https://doi.org/10.17221/126/2019-PPS

Larval feeding of *Cydalima perspectalis* on box trees with a focus on the spatial and temporal distribution

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Citation: Kulfan J., Dzurenko M., Parák M., Sarvašová L., Saniga M., Brown P.M.J., Zach P. (2020): Larval feeding of *Cydalima perspectalis* on box trees with a focus on the spatial and temporal distribution. Plant Protect. Sci., 56: 197–205.

Abstract: The box tree moth *Cydalima perspectalis* is an invasive pest on box trees originating from Eastern Asia which spread throughout Europe. We assessed the efficacy of photo-eclectors (emergence boxes) for the detection of its larvae. We also investigated their spatial distribution on the hosts and the spatial and temporal distribution of the leaf damage caused by this pest in Slovakia. Our results showed non-uniform vertical distribution of the overwintering larvae and leaf damage on the trees. The larval abundance in the spring was significantly affected by the height of the branches above the ground. During spring, the larvae occurred most abundantly in the upper parts of the trees. The leaf damage was greatest in the lower parts throughout the growing season. During the progress of the infestation, the development of the damage in the lower and middle parts was similar. In the upper part, the initial increase in the damage was slow, but accelerated four months before the complete defoliation. The field estimation of the proportion of damaged leaves and the accurate assessment based on counting the damaged leaves suggest a consistency between these estimates. Hence, a quick field estimation of the leaf damage may be utilised by horticultural practices.

Keywords: box tree moth; *Buxus*; invasive species; photo-eclectors; leaf damage

The box tree moth *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae), an invasive species native to Eastern Asia (Inoue 1982), currently occurs in most parts of Europe (Bras et al. 2019). This species is causing serious damage to natural box tree stands (Kenis et al. 2013; Raineri & Mariotti 2017) and ornamental box trees in urban areas of many European countries (Van der Straten & Muus 2010; Salisbury et al. 2012; Kenis et al. 2013; Matošević 2013; Fora & Poşta 2015).

The development of these moths, from oviposition to pupation, takes approximately 37 days at a temperature of 25 °C (Maruyama & Shinkaji 1987). The moth overwinters as young larvae between spun leaves (Nacambo et al. 2014) and can have up to three generations per year within Europe (Nacambo et al. 2014; Raineri & Mariotti 2017). In Central Europe, *C. perspectalis* usually has two generations per year and adults occur from May to July and from August to October (Nacambo et al. 2013; Šefrová et al. 2019).

Supported by the research Grants Nos APVV-14-0567, VEGA 2/0012/17 and VEGA 2/0032/19.

https://doi.org/10.17221/126/2019-PPS

To effectively protect ornamental box trees from *C. perspectalis* attacks, knowledge of the spatial and temporal distribution of the larvae on the infested trees is crucial. This will facilitate the early detection of the infestations and a more effective control of this pest. The tiny young larvae hiding between the leaves and their feeding marks can easily be overlooked (Salisbury et al. 2012; Leuthardt & Baur 2013; Matošević 2013). The common methods for sampling insects such as beating branches or sweeping trees (Basset et al. 1997), are ineffective for collecting the larvae of *C. perspectalis* developing in trimmed bushy box trees with dense foliage. However, the small inconspicuous larvae of certain moth taxa (e.g., Batrachedridae, Tortricidae) can be obtained from their host plants using photo-eclectors (emergence boxes) (Kulfan et al. 2010, 2016; Parák et al. 2015), and these tools could be utilised to record the density of *C. perspectalis.*

Herein, we focus on (1) the efficacy of the photo-eclectors for the early detection of the presence and abundance of the *C. perspectalis* larvae on the box trees, (2) the effect of the height level (height of branches above the ground) and the aspect (orientation) of the box trees (northern and southern) on the larval abundance and leaf damage, (3) the temporal development of the leaf damage by the larvae, and (4) the suitability of the field estimation of the leaf damage for horticultural practices. Emphasis was placed on large and old box trees.

There is evidence of the uneven distribution of Lepidoptera larvae on host plants (Ali et al. 1990; Torres et al. 2001; Paula-Moraes et al. 2012; Kulfan et al. 2016). We hypothesise that the larvae of *C. perspectalis* and/or their feeding marks could be present in certain parts (height levels) of box trees more abundantly than in other parts. We expected that the larval abundance and severity of the leaf damage on the box trees could change specifically at the different height levels over time (from generation to generation).

