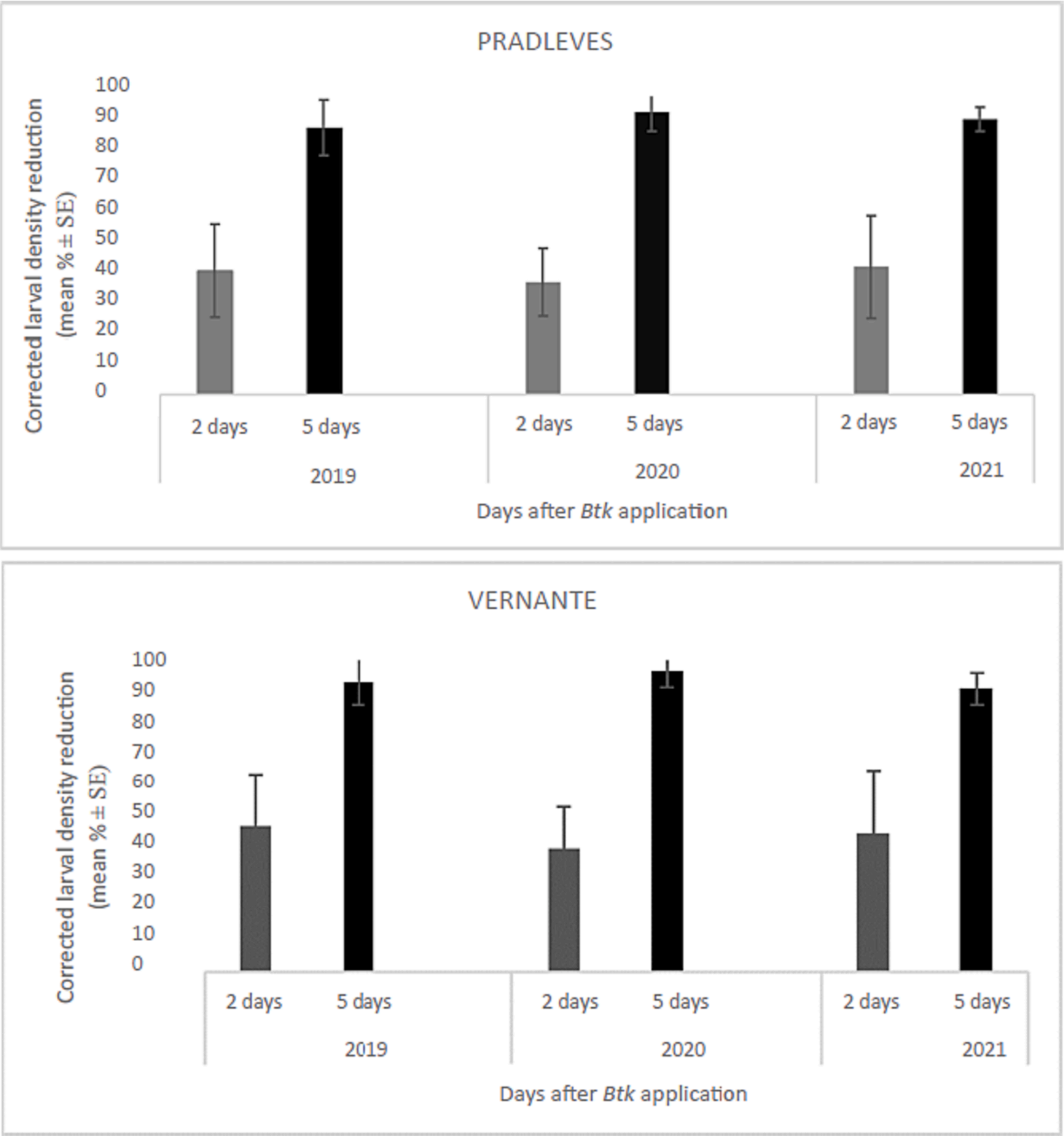

Fig. 1. Percentage reduction of *Cydalima perspectalis* larvae (L1, and L2 instars) 2 and 5 days after *Btk* treatment in Pradleves (top graph) and Vernante sites (bottom graph) in the three-year period 2019–2021. Larval reductions were corrected with the Schneider–Orelli formula.

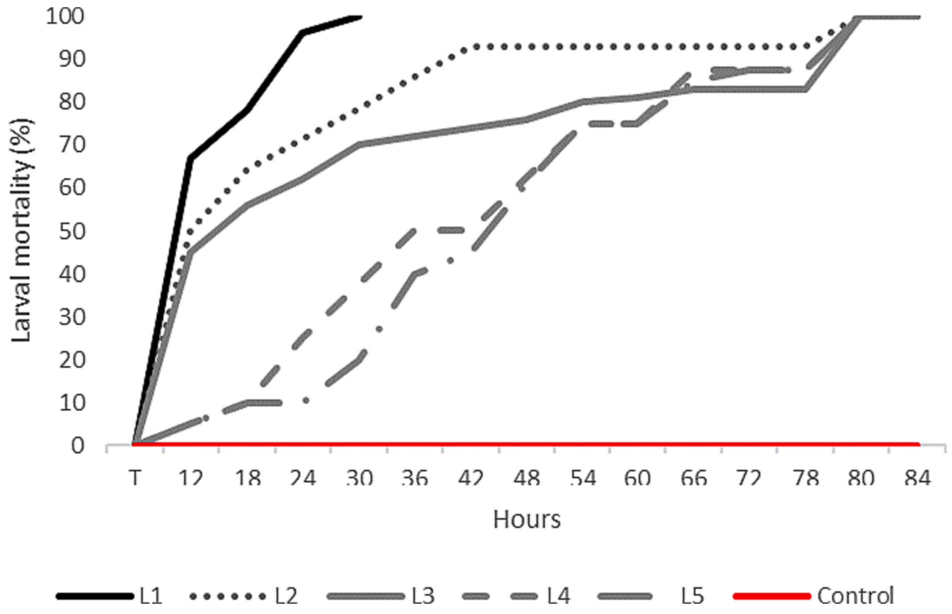

Fig. 2. Mortality (%) of *Cydalima perspectalis* larvae according to the instar (L1, L2, L3, L4, L5) and the number of hours since the *Btk* treatment (T) in laboratory conditions. The values shown are mean among the replicates (5 larvae per 4 replicates per 5 instars).

