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Computer tomography-assisted visualization of the movement triggered by frost in *Ostrinia nubilalis* overwintering in maize stalks

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Abstract. The damage of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), causes is mainly determined by the success of its overwintering. The aim of our study was to assess the consequences of the artificial cooling on the movement and survival ability of overwintering larvae of *O. nubilalis* by using computer-assisted tomography. The in situ movement of the examined larvae in icy media of different thickness (5, 15, and 30 cm) and during freezing periods of 5, 10, 20, 40, and 60 min was determined in maize stalks using CT and the positioner-laser of the CT apparatus. It has been found that the thickness of the ice had a significant effect on the displacement of the overwintering larvae, however, the impact of the duration of freezing on the moving of the larvae could not be proven statistically. Enhanced larvae activity due to thinner ice layers (5, 15 cm) were of exponential type, which was more pronounced just prior to the freezing point. In contrast, thicker ice covering (30 cm) caused complete immobility. According to our results, the diapausing larvae were still able to move and albeit it appeared to be capable of surviving the direct impact of extreme cold, it could even leave its overwintering place as a result of low temperature. Furthermore, the maize stalk tissue contributes to the survival-success of the larvae as it seems to act as a temperature-buffer moderating the severe impact that low temperature exerts on living tissue.

Key words. Computed tomography, European corn borer, freeze, imaging, maize stalk, non-destructive method, survival, temperature changing.

Introduction

European corn borer (ECB), *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), is a major pest of maize in vast Palearctic and Nearctic agricultural areas. In fact, it is one of the most widely studied agricultural pests in the world, which is attributable to its outstanding phytosanitary status (Brindley *et al.*, 1975; Saladini *et al.*, 2008). The damage degree in the given vegetation cycle of this pest is fundamentally determined by the survival success of the overwintering larval population, which can be considered a starting point of dangerous non-diapausing larvae mass (Kojić *et al.*, 2010; Keszthelyi and Holló 2019). *Ostrinia nubilalis* has a very wide host range. Nevertheless, it can survive by feeding on many different plants. The economic impact of *O. nubilalis* is mainly due to the damage it causes to maize, the primary host of *O. nubilalis* (Bohn *et al.*, 1999).

The ECB develops in different generation numbers depending on its distribution territory (Got *et al.*, 1996). The overwintering of *O. nubilalis* occurs in fully developed larva stadium in damaged plant remains, for example, in maize stalks. This diapausing stage is rather resistant against different ecological effects, which is preceded a physiological and enzymatic transition. During this essential process, the larva accumulates long-chain carbohydrates (trehalose, ribulose, etc.) and glycerol in its body (Nordin *et al.*, 1984; Beck, 1989; Uzelac *et al.*, 2020).

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In late summer or early autumn, when the daylength begins to shorten, mature larvae of the last generation move down to the base of the stem where they excavate a cell and overwinter (Stavrakis, 1967). This diapausing stage of the pest is usually static. However, the larvae can move during this overwintering stage if the conditions become unfavourable (Stavrakis, 1967; Andreadis *et al.*, 2008). According to Keszthelyi & Holló's (2019) study assisted by CT, the overwintering larvae are located closer to the brace root (and to the soil), possibly because of having moved downwards inside the stalk, where the temperature is slightly milder than at the upper part of the stalk.

The relationships between undercooling capacity, tissue freezing, and survival at sub-zero temperatures were studied in mature larvae of *O. nubilalis* by Hanec & Beck (1960). According to their results, cold-hardy winter larvae survived up to 3 months at −20 °C in the absence of contact moisture. Chilling in the presence of contact moisture caused freezing of the larvae and reduced their ability to survive cold conditions. Despite the formation of ice in their tissues, fully cold-hardy borers survived several weeks while frozen at −20°C.

