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MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

Hungarian University of Agriculture and Life Sciences

Improving methods in studying the *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) under laboratory and field conditions to better assess successes and failures of diverse control methods

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1. Introduction

1.1 Maize (*Zea mays* L.) and its importance

Together with rice and wheat, maize is one of major food sources for humans, it has been estimated that it's accounting for 42% of world's food calories through human diet (FAO 2021, http1). The worldwide area of maize cultivation was around 197 million ha and the maize grain production from that is 1.137 million tons in 2020 (FAO 2021, http1). Maize use is more versatile compared to wheat and rice. For example, in the developed economies it is used mainly for livestock feeding (~75% of the harvest) (Erenstein et al., 2022).

In Europe, in 2020 the total area sowed with maize to harvest grain maize and corn-cob-mix (silage) was around 9.2 million ha (Eurostat 2022, http2). The yield from these areas in 2020 was 67 million tons (Eurostat 2022, http2), which is equals to 24% of the total grain production among the main cereals in Europe (including seed production) (Eurostat Statistics Explained 2022, http3). Despite this high volumen of production, the EU member countries still import (19 million tons in 2020) more maize grains than they export (5.5 million tons in 2020) (EC-Agridata 2022, http4).

Lastly, in Hungary the sowing area of maize hybrids for grains was 927 thousand ha and from that farmers harvested 8.4 million tons of yield from it in 2021 (KSH 2021, http5).

1.2 The maize pest *Diabrotica virgifera virgifera* LeConte

1.2.1 Origin

Diabroticites (Chrysomelidae: Galerucinae: Luperini) is a section of the leaf beetles which has 823 species all together, among this species the richest is the *Diabrotica* genus (Eben, 2022). The majority belongs to the fucata group with 354 species (polyphagus, multivoltine species), the second richest group is the virgifera group with 24 species (oligophagus, univoltine species) and the third one is the signifera group with 11 species (Smith, 1967; Branson and Krysan, 1981; Derunkov and Konstantinov, 2013).

Diabrotica virgifera ssp. *virgifera*, or Western Corn Rootworm became a major pest of the maize-growing areas in North America (Krysan and Miller, 1986; Levine and Oloumi-Sadeghi, 1991; Lombaert et al., 2018). It is hypothesized that it is originated from Central America, probably from Mexico (Krysan and Smith, 1987; Campbell and Meinke, 2006).

1.2.2 Lifecycle and biology

Diabrotica v. virgifera is an univoltine species with eggs that overwinter in the soil (Ball, 1957; Chiang, 1973; Krysan and Miller, 1986). After maize has germinated in the spring, the eggs hatch, its three larval instars feed mostly on maize roots (Branson and Ortman, 1970), the average

development time for males is 29 days and for females is 32 days (Musick and Fairchild, 1971) (Krysan et al., 1984). The adults emerging from the soil end of June and beginning of July, depending on the weather conditions (Quiring and Timmins, 1990; Darnell et al., 2000; Bayar et al., 2003).

To distinguish between sexes of adults, the antenna length is the most important morphological feature, the males has longer antenna than the females (Krysan and Smith, 1987). Moreover, the last segment of the abdomen of the males are sclerotized, while female's abdomen is pointed (Krysan and Miller, 1986). The adults body appearance is bright yellow, exception the elytra and the head of the beetles, these are consisting black, longitudinal lines. On the male specimen these lines are fused together, but females has distinct black stripes on their elytra (Hammack and French, 2007) In the field, proterandry occurs between the sexes, meaning that the males are coming a 2-3 days earlier than females (Darnell et al., 2000; Bayar et al., 2003).

Egg laying by female WCR starts from late June to autumn (Short and Hill, 1972; Krysan and Miller, 1986; Komáromi et al., 2001). The eggs have yellowish color and oval shape and they are approximately 0.6 mm in length (Atyeo et al., 1964; Krysan and Miller, 1986). The eggs needs an obligate diapausing phase on cool temperatures (~10 °C) to develop, which is last about 70 to 160 days (Branson, 1976; Krysan, 1982).