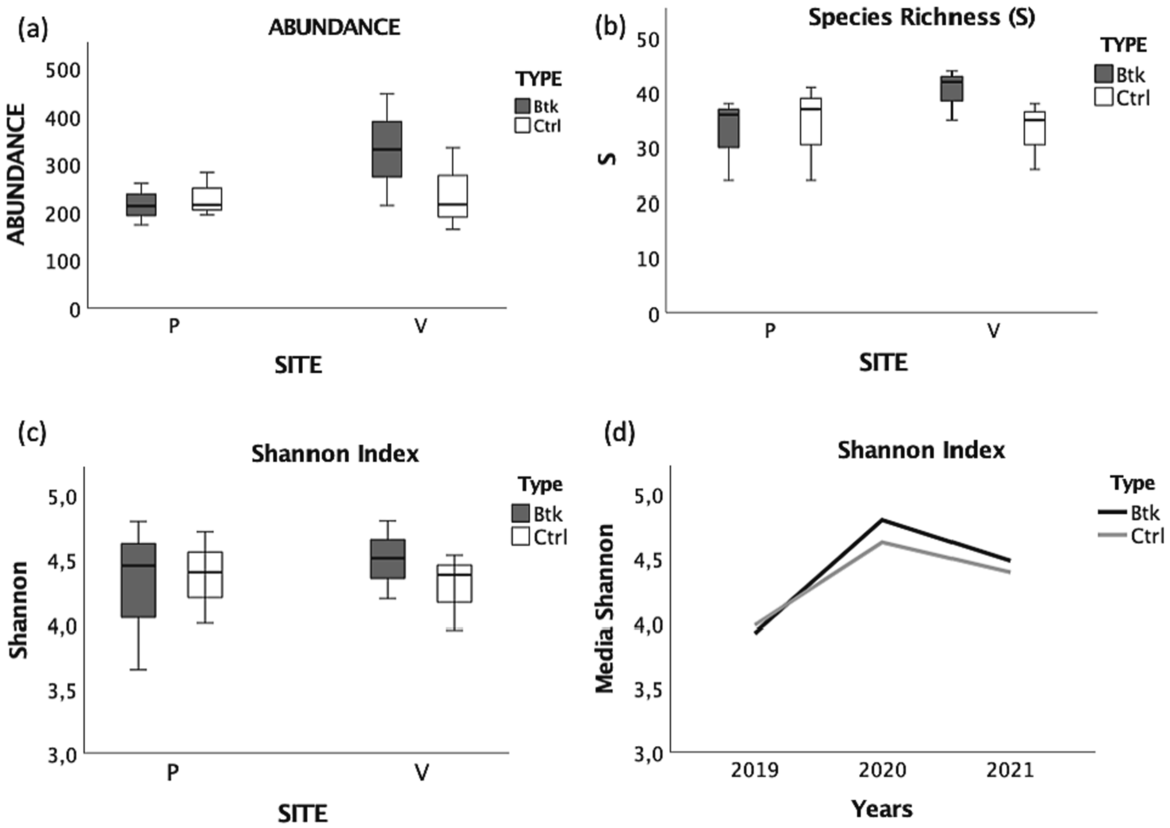

Fig. 3. Comparisons in the Abundance of individuals (A), Species Richness (B), and Shannon index (C) of non-target butterfly communities occurring in *Btk*-treated sites (dark gray box-plots) and control areas (white box-plots) at the two study sites (P = Pradleves; V = Vernante). Horizontal line = average value; box = 25th–75th percentiles; whiskers = minimum and maximum values. Variation of mean Shannon Index over time (D). The black line refers to *Btk*-treated sites, gray line refers to control areas.

potentially face moderate to high impact due to the *Btk* treatment (Fig. 5).

However, no local extinctions or variation in the abundance of these potentially high-impacted species were observed in treated areas that can be correlated to the *Btk*-spraying. When comparing the three years, if decreases in one species' abundance were found in the treated areas, they were also observed in the control areas.