MATERIAL AND METHODS

Study area and habitat of *Cydalima perspectalis*

To conduct the study, old and large box trees not treated with pesticides had to be found in a sufficient number. The trees had to be in the early stages of infestation by *C. perspectalis*. We expected that the moth would kill them over time and that the owners would accept the possible loss of their valuable trees. Considering these conditions, we found two ornamental hedges of the box tree *Buxus sempervirens* Linnaeus var. arborescens in the town cemetery in Zvolen in Central Slovakia (48,581456N, 19,140946E, 340 m a.s.l.). They each were approximately 3 m tall, 20 m long and 1.5 m wide, and were neither trimmed nor treated with pesticides. Both hedges followed a west-east direction. The hedges were comprised of trees thicker than 3 cm (the stem diameter measured at ground level) with an average density of approximately 10 stems/m. Until 2013, no damage was observed on these hedges in the Zvolen cemetery, and minor signs of larval feeding of *C. perspectalis* were first observed here in June 2014. The permit to conduct studies in the Zvolen cemetery was obtained in the summer of 2015 and our study could start soon thereafter. In the same year, the first signs of serious damage to the box trees were recorded. Most of our investigations, including all the destructive methods applied, were made on the two selected ornamental hedges in the Zvolen cemetery.

To gain broader insights into the performance of *C. perspectalis*, additional observations were conducted on solitary box trees in another 15 cemeteries in Slovakia within an area between Bratislava (48.148473N, 17.122221E), Plášťovce (48.163806N, 18.979581E), Gemerská Panica (48.473124N, 20.357510E), Betliar (48.701519N, 20.514467E), and Dubnica nad Váhom (48.976474N, 18.201468E) in the summer of 2016 and 2017. In these cemeteries, we estimated the leaf damage by *C. perspectalis* by visual inspection. The estimations were made for the lower, middle and upper parts of the trees.

Collecting data about *Cydalima perspectalis*

The efficacy of photo-eclectors for recording the occurrence and distribution of C. perspectalis larvae. To judge the efficacy of the photo-eclectors to detect the overwintering larvae and to determine the spatial distribution of larvae in the early spring, branches of the box trees (each 50 cm long) were collected from one infested hedge in the Zvolen cemetery on 21 March 2016. Forty-two branches were randomly sampled from each of the three height levels – lower (0.5 m), middle (1.5 m) and upper level (2.5 m above the ground) – and from two hedge aspects (southern and northern). In total, $42 \times 3 \times 2 = 252$ branches were sampled and stored separately in plastic bags for transportation and then placed in the photo-eclectors in the laboratory (see Zach 1991 for the eclector design), each eclector contained six branches from the same height and aspect.

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Additionally, in the same location, on 26 April 2016, three branches (each 50 cm long) were sampled from each height level and aspect to verify the efficacy of the photo-eclectors to record the older active larvae. In total, 3 × 3 × 2 = 18 branches were sampled, each eclector contained one branch. The larvae were collected from the glass collectors of the eclectors at 1 to 3-day intervals over one month. This enabled one to also record the adult moths of the larvae, which were consuming the drying leaves of the box trees and terminated their development in the photo-eclectors. The investigations took place in LD 12 : 12 (alternating 12 h light : 12 h dark), corresponding to the actual photoperiod during spring at 22–24 °C.

Distribution of leaf damage by the C. perspectalis larvae. To evaluate the infestation of the hedges by the *C. perspectalis*, the damage to the foliage was recorded from 23 to 30 July 2015 (the first signs of severe damage), 6 to 7 April 2016 (leaf damage after winter), and 2 August 2016 (the progress in the temporal development of the leaf damage) in the Zvolen cemetery. The percentage of the damage was assessed separately for each of the two hedges, the height levels and aspects. The damage was assessed on 15 spots. Each spot represented a circular area (a diameter of 30 cm) on the crown surface. It was located within each of the three height levels. The horizontal distance between the spots within the particular height level was 0.5 m. In total, 180 spots (15 spots × 3 height levels × 2 aspects × 2 hedges) were analysed.