The freshly hatched larvae are almost colorless, older larvae first turn into white color and later on they get creamy yellowish color, the larvae stages are last long about 4-6 weeks (Krysan, 1982). To differentiate between the stages of the instars the length of the larvae and the width of the head capsule could be used (Hammack et al., 2003; Becker and Meinke, 2008).

1.2.3 Invasion in Europe

Diabrotica v. virgifera was accidentally introduced from North America into Europe at least five occasions between the 1980s and the early 2000s (Guillemaud et al., 2005). The first economic damage was detected in 1992 near Belgrad, Serbia, but for this the population needed to build up for years so probably the beetles were there since the 1980s (Baca, 1993; Edwards et al., 1999; Kiss et al., 2001; Szalai et al., 2011). In Hungary, the species was captured in 1995, and needed nearly 10 years to cover the whole country according to the extensive monitoring programs (Princzinger, 1996; Barna, 2001, http6; Kiss et al., 2005; Szalai et al., 2011; EPPO, 2012, http7). Over the last 27 years *Diabrotica v. virgifera* has been invaded most maize growing areas of Central Europe, parts of Eastern Europe, parts of the Balkan, as well as Italy (Kiss et al., 2005; Meinke et al., 2009; Bazok et al., 2021). Altogether, it invaded 32 European countries, but there were some countries where the eradication programs were successful, like Belgium, Netherlands, and United Kingdom, they successfulness probably due to the fact that the climatic environment is not optimal for *Diabrotica v. virgifera* population increase (CABI 2021, http8).

1.2.4 Damage on maize

The primary damage on the plants comes after larvae starts to feed on the maize roots. Larvae injury causing reduced water and nutrient uptake, reduced yield, plant lodging and overall poor plant health (Kahler et al., 1985; Riedell, 1990; Maredia and Landis, 1993). High number of larvae combined with low soil moisture reducing the water uptake by the roots can kill a small plant (Steffey et al., 1999). The amount of yield losses really depend on the stress level of the hybrid infested with larvae (Urias-Lopez and Meinke, 2001), it can mean 10% yield losses in moderate year (Schaafsma et al., 1999; Princzinger and Ripka, 1999), but has been reported average 40% yield loss in continuous maize fields in Hungary (Tóth, 2005). Plant lodging or „goose necking" is a phenomenon usually occurs after when the severed pruned and chewed rooted plants starts to lay on the ground, sometimes strong winds on the field aids this phenomenon (Steffey et al., 1999). The consequences of plant lodging are could be bad pollination, later harvest because of the wet cobs and lower cobs cannot be picked up by the combine (Spike and Tollefson, 1989; Spike and Tollefson, 1991). For the root damage assessment two damage scale has been developed. The first is the Iowa 1-6 scale which has been described in 1971 (Hills and Peters, 1971). It is considered to better detect general root damage, because its recording feeding scars and softer pruning. The second one is the node-injury 0.00 to 3.00 which has overcome some deficiencies of the other scale, but only better assess heavy root damage only (Oleson et al., 2005). However, under favorable conditions, there is a poor relationships between the node-injury scores and yield, compared to a drier, environmentally stressful year (Oleson et al., 2005).

Adults can also cause damages in maize. After the male and female flowers of maize are appearing in the field adults usually feed on the pollen and silks, this corn silk clipping can cause un-pollinated kernels, which reduces yield (Tuska et al., 2002; Gyeraj et al., 2021). If fresh silks and pollen is not available anymore for the adults, they will migrate to other maize fields or they seek for pollens of weeds, alfalfa, sunflower or cucurbits to feed on (Moeser and Vidal, 2004; Spencer et al., 2009).