Among all the species censused, only *Parnassius mnemosyne* (L.,

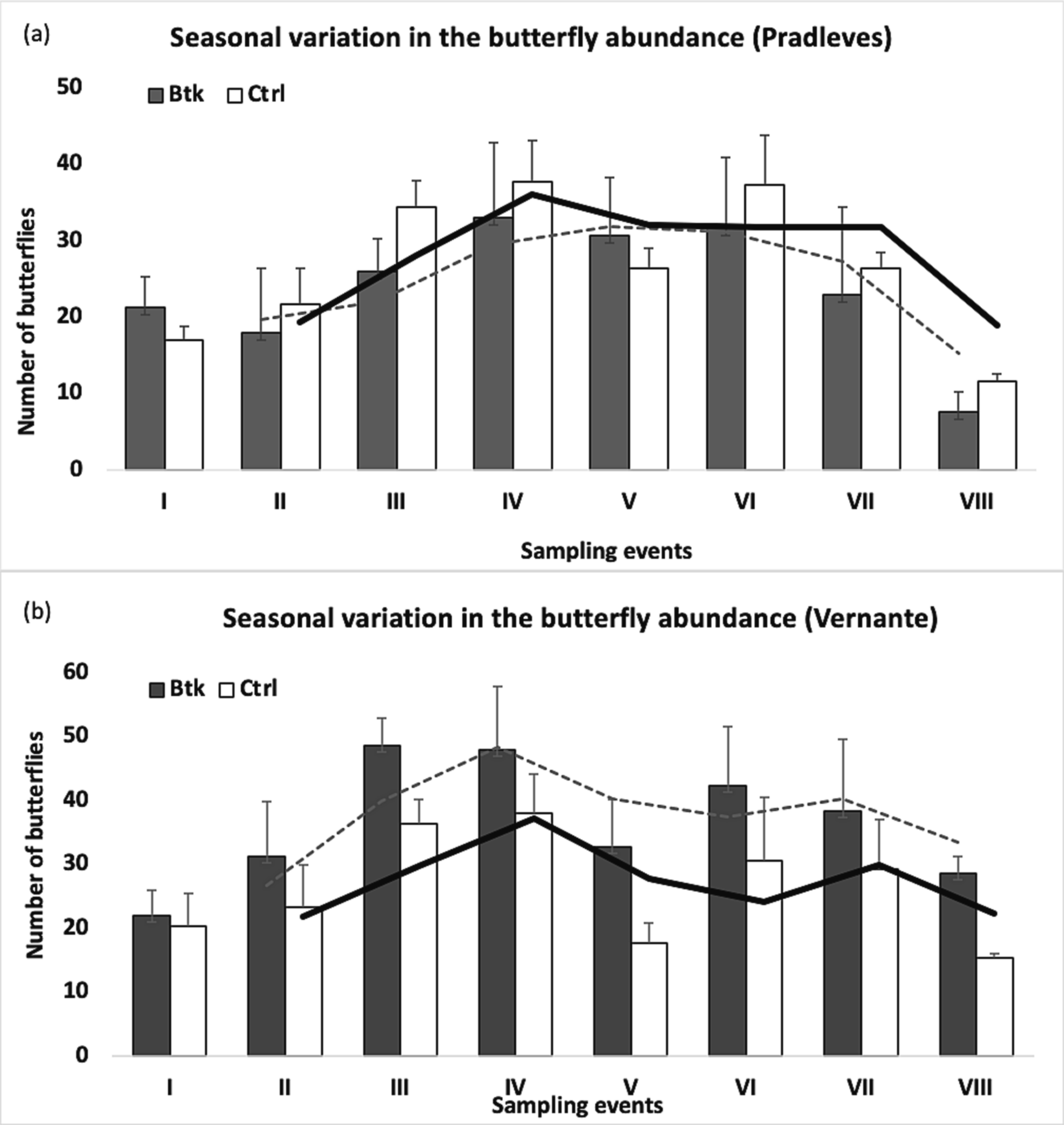

Fig. 4. Seasonal variation in the non-target butterfly abundances in Pradleves (A) and Vernante (B) sites. Average values calculated over the three years and standard deviations for each sampling occasion are shown. Dark gray bars report data collected in *Btk*-treated areas, while white bars illustrate data collected in control areas.

1758) is a Habitats Directive protected species, listed in annex IV. Even if its host plant (*Corydalis solida*) is abundant in the treated areas, the larval stages are present and feed only until the end of May (Cini et al., 2020), so the species is not threatened by *Btk* treatments planned for controlling the BTM (mid-July, early-September).

Euchloe tagis (Hübner, 1804) is considered a Least Concern species at the European level, whereas it is Near Threatened according to the Italian Red List (Bonelli et al., 2018) due to the fragmentation of its populations occurring only in the Maritime Alps and Tuscany. However, the impact of the *Btk* scheduled to optimize the control on BTM can be considered null, as only pupae are present when microbiological treatments are performed.

All the other species occurring in the two sites are of Least Concern at Italian level (Bonelli et al., 2018).

4. Discussion

Microbial bioinsecticides, particularly based on *B. thuringiensis*, have increased their importance as pest resistance and environmental concerns reduce the usefulness of conventional broad-spectrum insecticides (Ranjbari et al., 2011). Most of the *Bt* formulations are used to control many common leaf-feeding caterpillars. The most common applications include the larvae of the gypsy moth, *Lymantria dispar* (L., 1758) and the pine processionary moth *Thaumetopoea pityocampa* (Denis and Schiffermüller, 1775) in forestry, and the European corn borer larvae, *Ostrinia nubilalis* (Hübner, 1796) in agriculture (Sample et al., 1996; Sanchis and Bourguet, 2008; Ferracini et al., 2020).

However the implementation of *Btk* control strategies in nature must consider risk management and need to take into account two essential questions (i) how will the treatment affect the non-target species? (ii) Could this potential detrimental effect be mitigated or minimized?

These two questions become even more crucial when pest control strategies have to be carried out in natural protected areas.

Even though the efficacy of *Btk* treatments in the field can be affected by the spray droplet size, weather conditions, larval instar, and density (Boulton and Otvos, 2004; Matošević 2013), the protocol used in this

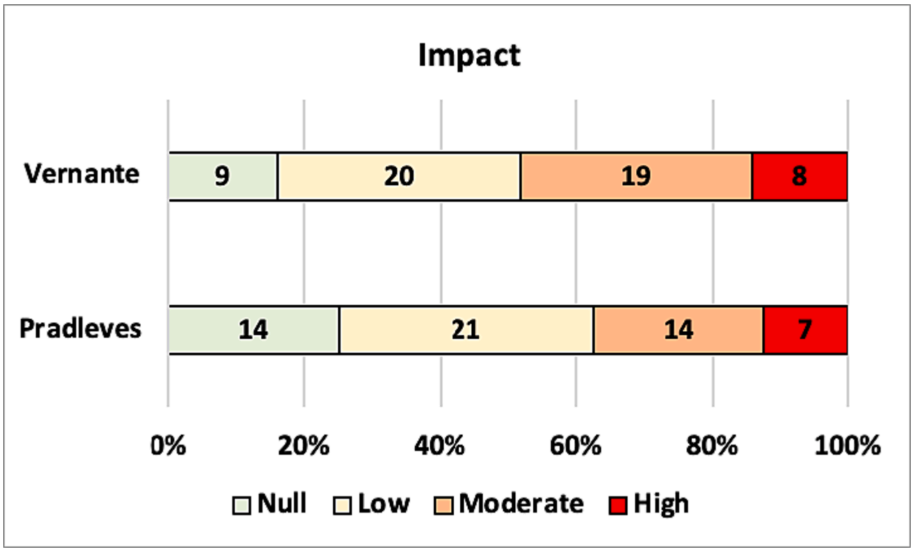

Fig. 5. Impact categories. Numbers and proportions of non-target butterfly species are reported for each category, ranging from Null to High impact.

work proved to strongly reduce the number of BTM larvae to such an extent that the boxwood was able to maintain lush, green foliage throughout the growing season. Even plants seriously damaged in the previous years, started resprouting thus recovering in the following season. The regeneration capability of boxwood is argued to be high enough to ensure the viability of the species under different disturbance pressures (pest, fire, and drought impacts); however, it is still unknown whether the plant is capable to resprout after repetitive BTM attacks (Canelles et al., 2022). The monitoring carried out in a close area (Tanaro Valley) where *Btk* treatments were not implemented showed that box tree shrubs could not recover after three consecutive years of BTM pressure (authors' personal observation).