Two methods were used to assess the *C. perspectalis* leaf damage in the Zvolen cemetery. (*i*) The precise counting of the leaves on the sample twigs. Twigs (each 30 cm long) were sampled from each spot, placed separately in a plastic bag and transported to the laboratory. On each twig, we first counted all the leaves and subsequently counted the leaves with more than 50% damage. The completely consumed or shed leaves were determined by the leaf scars persisting on the twig (undamaged leaves do not fall off spontaneously) and were also counted. The percentage of defoliation was based on the number of leaves shed and damaged more than 50%. The leaves were counted separately for the terminal (last non-branching shoot) and the basal (remaining) part of the twig. (*ii*) The visual estimation of the percentage of the leaf damage on the hedges in the field. The estimation (accuracy 10%) was made for each spot (as defined above) over one minute by three people and the individual estimations were averaged. Examples of the leaf damage estimation are shown in Figure 1.

To generalise the information about the spatial distribution of the leaf damage by *C. perspectalis*, the larvae infestations were also recorded on the individual box trees in 15 other cemeteries over the periods June–July 2016 and June–July 2017. In each cemetery, the damage was assessed for a single tree that was at least 3 m tall and showed moderate damage (40–60% of the leaves consumed). Strongly damaged trees could not be studied due to excessive damage to the whole tree crown. On each tree, damage to the foliage was assessed separately for the height levels 0.5, 1.5 and 2.5 m using the visual estimation method described above (Figure 1).

Statistical analyses

Distribution of the C. perspectalis larvae. The negative binomial generalised linear model (NB GLM; Venables & Ripley 2002) was applied to examine

Figure 1. The estimation of the *Cydalima perspectalis* leaf damage by the visual inspection in the field (A) 0%, (B) 50%, and (C) 100% damage

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whether the abundance of the *C. perspectalis* larvae is affected by the hedge's height level (lower, middle and upper level as defined above) and the hedge aspect (southern, northern). The abundance data (the counts of the larvae obtained through the photo-eclectors) were modelled using the log link function. The effects of the factors and their interaction were tested at the level of significance $P = 0.05$ using a stepwise backwards selection. The adjusted *P*-values were used in the multiple comparisons.

Distribution of the leaf damage by the C. perspectalis larvae. The linear mixed effects model (LME; Laird & Ware 1982) was used to assess the effect of the hedge's height level (lower, middle, upper), the hedge aspect (southern, northern) and the collecting period (fixed effects) on the leaf damage by the *C. perspectalis* larvae in the Zvolen cemetery, and the hedge (hedge id) was considered a random effect. A separate LME model was used to determine the effect of the twig part (basal, terminal) on the leaf damage by the *C. perspectalis* larvae, the hedge (hedge id) and twig sample (twig id) being considered random effects. An LME was also used to model the leaf damage as a function of the height level in the other cemeteries, with the inclusion of the effect of the box tree (tree id) as a random effect. The data (percentages of the leaf damage) were transformed using arcsine transformation. The effects and their interaction (height level × aspect) were tested at the significance level of 0.05 using a stepwise backwards selection. The mean damage was estimated along with approximately 95% confidence intervals for each combination of the height and aspect. Comparisons of the leaf damage between the collecting periods of July 2015, April 2016 and August 2016 were performed separately for each height level. For multiple comparisons, adjusted *P*-values were used. The statistical analyses and graphics were performed in R software (version R 3.6.1.) (R Core Team 2019).

RESULTS

Recording the *C. perspectalis* larvae via the photo-eclectors. On 21 March 2016, nine branches from the box tree hedges (three branches from each height level) were checked in the Zvolen cemetery to determine the instar of *C. perspectalis* larvae before placement in the photo-eclectors. The branches hosted 3rd instar larvae (determined by the head capsule width, Nacambo et al. 2014), mostly in hibernacula.

A total of 252 branches placed in the photo-eclectors yielded a total of 2 667 larvae. The larvae started leaving the eclectors as early as one day after storage, and much higher numbers followed after two days. Over 98% of all the larvae left within the first 8 days of the 14-day observation period (Figure 2A). Additional observations on the post-4th instar active larvae conducted one month after the end of hibernation in April 2016 ($N = 284$) showed a similar temporal trend in leaving the photo-eclectors (Figure 2B). The emergence of several adult moths indicated the successful development of the larvae in the eclectors.