1.3 Management options for *Diabrotica v. virgifera*

1.3.1 Cultural control

Firstly, good hybrid selection with high vigor to regenerate damaged roots, good weed management, early planting and high soil fertility are all contribute to healthy plants thus there is a higher chance to avoid huge *Diabrotica v. virgifera* damage (Steffey et al., 1999). Usually, females lay their eggs in maize fields, planting a non-host plant for *Diabrotica v. virgifera* in the next year disrupt the life cycle of the beetle, this practice so called crop-rotation has been used more than a 100 years now both in the USA (Spencer et al., 2014) and in Europe also (Széll et al.,

2005; Kiss et al., 2005; Vasileiadis et al., 2011). However, it seems that this strategy cannot be used forever. In the USA Corn Belt, where is the rotation of maize with soybean used for decades a new, rotation-resistant population emerged in which case females are losing their preference for oviposition in maize, and they rather lay eggs in soybean, thus the next year hatching larvae are secured (Barna et al., 1998, 1999; Levine et al., 2002; Komáromi, 2008). Due to lower maize field ratio and different agricultural practices in Europe than in the USA the emergence of this kind of rotation-resistant population is slower, but inevitable (Onstad et al., 2003).

1.3.2 Chemical control

From the 1940s chemical insecticides against the adults of the *Diabrotica v. virgifera* and the larvae below-ground started to became a general plant protection practice (Muma et al., 1949; Ball, 1983; Levine and Oloumi-Sadeghi, 1991). Since then, four groups of insecticides has been used prominently against the pest: the pyrethroids (e.g. tefluthrin, bifenthrin, cypermethrin); organophosphates (e.g. terbufos, clorpyriphos), carbamate (e.g. carbofuran), which can be formulated as a granule or a liquid both placed into the furrow usually at sowing time (Rozen and Ester, 2010). The latest one is the neonicotinoid group (i.e. clothianidin, thiamethoxam and imidacloprid). These chemical components used as seed coatings, and they are so-called systemic insecticides, which means they can be transported to the plants and thus they longevity and protective effect are longer and the active ingredient amount what is released to the environment could be reduced radically (Rozen and Ester, 2010). Although we have add that some of this insecticides are banned from the European market and cannot be used anymore against *Diabrotica v. virgifera*, yet in other countries in the world the majority of the listed insecticides are still part of the common plant protection practices (Mitchell et al., 2020).

1.3.3 Biological control

Due to the fact that there are several insecticides/insecticide group has been banned recently (especially in Europe) and the political and social pressure from the society to cultivate pesticide free or organic crops urged the appearance for biological solutions against *Diabrotica. v. virgifera*. Shortly, biological control uses species from the pest's natural enemy complex to fight against the pest itself.

There are several entomopathogenic fungi species was found to be effective against *Diabrotica. v. virgifera* larvae and adults the most effective ones are from the *Metarhizium* and *Beauveria* genera (Walsh et al., 2020). In laboratory bioassays *Metarhizium anisopliae* could infect 43% of *Diabrotica. v. virgifera* larvae and 62% of *Diabrotica. v. virgifera* adults (Pilz et al., 2007). *Metarhizium brunneum* reduced 31% of adult emergence from the soil under field conditions in maize (Pilz et al., 2009). Another study showed that several strains of *Beauveria*

bassiana, *Beauveria brongniartii* and *M. anisopliae* were effective in controlling *Diabrotica v. virgifera* larvae for up to 21 days after application (Cagáň et al., 2019). Nowadays there are also attempts to show exactly which protein is causing toxicity of the fungi. For example, aegerolysins group from the fungal genus *Pleurotusnematodes* could be promising pool of candidates for bioinsecticide against *Diabrotica* (Panevska et al., 2021).