One of the most important aspects of pest management when applying *Bt* is the host stage. It has been shown in laboratory and field bioassays that third instar larvae of Lepidoptera and Coleoptera are less susceptible to *Bt* products than younger larvae (preferably neonates) (Navon, 2000). Our data corroborate the findings of previous investigations (Burjanadze et al., 2019; Salioğlu and Göktürk, 2021; Tozlu et al., 2022), highlighting a larval density reduction (L1 and L2 instar) over 90 % in all sites and years, five days after spraying. Similar mortality values were achieved in controlled conditions, and mortality times proved to be longer, the higher the larval age. Specifically, L4 larvae took three times longer to die than L1 larvae.

As well as the use of microbiological treatments, various strategies were implemented to control the BTM in the native areas, such as biological control by nematodes (Choo et al., 1991; Lee et al., 1996) and mating disruption (Kawazu et al., 2007). In terms of chemical control, broad-spectrum insecticides proved to be the most effective (Zhou et al., 2005) , although responsible for undesirable effects on pollination and non-target organisms, and thus applicable only under limited conditions (Oberemok et al., 2015). The use of natural enemies, introduced from the native region or present in the newly colonized area, and specific pathogens of BTM have been proposed to halt the outbreak of BTM. Still, they need further investigation to assess their specificity, effectiveness, and side effects (Rose et al., 2013; Wan et al., 2014; Oberemok et al., 2017; Morel et al., 2021). On the other hand, less invasive methods comprise the mechanical removal of BTM larvae manually (Kenis et al., 2013), but this practice is only feasible for small trees and it is very laborious, requiring the involvement of lots of people to cover enough *Buxus* plants. Indeed, the BTM could be an outstanding example of a pest that can be not only monitored but also controlled by citizen science programs since it is morphologically distinctive from other species and does not face any identification issues (Bereś et al., 2021; Crow et al., 2020; Kazilas et al., 2021; Guarrasi, 2019). However, this control strategy is affected by the toughness of getting people involved and maintaining its commitment over a long period (Crow et al., 2020).

Microbiological treatments based on *Btk* spraying are not flawless as they (i) require professional use; (ii) can be costly; (iii) could negatively impact non-target species; (iv) must be strictly scheduled in close relation to monitoring; (v) could be less effective when plants are found in dense forests (incomplete spray coverage); (vi) can be subject to rapid degradation by UV radiation, or by washing off by rain or overhead irrigation. Moreover, an excessive use of *Bt* in pest control may induce resistance in target organisms, as increasingly reported for agricultural pests (Navon, 2000).

Nevertheless, bioinsecticides based on *B. thuringiensis* have been proven highly effective and they are usually the preferred option on ornamental box trees because of their limited impact on the environment, and their use in field applications has been generally accepted (Yaman, 2023).

Findings gathered over a three-year sampling period suggest that the protocol followed in Pradleves and Vernante to disperse the *Btk* is effective against BTM larvae and does not negatively impact the diversity or abundance of the local butterfly community.

Results on the effect of *Btk* treatment on non-target species in the field are controversial and primarily related to the strategies implemented to control the gypsy moth in North America (Solter and Hajek, 2009). These surveys revealed both detrimental (e.g., Miller 1990a,b; Whaley et al., 1998; Rastall et al., 2003) and positive effects (Manderino et al., 2014) on other Lepidoptera because the susceptibility to *Btk* varies with species, the extent of treated areas and the frequency, spray modalities or timing of the treatments (e.g., Herms et al., 1997; Boulton and Otvos, 2004). Therefore, in this work, the effect of *Btk* was evaluated on the most sensitive component of the lepidopteran community, i.e., butterflies. Several studies concur in reporting a higher sensitivity to *Btk* of butterflies than moths (e.g., Wagner et al., 1996; Peacock et al., 1998). The difference in tolerance could be physiological but also partially explained by larval behavior that, in the case of several moth species, involves using shelters and prevent the ingestion of *Btk*-contaminated food resources (Martinat et al., 1988; Wagner et al., 1996).

The spatial scale and the period under consideration to assess the impact of *Btk* on non-target species are other key factors that can strongly affect results. Numerous investigations showed significant shortcomings by comparing non-target Lepidoptera communities between treated and control areas, which showed *a priori* differences due to their habitat composition and broad spatial distance (see Miller 1990a,b). Also, when aiming at assessing the long-term impact of the *Btk*, researchers have to consider that the variation in species abundance can be masked (Sample et al., 1996) by density-dependent or stochastic fluctuations known to occur in lepidopteran populations (Nowicki et al., 2009). Thus, sites were carefully selected close enough to be characterized by the same habitat and butterfly communities while avoiding treatment drift effects, and the data collection spanned over three years in both control and treated sites. Lastly, adult butterflies were monitored instead of larvae since increased larval mortality caused by *Btk* should be mirrored in short- and long-term reduction of species richness and/or adult abundance. Methods to assess butterfly population dynamics are standardized and allow robust comparison between areas and across years (Pollard, 1977), whereas larval presence could be underestimated, and species identification could easily lead to mistakes (Ehrlich and Raven, 1964).

In the absence of other large-scale disturbance events and considering seasonality, synchronized declines in most of the butterfly species in a community are not expected, but their occurrence can be explained if *Btk* treatment severely affects non-target species (Severns, 2002). No significant decrease or difference was found when comparing the within-season and among-year variations in abundances or diversities between the butterfly assemblages occurring in the *Btk*-sprayed and control areas. Furthermore, no decreases or local species extinctions were observed in the treated areas not revealed in the control zones. All the butterfly communities showed increased diversity from the first year of treatments, and trends in abundance were expected as intrinsic butterfly fluctuations (Nowicki et al., 2009) since they paralleled in treated and control populations.