CT is one of the most useful non-destructive techniques providing qualitative and quantitative results using ionized radiation. It can visualize the texture and volume fractions of examined objects. However, there are some limitations of this technique. Naturally, the ionizing radiation has been used to induce the sterility and mortality of some pests, because of its physically cell destructive effect (Buscarlet, 1982; Hallman, 2013). In contrast, there are more studies employed in insect morphology and physiology that have already created, without any deteriorative effects of radiation upon the vitality of target organisms. The possibility of non-destructive insect observation can be traced back to the minor effects of a low dose of CT irradiation on insect cells (Wipfler *et al.*, 2016; Hall & Martín-Vega, 2019).

The purpose of our laboratory examination was the assessment of the consequences of the artificial cooling on the movement and survivability of overwintering larvae of *O. nubilalis*. In additional, our work was motivated to the exact cognition of this winter population influencing factor. These achieved results can contribute to the optimal practical treatment of preceding maize remains. We were particularly interested in the buffering effects of the stalk surrounding the larvae, which could be observed by a non-destructive method. Besides, we addressed the potential usability of CT imaging for the physiological study of this important agricultural pest.

Materials and methods

Sampling and experimental settings

The 100 pieces of maize plants damaged by *O. nubilalis* were collected at the end of the vegetation cycle (on October 18, 2019). The samples derived from first-year maize acreage in the periphery of Csokonyavisonta (Somogy county, Hungary; geographical coordinates: 46°30′92.88″N 17°28′34.78″E). For selecting the plants to be studied, we have relied on the clearly visible holes and mastication signs triggered by the boring larvae in the stalks. Uniformly 170 cm long stalks were cut above the brace root; subsequently the ears and all of the leaves were detached. These stalk samples were stabilized by a styrofoam holder in a 5 × 5 cm bond in order to image them by CT (Fig. 1). These prepared bundles were stored in the open until the CT analysis was performed to ensure natural environment conditions.

CT imaging and processing

The CT acquisitions were performed by Siemens Somatom Definition AS+ (Siemens, Erlangen, Germany) using the following scanning parameters: tube voltage 120 kV, exposure 120 mAs, and spiral data collection mode with 0.8 pitch factor. Images were reconstructed with 300 mm field of view and 1 mm slice thickness. The location level of the larvae was determined and signed on the surface of the stalks using the CT images and the positioner laser of the CT apparatus in situ. The stems containing the corn borer larvae were cut 15–15 cm away from the larval site. These 30 cm stems, containing the larvae uniformly in the middle, were placed vertically in boxes containing water with ice pieces at different heights using a perforated styrofoam holder arranged 5 × 3. The height of the ice in the boxes into which the stems were immersed was 5, 15, 30 cm (Fig. 1). During the freezing the changing of the inner temperature of stalk was continuously measured by Ebro Electronic TTX 100 type core thermometer. CT scans were taken at 0 as baseline and at 5, 10, 20, 40, 60 min using the same acquisition protocol as previously described. The displacement of the larvae from baseline was determined by CT scan at different time points using the 3D Slicer 4.11.0 programme.

A control group (n = 10) was formed from specimens, which were directly immersed into the icy medium (froze icy substance) in order to assess the effect of chilling on larvae-overwintering stages without stalk. Insect mortality was controlled hourly for 2 days.

Statistical analysis

In order to test the displacement and survival data of the examined overwintering larvae ($n > 50$), the Shapiro–Wilk test was used. The effect of cooling on the displacement of overwintering larvae in the stalk as well as the consequences of direct freezing on the survival of these larvae was statistically examined by two-way ANOVA ($P < 0.05$) and by correlation-regression analysis using the Microsoft Excel 2016 software.