Distribution of the *C. perspectalis* larvae. Since the 3rd instar larvae were mostly in hibernacula during the sampling in Zvolen at the end of March 2016, our results reflect their distribution during overwintering. The abundance of the larvae was significantly affected by the height level of the hedge

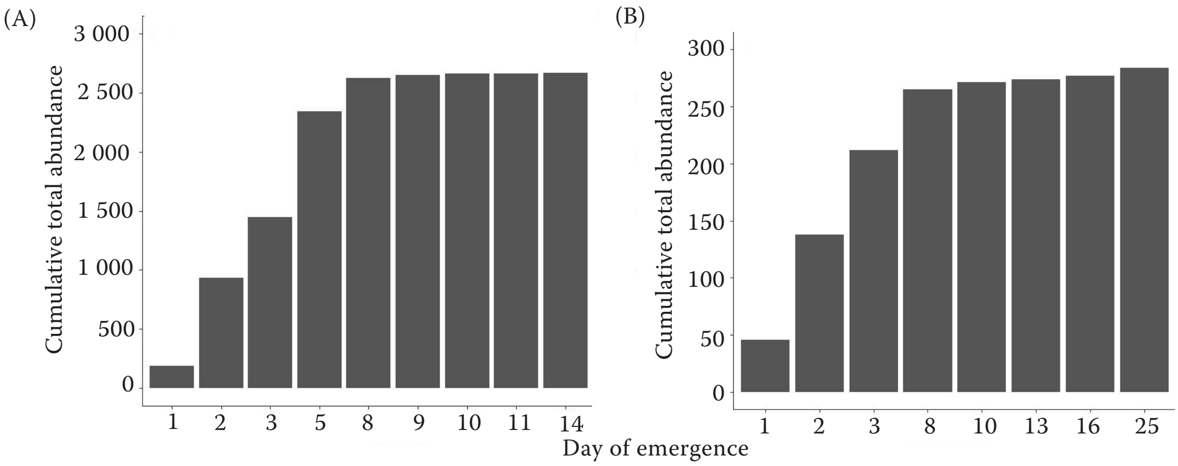

Figure 2. The cumulative abundance of the *Cydalima perspectalis* larvae that emerged from the photo-eclectors in (A) March 2016 ($N = 2667$) and (B) April 2016 ($N = 284$)

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Table 1. The significant differences (at a level of significance of 0.05) of the comparisons of the leaf damage by the *Cydalima perspectalis* larvae between the three height levels (lower, middle, upper) of the box trees at the two sampling occasions

Period	Lower vs. middle	Middle vs. upper	Lower vs. upper
Summer 2015	t = 3.814, P = 0.0005	t = 6.336, P < 0.0001	t = 10.150, P < 0.0001
Spring 2016	t = 2.435, P = 0.0418	t = 6.344, P < 0.0001	t = 8.780, P < 0.0001

(χ^2 = 7.181; P = 0.0276), while the hedge aspect was not significant (χ^2 = 1.579; P = 0.2089). Multiple comparisons showed that the abundance of the larvae in the upper hedge height level (a mean of 74.6 per six branches) was significantly higher than in the lower level (a mean of 42.5 per six branches) (Z = 2.346; P = 0.0498). The number of larvae in the middle level (a mean of 73.4 per six branches) was not significantly different from that both in the lower level (Z = 2.281; P = 0.0584) and the upper level (Z = 0.065; P = 0.9977).

Distribution of the leaf damage by the *C. perspectalis* larvae. The accurate laboratory counts of the damaged leaves (actual damage) from the hedges in Zvolen showed no interaction of the height level of the hedge and the hedge aspect on the severity of the damage by the *C. perspectalis* larvae in the summer of 2015 ($F_{2,173}$ = 0.503, P = 0.6054) and the spring of 2016 ($F_{2,173}$ = 0.333, P = 0.7170). The proportion of damaged leaves was significantly affected by the height level ($F_{2,173}$ = 53.244, P < 0.0001 and $F_{2,173}$ = 52.772, P < 0.0001, respectively) (Figure 3A, C). The leaf damage was most serious in the lower height level, less in the middle level, and the least in the upper level. Multiple comparisons detected significant differences in the leaf damage between all the height levels over time (Table 1). In the summer of 2016, the leaf damage was not significantly affected by any factors (P > 0.05). The