Although these results suggest that a microbiological treatment narrowed spatially and temporally will not be detrimental for non-target species, both at species and community levels, about 40 % of species occurring in the investigated sites could be impacted negatively (from moderate to severe) by *Btk* spraying. Based on ecological and functional traits of non-target species, this finding should not be underrated while conceiving a control strategy based on *Btk*. Indeed, the potential sensitivity of several species, which in this work account for almost half of the community, explained why treating thousands of hectares (o acres) with *Btk* caused a severe decline in non-target species such as those observed, for instance, in the studies by Miller (1990a,b). In contrast, the need to preserve the local butterfly community by treating several small patches scattered on the surface where *B. sempervirens* bushes occur is highlighted by this work.

If implemented when voracious first larval stages of BTM are present, the patchy application of *Btk* effectively controls the pest outbreak. It allows the full resprouting of plants in the following year, as witnessed in treated areas, without causing detrimental effects on other lepidopteran components. Overall, a rigorous plan for the application, detection and surveillance of the effects needs to be implemented whenever *Btk* is applied. Based on this three-year survey, an *a priori* monitoring on non-target Lepidoptera turns out to be necessary to avoid detrimental effects on biodiversity caused by microbiological treatments, with special regard to Habitats Directive species.

5. Conclusions

The failure to implement effective control strategies to halt the outbreak of *C. perspectalis* has already brought to worldwide loss of priceless box forests and, consequently, of other biotic components related to protected EU Habitat 5110.

Results reported here suggest that *Btk* treatments dispensed twice a year in July and September in limited *Buxus* patches could provide the best control strategy to limit the expansion and the damage caused by *C. perspectalis*. Indeed, the *Btk* spraying operations proved effective in killing the BTM larvae, whereas there was no evidence of significant short- and long-term impacts on the non-target butterfly fauna. Our findings suggested that plots suitable for *Btk* treatments should be preferentially small, shaped as stripes, and scattered across the habitat to minimize the impact on non-target species. Before considering a plot feasible for *Btk* treatments, a deep survey on the potential occurrence of threatened Lepidoptera species is required. If (i) adults of rare species are captured, (ii) their host plants are present, and (iii) the phenology of their larval stages overlaps with BTM, then the *Btk* treatment should be avoided.

Ledru and colleagues (2022) described a scenario where the trade-off between the BTM colonization/extinction of patches could lead to the persistence of *Buxus* in natural areas. In other words, the metapopulation dynamics of the moth and spatial effects are of pivotal importance in explaining how the pest and its resource might coexist. Although spatial factors seem crucial in favoring this coexistence, in general, they have not been adequately considered in the context of biological invasion (Melbourne et al., 2007; Ledru et al., 2022). The findings reported here showed that causing the local extinction of the pest from patches without affecting other valuable components of the biotic fauna is feasible by employing *Btk* treatments. Therefore, this research provides insights to support the development of BTM management aiming to affect the metapopulation dynamics of the pest by promoting the reduction of moths locally. In this way, the *Buxus* trees will not be fully depleted and will have time to recover. Instead of pledging for BTM eradication, controlling the coexistence between *C. perspectalis* and its *Buxus* resources by implementing the *Btk* treatments in patches could be the adequate management design to make the impact of the BTM sustainable, thus effectively preserving the box forests.

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CRediT authorship contribution statement

Francesca Barbero: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review and editing. **Cristina Pogolotti:** Data curation, Formal analysis, Writing – review & editing. **Simona Bonelli:** Conceptualization, Methodology, Writing – review & editing. **Chiara Ferracini:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105427.

References

Balletto, E., Kudrna, O., 1985. Some aspects of the conservation of the butterflies (Lepidoptera: Papilionoidea) in Italy, with recommendations for the future strategy. Boll Soc. Entom. Ital. 117, 39–59.

Bereś, P.K., Ziętara, P., Nakonieczny, M., Kontowski, Ł., Grzbiela, M., Augustyniak, M., 2021. *Cydalima perspectalis* in Poland - 8 years of invasion against the background of three other invasive species. Diversity 14 (1), 22.

Bird, S., Raper, C., Dale-Skey, N., Salisbury, A., 2020. First records of two natural enemies of box tree moth, *Cydalima perspectalis* (Lepidoptera: Crambidae), in Britain. British J. Entomol. Natural Hist. 33, 67–70.

Bonelli, S., Casacci, L.P., Barbero, F., Cerrato, C., Dapporto, L., Sbordoni, V., Balletto, E., 2018. The first red list of Italian butterflies. Insect Conserv. Divers. 11 (5), 506–521. https://doi.org/10.1111/icad.12293.

Boulton, T.J., Otvos, I.S., 2004. Monitoring native non-target Lepidoptera for three years following a high dose and volume application of *Bacillus thuringiensis* subsp. *kurstaki*. Int. J. Pest Manag. 50 (4), 297–305.

Bras, A., Avtzis, D.N., Kenis, M., Li, H., Vétek, G., Bernard, A., Auger-Rozenberg, M.A., 2019. A complex invasion story underlies the fast spread of the invasive box tree moth (Cydalima perspectalis) across Europe. J. Pest Sci. 92, 1187–1202. https://doi.org/10.1007/s10340-019-01111-x.

Braun-Blanquet, J., 1964. Pflanzensoziologie, Grundzüge der Vegetationskunde [Plant Sociology, Basics of Vegetation Science], 3rd ed. Springer, Berlin/Heidelberg, Germany.

Burjanadze, M., Supatashvili, A., Gorkturk, T., 2019. Control strategies against invasive pest Box Tree Moth - *Cydalima perspectalis* in Georgia. SETSCI Conference Indexing System 4 (1), 1–4.