Results

The detection of *O. nubilalis* larvae present in the analysed maize stalks has been possible by CT. The overwintering stage of the pest in the plant remains could be well visualized in the transversal recordings (Fig. 2). The average distance of *O. nubilalis* larvae from the brace root in samples detected by CT imaging was 607.33 ± 37.90 mm. The average number of

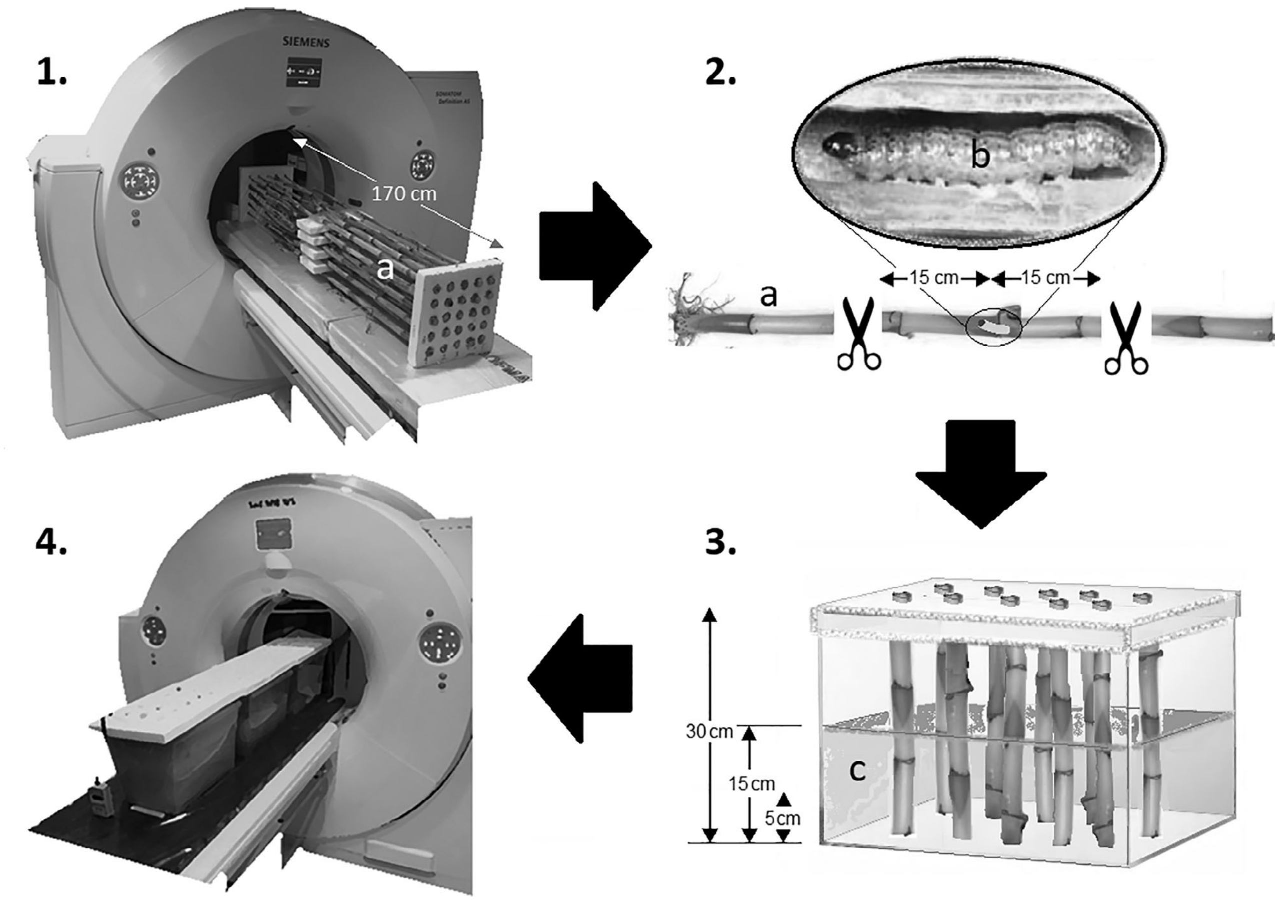

Fig. 1. Steps of computer tomography imaging and the associated experimental processing. 1: Determination of the larvae locations by CT; 2: Set-up of the samples; 3: Placement of the samples into icy media; 4: Determination of the larvae displacements by CT; a: Maize stalk; b: *O. nubilalis* larva; c: Icy medium.