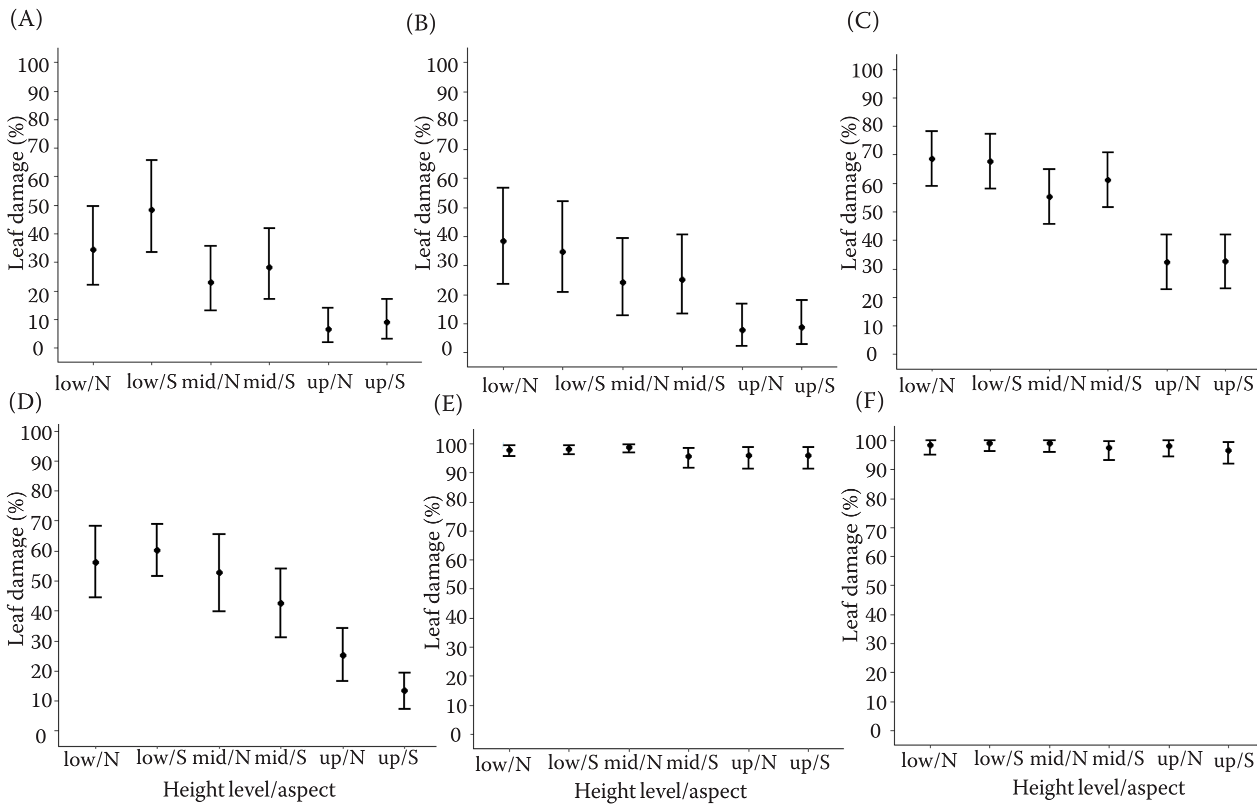

Figure 3. The spatial distribution of the leaf damage by the *Cydalima perspectalis* larvae on two box tree hedges, (A, C, E) the damage assessed by the precise counting of the leaves on the sample twigs; (B, D, F) the damage observed on the hedges in the field, (A, B) July 2015, (C, D) April 2016, (E, F) August 2016

The points (N = 30) represent the mean leaf damage; the line segments denote approximate 95% confidence intervals (CIs) for the means; height level: lower – low (50 cm), middle – mid (150 cm), upper – up (250 cm above the ground); aspect: N – northern; S – southern; non-overlapping 95% CIs indicate the statistically significant differences between the means

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leaf damage in the middle (98%), upper (96%) and lower height levels (98%) were almost equal (Figure 3E).

The visual assessment of the leaf damage in the field yielded similar results to those obtained in the laboratory, meaning that the visually estimated damage corresponded well to the actual (accurately estimated) damage (Figure 3B, D, F). The effect of height level on the leaf damage was significant in the summer of 2015 ($F_{2,173}$ = 47.126, $P < 0.0001$) and the spring of 2016 ($F_{2,173}$ = 45.379, $P < 0.0001$), but was not significant in the summer of 2016 ($P > 0.05$).

The damage to the basal parts of the shoots was higher compared to that in the terminal parts, although this was not always significant (summer 2015 – $F_{1,179}$ = 0.350, P = 0.5549; spring 2016 – $F_{1,179}$ = 21.357, $P < 0.0001$; summer 2016 – $F_{1,179}$ = 2.290, P = 0.1321).