Canelles, Q., Bassols, E., Vayreda, J., Brotons, L., 2021. Predicting the potential distribution and forest impact of the invasive species *Cydalima perspectalis* in Europe. Ecol. Evol. 11 (10), 5713–5727. https://doi.org/10.1002/ece3.7476.

Canelles, Q., Aquilué, N., Brotons, L., 2022. Anticipating *B. sempervirens* viability in front of *C. perspectalis* outbreaks, fire, and drought disturbances. Sci. Total Environ. 810, 151331.

Choo, H.Y., Kaya, H.K., Lee, S.M., Kim, T.O., Kim, J.B., 1991. Laboratory evaluation of entomopathogenic nematodes, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* against some forest insect pests. Korean J. Appl. Entomol. 30, 227–232.

Cini, A., Barbero, F., Bonelli, S., Bruschini, C., Casacci, L.P., Piazzini, S., Scalercio, S., Dapporto, L., 2020. The Decline of the Charismatic *Parnassius mnemosyne* (L.) (Lepidoptera: Papilionidae) in a Central Italy National Park: A Call for Urgent Actions. J. Insect Biodivers. 16, 47–54.

Colombel, E., Tabone, E., Morel, E., Eyssan, C., Deogratias, J. M., Guérin, M., 2022. Biocontrol of the box tree moth, *Cydalima perspectalis*, with macro-organisms. International Horticultura Congress, ISHS, Aug 2022, ANGERS, France.

Crawley, M.J., 1983. Herbivory: the dynamics of animal-plant interactions. Blackwell Scientific, Oxford, UK.

Crow, P., Perez-Sierra, A., Kavčič, A., Lewthwaite, K., Kolšek, M., Ogris, N., De Groot, M., 2020. Using Citizen Science to monitor the spread of tree pests and diseases: outcomes of two projects in Slovenia and the UK. Manag. Biol. Invasions 11 (4), 703.

di Domenico, F., Lucchese, F., Magri, D., 2012. *Buxus* in Europe: Late quaternary dynamics and modern vulnerability. PPEES. 14 (5), 354–362.

Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants: a study in coevolution. Evol. 18 (4), 586–608.

Er, M.K., Karadağ, S., Mart C., 2007. Effectiveness of *Bacillus thuringiensis* var. *kurstaki* on *Thaumetopoea solitaria* Frey. (Lepidoptera: Thaumetopoeidae) larvae in laboratory conditions. Turk. J. Agric. For. 31(4), 255-261.

Espluga, B.G., Garcia-Reàdigos, M.À., 2020. Invasive box-tree moth *Cydalima perspectalis*, a new food resource for Great Spotted Cuckoo *Clamator glandarius*. Rev. Catalana D'ornitologia. 36, 70–73. https://doi.org/10.2436/20.8100.01.23.

Ferracini, C., Saitta, V., Pogolotti, C., Rollet, I., Vertui, F., Dovigo, L., 2020. Monitoring and Management of the Pine Processionary Moth in the North-Western Italian Alps. Forests. 11, 1253. https://doi.org/10.3390/f11121253.

Ferracini, C., Pogolotti, C., Mancardi, P., Miglio, M., Bonelli, S., Barbero, F., 2022. The box tree moth: an invasive species severely threatening *Buxus* natural formation in NW Italy. Forests. 13 (2), 178.

Gandhi, K.J.K., Herms, D.A., 2010. Direct and indirect effects of alien insect herbivores on ecological processes and interactions in forests of Eastern North America. Biol. Invasions 12, 389–405.

Göttig, S., Herz, A., 2016. Are egg parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) promising biological control agents for regulating the invasive box tree pyralid, *Cydalima perspectalis* (Lepidoptera: Crambidae)? Biocontrol Sci. Technol. 26(11), 1471-1488. https:/doi.org/ 10.1080/09583157.2016.1211990.

Göttig, S., Herz, A., 2018. 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. JPDP 125 (4), 365–375. https://doi.org/10.1007/s41348-018-0154-8.

Guarrasi, M.A., 2019. Potential Impacts and Control of the Non Native Box Tree Moth in Canada. https://www.cififc.org/wpcontent/uploads/2019/10/2019ConfPres_S05P2_Potential-Impacts-and-Management-of-theNon-Native-Box-Tree-Moth-in-Canada-_Mariaelana-A-Guarrasi.pdf 2018 Available online: accessed on 9 September 2023.

Herms, C.P., McCullough, D.G., Baue, L.S., Haack, R.A., Miller, D.L., Dubois, N.R., 1997. Susceptibility of the endangered Karner blue butterfly (Lepidoptera: Lycaenidae) to *Bacillus thuringiensis* var. *kurstaki* used for gypsy moth suppression in Michigan. Gt. Lakes Entomol. 30(4), 1.

Kawazu, K., Honda, H., Nakamura, S., Adati, T., 2007. Identification of sex pheromone components of the box tree pyralid, *Glyphodes perspectalis*. J. Chem. Ecol. 33, 1978–1985.

Kawazu, K., Nakamura, S., Adati, T., 2010. Rearing of the box tree pyralid, *Glyphodes perspectalis*, larvae using an artificial diet. Appl. Entomol. Zool. 45 (1), 163–168.

Kazilas, C., Kalaentzis, K., Demetriou, J., Koutsoukos, E., Strachinis, I., Andriopoulos, P., 2021. Utilization of citizen science data to monitor alien species: the box tree moth *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) invades natural vegetation in Greece. BioInvasions Rec. 10 (4), 1032–1044. https://doi.org/10.3391/bir.2021.10.4.28.

Kenis, M., Auger-Rozenburg, M.-A., Roques, A., Timms, L., Péré, C., Cock, M.J.W., Settele, J., Augustin, S., Lopez-Vaamonde, C., 2009. Ecological effects of invasive alien insects. Biol. Invasions 11, 21–45.

Kenis, M., Nacambo, S., Leuthardt, F.L.G., di Domenico, F., Haye, T., 2013. The box tree moth, *Cydalima perspectalis*, in Europe: horticultural pest or environmental disaster? Aliens: the Invasive Species Bull. 33, 38–41.

Lambert, B., Peferoen, M., 1992. Insecticidal promise of *Bacillus thuringiensis*. BioScience 42 (2), 112–122.