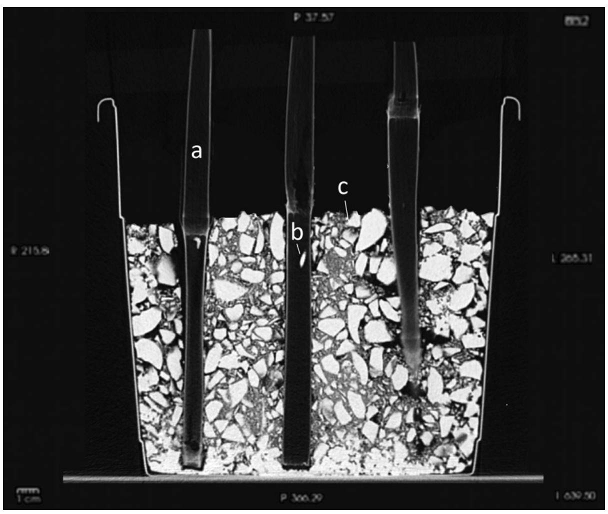

Fig. 2. Transversal CT recordings of examined maize stalk containing *O. nubilalis* larvae in icy medium. a: Maize stalk; b: *O. nubilalis* larva; c: Icy medium.

larvae/stalk was 1.92. In most cases the larvae in these samples occurred in the fifth (22.22%) and the neighbouring (4th and 6th) internodes (in the case of internodes 4th and 6th the occurrence was equally 15.55%). The major location of the other larvae was between the fifth internode and brace root.

The effects of thickness and the duration of the freezing on the degree of displacement of the overwintering larvae in the maize stalks are shown in Figure 3. The directions and the intensities of displacement differed as a function thicknesses applied. Movement of the larvae started in the case of thinner ice (5 cm), however, the main direction was away from the ice. In contrast, in the treatment with thinner ice (15 cm), the movement towards the ice has moderately taken place, and the effect of cooling was more pronounced in terms of larvae displacement. In few samples the larvae activity intensified app. Twenty minutes upon chilling, which decreased after reaching the appropriate distance (Fig. 4). Nevertheless, a significant change in the intensity of the motion could not be proven among the applied cooling periods. In the case of the thickest ice covering the entire stalk (30 cm), no movement could be detected. The larvae in this experimental samples have uniformly stayed motionless most probably due to drastic freezing. The same phenomenon could be observed in