The second and most successful biological control group is come from the families of Steinernematidae and Heterorhabditidae entomopathogenic nematodes. They attack and kill different arthropods effectively, after the infection the nematodes release they bacteria hosts (Xenorhabdus and Photorhabdus, respectively) and use the insect larvae as cadaver to raise and release the next generation (Jackson, 1995, 1996). Screening programs with biotests under laboratory conditions showed that most virulent species against *Diabrotica* larvae were the: *Heterorhabditis bacteriophora*, *Heterorhabditis megidis*, *Steinernema feltiae*, *Steinernema arenarium*, and *Steinernema kraussei* (Journey and Ostlie, 1994; Toepfer et al., 2005; Hiltpold et al., 2010). In field trials, it became evident that the best candidates for large scale use is the *H. bacteriophora* and *H. megidis*, because their efficacy against *Diabrotica v. virgifera.* larvae can be up to 81% and they can prevent 80% of the root damage (Toepfer et al., 2008; Pilz et al., 2011). However, they can be persist in the soil for months after application still they efficacies can be variable in the field against the pest (Tóth et al., 2020). One product based on *H. bacteriophora* is currently available in the EU for four member countries according to the producer (e-nema GmbH) and can be used by the farmers (Toepfer and Tóth, 2020).

Bacillus thuringiensis strains has been long known to be infectious to different insect pests including *Diabrotica. v. virgifera* (Feitelson et al., 1992). However other species and strains of bacteria has been proved to could be used to control this insect species or group like the *Serratia* species (Prischmann et al., 2008) or different *Pseudomonas* species (Jaffuel et al., 2019).

These biological control agents were extensively tested also in Europe against *Diabrotica. v. virgifera* (Balog et al., 2013) under field conditions. Lastly, biopesticides are important part of the integrated pest management strategy and in the sustainable agriculture (Boriani et al., 2006; Kiss and Delos, 2020).

1.3.4 Maize hybrids based on GM technology and new breeding techniques

Proteins from different species of *Bacillus thuringiensis* species have been proven to be toxic to different lepidopteran and coleopteran pests, and it also been found to be effective against *Diabrotica. v. virgifera* (Donovan et al., 1992). With this genes maize plants were transformed and thus they produce these crystal proteins all of their lifetime and when the larvae attacks the roots, they eat up this proteins, the protein attaches to a specific receptor in the larvae gut and

making pores on it, thus the ion exchange of the larvae are disrupted and they will die in sepsis (Sanahuja et al., 2011). The first commercialized transgenic hybrid introduced into the market in 2003 which contained the Cry3Bb1 insecticidal toxin (Vaughn et al., 2005). After the first protein, there were others which could be used against *Diabrotica. v. virgifera* such as the Cry34Ab1/Cry35Ab1 and mCry3A and the eCry3.1Ab (Moellenbeck et al., 2001; Rice, 2004; Narva et al., 2013). Later on to delay establishment of the resistance against these proteins they introduced more than one into hybrids in a process called pyramiding (Carrière et al., 2015). However, resistance to all Cry proteins has been detected since than (Gassmann, 2012; Gassmann et al., 2014; Gassmann et al., 2016). There is no genetically modified maize hybrid against coleopteran species in the EU market for cultivation (Meissle et al., 2011; ISAAA database, 2022, http9).

Since then, new molecular techniques have been used to create transgenic maize plants which can effectively kill *Diabrotica. v. virgifera* larvae. Based upon double stranded RNAs RNA interference has been used to develop such hybrids (Whangbo and Hunter, 2008). The most successful one has been already commercialized and targeting the *Diabrotica. v. virgifera* DvSnf7 gene (*Diabrotica v. virgifera* sucrose-non-fermenting genes SNF7) which is encoding a protein which is crucial to transmembrane protein sorting. (Baum et al., 2007; Levine et al., 2015). Again, resistance of the larvae against this newly targeted gene also has been demonstrated (Khajuria et al., 2018).