Ledru, L., Garnier, J., Gallet, C., Noûs, C., Ibanez, S., 2022. Spatial structure of natural boxwood and the invasive box tree moth can promote coexistence. Ecol. Modell. 465, 109844.

Lee, S.M., Lee, D.W., Choo, H.Y., 1996. Biology and pathogenicity of entomopathogenic nematodes, *Steinernema* spp., isolated from forest soil in southern Korea. JFES 53, 117–123.

Leuthardt, F.L.G., Baur, B., 2013. Oviposition preference and larval development of the invasive moth *Cydalima perspectalis* on five European box-tree varieties. J. Appl. Entomol. 137 (6), 437–444.

Leuthardt, F.L.G., Glauser, G., Baur, B., 2013. Composition of alkaloids in different box tree varieties and their uptake by the box tree moth *Cydalima perspectalis*. Chemoecology 23 (4), 203–212.

Manderino, R.O., Crist, T., Haynes, K.J., 2014. Lepidoptera-specific insecticide used to suppress gypsy moth outbreaks may benefit non-target forest Lepidoptera. Agric. For. Entomol. 16 (4), 359–368.

Martinat, P.J., Coffman, C.C., Dodge, K.M., Cooper, R.J., Whitmore, R.C., 1988. Effect of Dimilin 25-W on the canopy arthropod community in a central Appalachian forest. J. Econ. Entomol. 81, 261–267.

Matošević, D., 2013. Box tree moth (*Cydalima perspectalis*, Lepidoptera; Crambidae), newinvasive insect Pest in Croatia. South-East Eur. For. 4, 89–94.

Melbourne, B.A., Cornell, H.V., Davies, K.F., Dugaw, C.J., Elmendorf, S., Freestone, A.L., Hall, R.J., Harrison, S., Hastings, A., Holland, M., Holyoak, M., Lambrinos, J., Moore, K., Yokomizo, H., 2007. Invasion in a heterogeneous world: resistance, coexistence or hostile takeover? Ecol. 10 (1), 77–94.

Miller, J.C., 1990a. Field assessment of the effects of a microbial pest control agent on nontarget Lepidoptera. Am. Entomol. 36, 135–139.

Miller, J.C., 1990b. Effects of a microbial insecticide, *Bacillus thuringiensis kurstaki*, on nontarget Lepidoptera in a spruce budworm-infested forest. J. Res. Lepid. 29, 267–276.

Mitchell, R., Chitanava, S., Dbar, R., Kramarets, V., Lehtijärvi, A., Matchutadze, I., Mamadashvili, G., Matsiakh, I., Nacambo, S., Papazova-Anakieva, I., Sathyapala, S., Tuniyev, B., Vétek, G., Zukhbaia, M., Kenis, M., 2018. Identifying the ecological and societal consequences of a decline in *Buxus* forests in Europe and the Caucasus. Biol. Invasions 20 (12), 3605–3620.

Morel, E., Capelli, M., de Bodard, M., Colombel, E., Michel, T., Tabone, E., 2021. Research for native parasites and predators of the box tree moth *Cydalima perspectalis*, in natural boxwood forest in France. In *Int. Sci. Events-10th Int. Conf. Agr. Food*, *9* (1314-8591), 231-242.

Mostini, L., 2018. Bird predation on larvae of the box tree moth, *Cydalima perspectalis* (Walker, 1859). Res. Ornithol. 88 (2), 45–46.

Nacambo, S., Leuthardt, F.L.G., Wan, H., Li, H., Haye, T., Baur, B., Weiss, R.M., Kenis, M., 2014. Development characteristics of the box-tree moth *Cydalima perspectalis* and its potential distribution in Europe. J. Appl. Entomol. 138 (1–2), 14–26.

Navon, A., 2000. *Bacillus thuringiensis* insecticides in crop protection - reality and prospects. Crop Prot. 19 (8–10), 669–676. https://doi.org/10.1016/S0261-2194(00)00089-2.

Nentwig, W., Bacher, S., Kumschick, S., Pyšek, P., Vilà, M., 2018. More than "100 worst" alien species in Europe. Biol. Invasions 20 (6), 1611–1621.

Nowicki, P., Bonelli, S., Barbero, F., Balletto, E., 2009. Relative importance of density-dependent regulation and environmental stochasticity for butterfly population dynamics. Oecologia 161, 227–239.

Oberemok, V.V., Laikova, K.V., Gninenko, Y.U.I., Zaitsev, A.S., Nyadar, P.M., Adeyemi, T.A., 2015. A short history of insecticides. J. Plant Prot. Res. 55, 221–226.

Oberemok, V.V., Laikova, K.V., Shumskykh, M.N., Zaitsev, A.S., 2017. The first record of box tree moth in Crimea and a novel perspective of its biological control based on *Lymantria dispar* multicapsid nuclear polyhedrosis virus and DNA insecticides approach. Entomol. Gen. 36 (3), 207–217.

Peacock, J.W., Schweitzer, D.F., Carter, J.L., Dubois, N.R., 1998. Laboratory assessment of the effects of *Bacillus thuringiensis* on native Lepidoptera. Environ. Entomol. 27 (2), 450–457.

Poitou, L., Bras, A., Pineau, P., Lorme, P., Roques, A., Rousselet, J., Auger-Rozenberg, M. A., Laparie, M., 2020. Diapause regulation in newly invaded environments: termination timing allows matching novel climatic constraints in the box tree moth, *Cydalima perspectalis* (Lepidoptera: Crambidae). Insects 11 (9), 629.

Pollard, E.A., 1977. Method for assessing changes in the abundance of butterflies. Biol. Conserv. 12, 115–124.

Pollard, E., Yates, T.J., 1993. Monitoring Butterflies for Ecology and Conservation. Chapam and Hall, London, UK.

Pünterner, W., 1981. Manual for field trials in plant protection. In Agricultural Division; Ciba-Geigy, Ltd.: Basle, Switzerland.