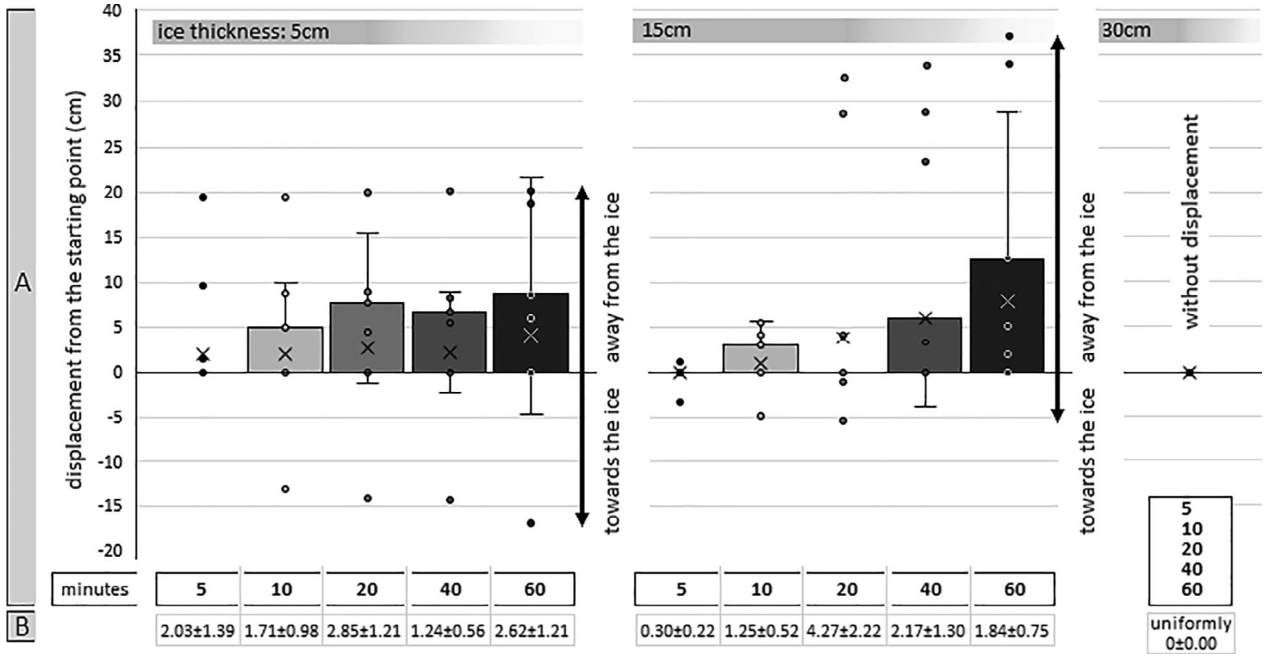

Fig. 3. The effect of the ice slabs of different thickness and that of the duration of freezing on the degree of displacement of the overwintering *O. nubilalis* larvae in the maize stalks. A: Cumulative displacement; B: Displacement (mm) of larvae between each recording.