2. Hypotheses and aim of the study

Our major goal was to improve our understanding on why the different control methods are succeeding or failing to control *Diabrotica v. virgifera*. One of the aims of this study was to investigate why soil insecticides and biological control agents, such as entomopathogenic nematodes, occasionally lead to variable efficacies at reducing *Diabrotica v. virgifera* populations and preventing root damage under field conditions. Because of their variable efficacies and because of the ongoing phase out of a number of pesticides, we also investigated an alternative, botanical derived control option to prevent root damage and to manage *Diabrotica v. virgifera* populations, this is the neem-derived azadirachtin. Moreover, to conduct proper plan trials and bioassays, precise hatching information of this pest's larvae is needed. Therefore, we investigated the effects of diapause length and post-diapause incubation temperature on egg hatching patterns

of a diapausing and a non-diapausing colony of *D. v. virgifera*, with the ultimate aim to shorten experimental periods The detailed research questions and objectives were the following:

1. **How does the efficacy of entomopathogenic nematodes and soil insecticides change against *Diabrotica v. virgifera* pest populations during the cropping seasons? In other words, what is the effect of time on treatment efficacies?** (**Chapter I.:** Missing temporal effects of soil insecticides and entomopathogenic nematodes in reducing the maize pest *Diabrotica virgifera virgifera*)

   To manage *Diabrotica v. virgifera* larvae in the soil treatments are usually applied 2-4 weeks before larvae hatching. After that, still weeks to pass until they are reaching to pupation and complete their lifecycle in the soil. This means soil insecticides and biological control agents have to be present and be effective in the control of larvae populations for a rather long time. Thus, it is crucial to understand whether agent efficacies change in time. Also, these effects should be reflected in changing temporal patterns of adult population emergence from the treated plots. For example, evidence shows that soil samples taken from clorpyriphos treated plots can kill 3rd instar larvae within 2 days at almost 100% (Sutter et al., 1989). Boetel et al. (2003) showed that delayed cumulative emergence of *Diabrotica v. virgifera* adults from terbufos -treated plots occasionally occurs. According to Michaelides and Wright (1997) sub-lethal dosage of tefluthrin *Diabrotica undecimpunctata howardi* has severely affected larvae development in the soil and thus adult emergence dynamics was affected. Commercial *H. bacteriophora* strains are bred under artificial conditions which can deteriorate some traits of the offspring generations (i.e. virulence, survival) (Bilgrami et al., 2006) which may have an effect on their efficacy under farming conditions against *Diabrotica. v. virgifera*. However, they are known to well-persist for months in the field (Kurtz et al., 2007; Pilz et al., 2014).

   Therefore, we analyzed adult capture data from 12 field-scale experiment between 2010 and 2018 in maize growing areas in South Hungary. We investigated the temporal effects on the efficacies of the entomopathogenic nematode *H. bacteriophora* and the granular soil insecticides chlorpyrifos, cypermethrin, and tefluthrin. **We hypothesized** that: • soil insecticides may rather kill the early hatching proportion of larvae than the later ones, due their degradation or/and depletion from the soil, while • the efficacy of the entomopathogenic nematodes may increase with time because they propagate in the larvae. We thought, these processes will be reflected in the temporal adult emergence patterns.

2. **Which abiotic and biotic factors may influence and how the efficacies of entomopathogenic nematodes and soil insecticides at reducing *Diabrotica v. virgifera* pest populations and preventing root damage under field conditions?** (**Chapter II.:** Limited influence of abiotic and biotic factors on the efficacy of soil insecticides and entomopathogenic nematodes when managing the maize pest *Diabrotica v. virgifera* (Coleoptera: Chrysomelidae)

Soil insecticides, seed coatings and entomopathogenic nematodes are in a frequently changing below-ground environment. Therefore, interactions of treatments with abiotic and biotic factors may negatively influence their efficacies for larvae population management and root protection. For example, the type of the soil could be a major factor. It seems that for some soil insecticides, a higher organic soil content positively influences their effectiveness (i.e. muck and clay-type soils), but more sand content may lower efficacies (i.e. quartz sand, plain field sand, sandy loam) (Harris, 1972). However, it seems soil type is not a major factor to influence *H. bacteriophora* efficacies against *Diabrotica. v. virgifera* (Toepfer et al., 2010). Low soil moisture level negatively affects organochlorines and organophosphates toxicity (Wolcott, 1970) against insect pests. Some studies indicate that this may also be true for *H. bacteriophora* (Grant and Villani, 2003).