The visual assessment of the defoliation of the moderately damaged (40–60% damaged leaves) individual box trees in the 15 other localities showed a similar spatial pattern in the leaf damage. The defoliation significantly differed between height levels ($F_{2,28}$ = 58.481, $P < 0.0001$). The highest damage was seen in the lower height level (74%), moderate damage was seen in the middle (51%), and the lowest damage was seen in the upper level (28%).

Progress of the defoliation. In June 2014, only a few isolated feeding marks of the *C. perspectalis* larvae were recorded on the investigated box tree hedges in the Zvolen cemetery. The hedges were further infested and increasingly consumed by the larvae which caused their almost complete defoliation by August 2016. A marked steady increase in the damage from moderate in July 2015 (Figure 4A) to almost complete in August 2016 (Figure 4B) was found in lower ($F_{2,176}$ = 203.986, $P < 0.0001$) and middle height levels of the hedges ($F_{2,176}$ = 267.863, $P < 0.0001$) (Figure 5A and B). The damage in the upper height level also increased over this period ($F_{2,176}$ = 516.819, $P < 0.0001$). However, the increase was only slight from July 2015 to April 2016, but accelerated by August 2016, the damage varying from 30 to 90% within four months (Figure 5C). In the autumn of 2016, the box hedges generated new shoots which desiccated completely in March 2017.

Figure 4. The progress of the defoliation on the box tree hedge from (A) 23 July 2015 to (B) 2 August 2016

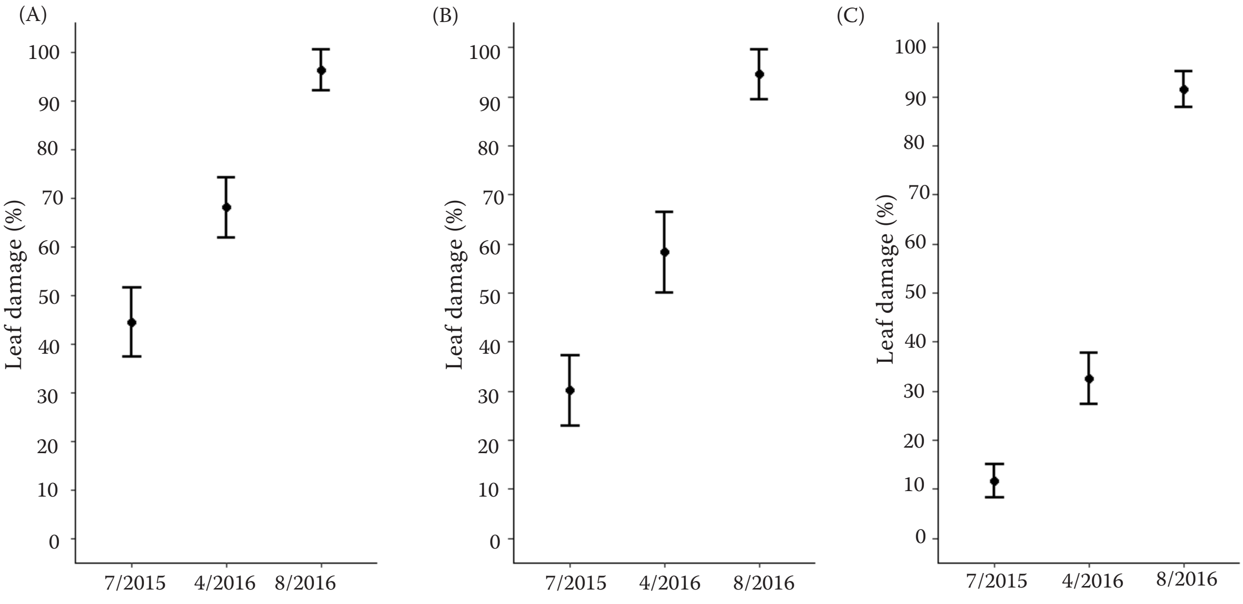

Figure 5. The temporal changes in the leaf damage by the *Cydalima perspectalis* larvae in the (A) lower, (B) middle and (C) upper height level of two box tree hedges

The points (N = 60) and line segments as shown in Figure 1; the non-overlapping 95% CIs indicate the statistically significant differences between the means

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The desiccation of these poorly developed shoots could be caused by the increased solar radiation, drought stress, or their combination.