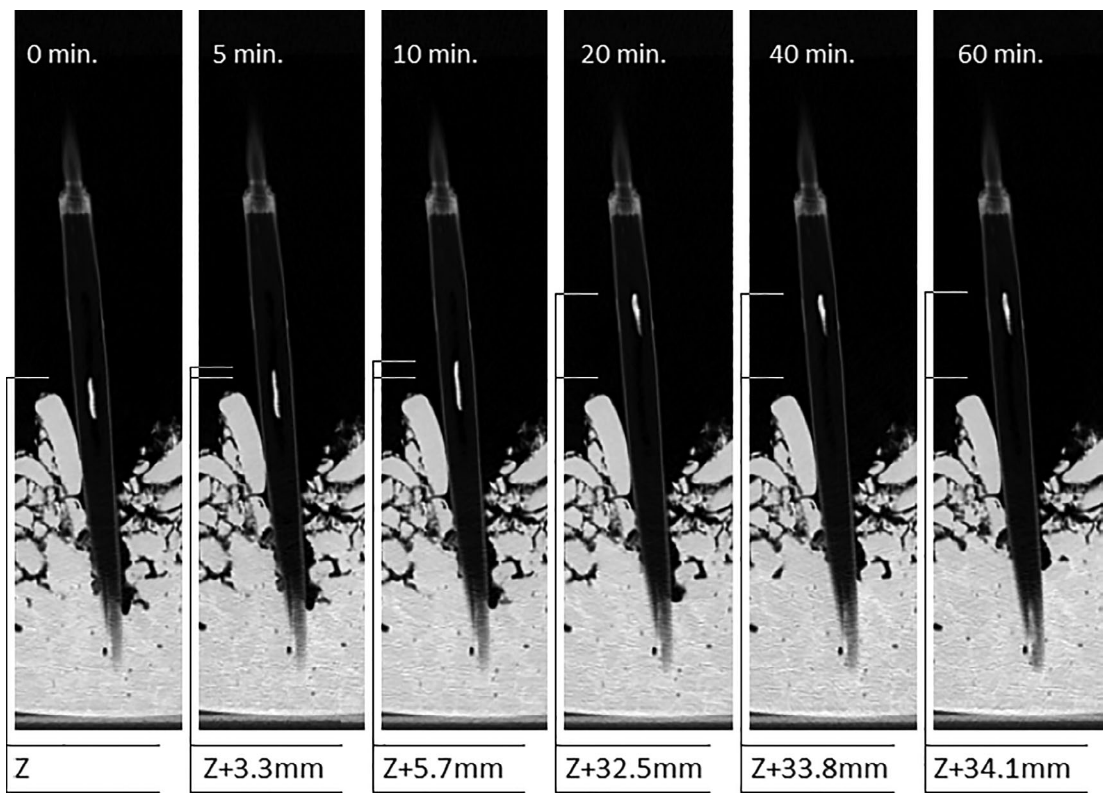

Fig. 4. CT image series representing larval moving as a result of chilling duration.

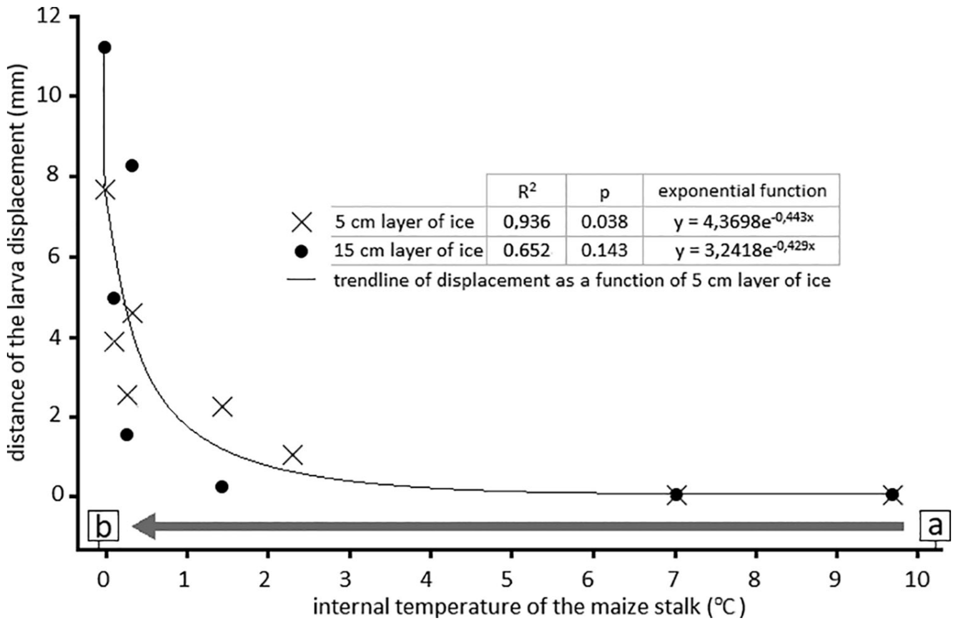

Fig. 5. Degree larva displacement as a result of the decreasing internal temperature of maize stalk upon freezing for 1 h. (a) Initial temperature of the stalk interior being equal to the ambient air temperature (10.3°C); (b) Final temperature of the stalk interior being equal to that of the freezing ice (−0.1 °C).

both treatments in which thinner ice was used. The percentages of these immobile larvae were 46.66% and 60% in the samples treated by 5 cm of ice and in the samples treated by 15 cm of ice, respectively.

The Shapiro–Wilk normality test showed that our CT-measured data are of a normal distribution, ($p > 0.05$). The significant effect of ice thickness on the displacement of the overwintering larvae ($P = 0.047$) was confirmed by two-way ANOVA. Nonetheless, the impact of the duration of stalk freezing on the displacement of the overwintering larvae could not be proven statistically ($P = 0.153$). The combined effect of ice thickness and freezing duration on the displacement showed a similar statistical relationship ($P = 0.445$).