Raineri, V., Bonechi, F., Caracciolo, D., Cresta, P., Mariotti, M., 2017. *Cydalima perspectalis* (Walker, 1859) (Lepidoptera, Crambidae) and the threats for the nature 2000 habitat 5110 in Liguria (NW-Italy). Boll. Mus. Ist. Biol. Univ, Genova, p. 79.

Ranjbari, S., Safaralizadeh, M.H., Aramideh, S., 2011. Insecticidal effect of *Bacillus thuringiensis* var *kurstaki* on the various instars larvae of *Plutellax ylostella* L. (Lep.: Plutellidae) under laboratory condition. Egypt Acad. J. Biol. Sci. 3 (1), 27–32.

Rastall, K., Kondo, V., Strazanac, J.S., Butler, L., 2003. Lethal effects of biological insecticide application on nontarget lepidopterans in two Appalachian forests. Environ. Entomol. 32, 1364–1369.

Redman, A.M., Scriber, J.M., 2000. Competition between the gypsy moth, *Lymantria dispar*, and the northern tiger swallowtail, *Papilio canadensis*: interactions mediated by host plant chemistry, pathogens, and parasitoids. Oecologia 125, 218–228.

Rose, J., Kleespies, R.G., Wang, Y., Wennmann, J.T., Jehle, J.A., 2013. On the susceptibility of the box tree moth *Cydalima perspectalis* to *Anagrapha falcifera* nucleopolyhedrovirus (AnfaNPV). J. Invertebr. Pathol. 11, 191–197.

Salioglu, S., Göktürk, T., 2021. Determination of the effects of some entomopathogens on *Cydalima perspectalis* (Lepidoptera: Crambidae) larvae. Turk. J. Biod. 4 (1), 31–35.

Sample, B.E., Butler, L., Zivkovich, C., Whitmore, R.C., Reardon, R., 1996. Effects of *Bacillus thuringiensis* Berliner var. *kurstaki* and defoliation by the gypsy moth [(*Lymantria dispar* (L.) (Lepidoptera: Lymantriidae)] on native arthropods in West Virginia. Can. Entomol. 128, 573–592.

Sanchis, V., Bourguet, D., 2008. *Bacillus thuringiensis*: applications in agriculture and insect resistance management. A Review. Agron. Sustain. Dev. 28, 11–20.

Santi, F., Radeghieri, P., Sigurtà, G.I., Maini, S., 2015. Sex pheromone traps for detection of the invasive box tree moth in Italy. Bull. Insectology 68 (1), 158–160.

Scriber, J.M., 2004. Non-target impacts of forest defoliator management options: Decision for no spraying may have worse impacts on non-target Lepidoptera than *Bacillus thuringiensis* insecticides. J. Insect Conserv. 8, 241–261.

Severns, P.M., 2002. Evidence for the negative effects of BT (*Bacillus thuringiensis* var. *kurstaki*) on a nontarget butterfly community in western Oregon, USA. J. Lepid. Soc. 56 (3), 166–170.

Solter, L.F., Hajek, A.E., 2009. Control of Gypsy Moth, *Lymantria dispar*, in North America since 1878. In: Hajek, A.E., Glare, T.R., O'Callaghan, M. (Eds.), Use of Microbes for Control and Eradication of Invasive Arthropods. Progress in Biological Control, vol 6. Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-8560-4_11.

Suárez-Muñoz, M., Bonet-García, F., Hódar, J.A., Herrero, J., Tanase, M., Torres-Muros, L., 2019. INSTAR: an agent-based model that integrates existing knowledge to simulate the population dynamics of a forest pest. Ecol. Modell. 411, 108764 https://doi.org/10.1016/j.ecolmodel.2019.108764.

Tozlu, E., Tozlu, G., Kotan, R., Tekiner, N., Dadaşoğlu, F., Göktürk, T., 2022. Eco-friendly control method against invasive pest box tree moth, (*Cydalima perspectalis* (Walker) (Lepidoptera: Crambidae)). Egypt. J. Biol. Pest Control 32, 96. https://doi.org/10.1186/s41938-022-00595-7.

van der Straten, M.J., Muus, T.S.T., 2009. The box tree pyralid, *Glyphodes perspectalis* (Lepidoptera: Crambidae), an invasive alien moth ruining box trees. Proc. Netherlands Entomol. Soc. Meeting 21, 107–111.

Wagner, D.L., Peacock, J.W., Carter, J.L., Talley, S.E., 1996. Field assessment of *Bacillus thuringiensis* on nontarget Lepidoptera. Environ. Entomol. 25, 1444–1454.

Wagner, D.L., Grames, E.M., Forister, M.L., Berenbaum, M.R., Stopak, D., 2021. Insect decline in the anthropocene: death by a thousand cuts. Proc. Natl. Acad. Sci. 118 (2).

Wan, H., Haye, T., Kenis, M., Nacambo, S., Xu, H., Zhang, F., Li, H., 2014. Biology and natural enemies of *Cydalima perspectalis* in Asia: is there biological control potential in Europe? J. Appl. Entomol. 138 (10), 715–722. https://doi.org/10.1111/jen.12132.

Warren, M.S., Maes, D., Van Swaay, C.A., Goffart, P., Van Dyck, H., Bourn, N.A., Ellis, S., 2021. The decline of butterflies in Europe: problems, significance, and possible solutions. Proc. Natl. Acad. Sci. 118 (2), e2002551117.

Whaley, W.H., Anhold, J., Schaalje, G.B., 1998. Canyon drift and dispersion of *Bacillus thuringiensis* and its effects on select nontarget Lepidoptera in Utah. Environ. Entomol. 27, 539–548.

Yaman, M., 2023. Potential of entomopathogens in biological control of the box-tree moth, *Cydalima perspectalis* (Lepidoptera: Crambidae). Balt. For. 29 (1), id603. https://doi.org/10.46490/BF603.

Zhou, W., Xia, C.Y., Sun, X.Q., Zhu, B., Liu, X.-P., Liu, Z.C., Wang, Y., 2005. Studies on the biological characteristics and control of *Diaphania perspectalis* Walker. J. Shanghai Jiaotong Univ. (agric. Sci.) 23, 52–56.