DISCUSSION

Our results indicate that the larvae of *C. perspectalis* can be obtained through photo-eclectors, as is the case with various other insect pests (Zach 1991; Parák et al. 2015; Kulfan et al. 2016). Approximately 98% of the collected larvae emerged from the photo-eclectors within 8 days. The larvae mortality in the eclectors could not be assessed due to the inconspicuousness of the tiny 3rd instar larvae (length less than 10 mm) on the dense foliage. Nevertheless, photo-eclectors may be useful tools for extracting and estimating the number of box tree moth larvae on the hosts, since the usual insect collecting methods are not reliable. Adverse weather (low temperatures, rainfall) during the winter and in the early spring limit the detection of the *C. perspectalis* larvae, so that extraction through eclectors in the laboratory could be useful. The early detection of the pest is crucial so that control measures can be applied quickly (Vétek et al. 2017).

Damage to box tree leaves by the *C. perspectalis* larvae was assessed by several authors. but was not studied in detail. Different protocols and methods (Fora & Poşta 2015; Raineri & Mariotti 2017; Načeski et al. 2018; Akıncı & Kurdoğlu 2019; Badano et al. 2019; Baur et al. 2019) were employed to assess the leaf damage. Herein, we provide an accurate method to determine the leaf damage on box trees, judged from the similar results of the estimation in the field and counting the leaves in the laboratory. A field observation, thus, provides a quick and sufficient approximation of the leaf damage. It is cost-effective and widely utilisable by horticultural practitioners.

Our data from the early spring (March) did not show a higher abundance of the overwintering *C. perspectalis* larvae in the lower height level of the box hedges compared to the middle and upper height levels. This contrasts with the leaf damage, which was highest in the lower height level, in accordance with previous studies (Matošević 2013; Pencheva & Yovkova 2016). This discrepancy could be due to the lower leaf biomass in the lower height level. According to Sternberg and Shoshany (2001), many shrub species have less leaf biomass in their lower parts. This could explain the higher damage to the leaves in the lower parts of the hosts even in cases where the larvae were less abundant. The active larvae, also, could move from their overwintering sites to other parts of the trees. For example, the vertical movement of larvae, lowering themselves on silk threads (Pencheva & Yovkova 2016) from the top to the bottom parts of the trees, could increase the number of larvae at the lower levels, and the subsequent damage there. Hence, we recommend recording the *C. perspectalis* larvae within the whole crown and the leaf damage primarily in the lower parts of the trees. Control measures, e.g., insecticides, should be applied to the whole box tree, not only to the parts where the damage is visible.

The non-significant influence of the aspect of the box trees (southern or northern exposition) on the abundance of *C. perspectalis* larvae may indicate the ability of this species to tolerate varying air temperatures and light intensities or microclimates, respectively (Nacambo et al. 2014).

Our results suggest that even in the case of heavy box tree infestations by *C. perspectalis,* the damage to the foliage could remain inconspicuous in the upper height level of the trees for a long time period (over two successive growing seasons). However, a considerable increase in the damage may quickly follow, leading to the almost complete defoliation within four months during the growing period. Completely defoliated box trees often exhibit a certain regeneration potential by producing new shoots in the late summer or autumn (Šefrová et al. 2019). New foliage masks the actual damage to the trees and increases the probability that the adverse effects of the larval feeding could be overlooked. According to our observations, the mortality of the damaged trees with the new foliage is high the following spring.

The use of the destructive methods in our study was limited by the availability and size of the experimental hedges (rare samples). Certain questions, such as recording the larvae of *C. perspectalis* and the damage to the host trees by this pest require further investigation: the use of photo-eclectors in different seasons of the year including winter, the activity and distribution of the particular larval instars over the growing period, etc.

In conclusion, photo-eclectors are an effective method for sampling the *C. perspectalis* larvae. Field observations of the box trees are adequate to assess the damage by *C. perspectalis* and useful for horticulturalists. Additionally, this may be impor-

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tant in the context of maximising the opportunities for non-native species to be detected, reported and controlled. For example, citizen scientists may be encouraged to look for invasive species such as *C. perspectalis*. Citizen science projects may have an important role in the monitoring of invasive insects, leading to the faster detection and effective control actions (Roy et al. 2015).

Acknowledgement: We thank M. Mikuš, P. Tuček and J. Kučerová which aided the research in the field and laboratory. We would also like to thank the two anonymous reviewers for their suggestions and insights.

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Received: December 13, 2019
Accepted: April 15, 2020
Published Online: May 15, 2020