The more intensive motion was triggered by the increasing duration of the freezing in the treatment of 5 and 15 cm of ice. Our supplementary experimentation done by using an internal thermometer confirmed that the maize stalks completely immersed into the crushed ice, therefore, they were continuously cooled to the temperature of the surrounding medium. Therefore, the stalk buffered the harmful impact of freezing on the overwintering specimens. The cold condition was triggered by thinner ice (5 cm).

The degrees of larval displacement occurring in the stalk upon freezing are shown in Figure 5. Larvae activity triggered by ice freezing was of exponential type, which intensified just prior to the freezing point. Moreover, a more steeply rising curve was observed in the case of 15 cm-thick ice, which can be explained by the impact of faster cooling. However, the R-squared values reveal a close relationship between the decreasing internal temperature of the stalk and the larval movement, therefore, a statistically significant correlation could only be demonstrated when 5 cm-thick of ice was used. The extreme buffering-ability of stalk is unequivocally evinced by the presented data. The cooling interval from outside temperature (10.3°C) to zero inside the stalk was on average 41 ± 3.5 min.

The effect of direct ice-freezing on the mortality of overwintering *O. nubilalis* cannot be determined. All larvae immersed into the ice (−0.1 °C) have survived without exception for 2 days.

Discussion

Diapause induction and related traits of *O. nubilalis* have been of interest to scientists since 1927 (Parker & Thompson, 1927). This species enters a well-defined, photoperiodically induced larval diapause as a mature fifth instar between 20 and 29 °C (Skopic & Bowen, 1976; Takeda & Skopik, 1985). *Ostrinia nubilalis* prepares for the winter from the end of August in temperate regions (Hanec & Beck, 1960). Finding an optimal overwintering place and a special physiological change (increasing the concentration of cryoproteins) help in the overwintering stage of the larvae for the survival of unfavourable conditions. Slightly before the onset of more favourable environmental conditions in the spring, clearance of cryoprotectants is usually activated at the onset of diapause termination. At diapause termination, glycerol and trehalose levels decreased while trehalose levels remained at the same level (Kojić *et al.*, 2010; Wadsworth *et al.*, 2015). The ability of an insect to survive sub-zero temperatures is termed cold-hardening (Hanec & Beck, 1960; Stanic *et al.*, 2004). According to our investigation, the location in lower internodes of *O. nubilalis* larvae in the stalk corresponded to the season, which is line with the results of the study of Keszthelyi & Holló (2019).

Ostrinia nubilalis can notoriously tolerate the formation of ice crystals in its tissues hence it can still survive under this condition (Lee, 2010). This species capitalizes on this special ecological trait especially because in many cases the overwintering location is very wet and the larva may be completely frozen and encased in ice (Stanic *et al.*, 2004; Kojić *et al.*, 2010). Independently of this adaptation strategy, our investigation has pointed out the effect of non-direct chilling triggering larvae displacement, the degree of which depends on the distance between the larvae and the ice. The direction of displacement and its intensity are rather controversial as the former results of Keszthelyi and Holló (2019) have also pointed out. According to the answer given in our main hypothesis, more intensive moving has been triggered by the impending frost effect, while the totally motionless state has occurred only by direct chilling (ice contacting larva).

The larva being in diapause could still move and seemed to be able to survive the direct impact of extreme cold as well. Further, it was able to change its overwintering place as a result of attempting to avoid an unfavourable environmental effect. Basically, these developmental stages can be depicted by the seeking for optimal conditions in order to ensure the survival of the larvae over the winter period (Chiang, 1964; Grubor-Lajsic *et al.*, 1991).

The inner tissue of maize stalk not only gives shelter but also offers a discernible temperature buffering-ability for the overwintering of the insect dwelling in it. Thus, the serious cell-destroying impact of low temperature can be moderated and at the same time, it can contribute to the successful overwintering of the insect.

In summary, the importance of *O. nubilalis* cold-hardiness in climate acclimatization was confirmed by our results, as well as this ecological property was supplemented by the necessity of displacement ensuring survival.

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Data availability statement

Data available on request from the authors.

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