Therefore, we analyzed datasets from experiments in maize fields in Hungary between 2010 and 2020, this is adult captures from gauze cages and root damage data according to two different damage rating scales one assessing general root damage, and one assessing heavy root damage. Then, we calculated efficacies for the entomopathogenic nematode *H. bacteriophora* and the seed treatment clothianidin and granular soil insecticides cypermethrin and tefluthrin. After that we investigated the correlations and regressions between 32 abiotic and biotic factors and the above-mentioned treatment efficacies. **We hypothesized** that • more rainfall early in the cropping seasons may „wash-out" soil insecticides • in opposite, more water/moisture of the soil may aid the entomopathogenic nematodes • higher air temperatures may cause nematodes to be more active, thus they will die earlier • higher temperatures may cause earlier larvae hatch, which may therefore be better reached by treatments.

3. **Can the botanical azadirachtin sufficiently kill *Diabrotica v. virgifera* larvae and prevent root damage to become an alternative, new candidate that can replace conventional soil insecticides?** (**Chapter III.:** Can the botanical azadirachtin replace

phased-out soil insecticides in suppressing the soil insect pest *Diabrotica virgifera virgifera*?)

Botanical insecticides are used a since long time against insect pests (Isman, 2005). Azadirachtin is known to be lethal to rootworm larvae, such as against *Diabrotica speciosa* (Boiça Júnior et al., 2017) or the here-studied *Diabrotica. v. virgifera* (Xie et al., 1991). They are also repellent to larvae of cucumber beetles such as *Diabrotica undecimpunctata howardi* (Landis and Gould, 1989). Estes et al. (2018) tried to control *Diabrotica. v. virgifera* larvae with different liquid and granular formulation of the azadirachtin, but their results were inconclusive. It seems that detailed information how to use azadirachtin against rootworms are still missing.

Therefore, we tested a novel granular formulation of azadirachtin against *Diabrotica. v. virgifera* larvae under laboratory conditions using artificial diet-based assays as well as under greenhouse conditions, using potted infested maize plants. **We hypothesized** that this azadirachtin treatment has a similar effect on larvae mortality as conventional insecticides. We collected data on larvae mortality and sub-lethal effects of the azadiracthin after 3 days and 5 days. We calculated the LD_{50} and LD_{90} values. After that, we conducted trials under greenhouse conditions with potted-maize plants and collected data on the number of the survived larvae, root damage (general root damage with IOWA scale and heavy root damage with node-injury scale) and above-ground plant biomass after 1.5 months infested the plants with the pest's eggs. We compared azadirachtin to other conventional insecticides such as: thiomethoxam, cypermethrin and tefluthrin. We calculated ED_{50} and ED_{90} values.

4. **What is the shortest diapause length and most practical incubation temperatures which still do not compromises proper hatching rates and a good hatching synchrony of *Diabrotica v.virgifera* eggs?** (**Chapter IV.:** On understanding and manipulating the hatching patterns of *Diabrotica virgifera virgifera* eggs to improve design of the experiments).

This insect's eggs overwinter in the soil naturally for 8-10 months (Ball, 1957). For effective and economic experimentation, continuous supply of ready to hatch eggs needs to be ensured. Consequently, numerous studies were conducted to provide information on hatching dynamics of this insect (Branson, 1978; Krysan, 1982; Schaafsma et al., 1991). Nevertheless, there is no clear overview on egg overwintering and hatching dynamics.

Therefore, in this chapter, we wanted provide detailed information on the survival and temporal hatching patterns of the pest's eggs depending on diapause length as well as post-diapause incubation temperature. This will allow better planning of experimentation with the pest's eggs and the hatching larvae We established a dataset on effects of diapause lengths and post-diapause incubation temperatures on egg overwintering survival, as well as the start, peak, duration, and end of egg hatching and hatching success. This was done for a diapausing and a non-diapausing colony of *Diabrotica. v. virgifera*. **We hypothesized** that diapause length may be shortened to a certain extent without compromises the hatching rates and hatching synchrony, which would allow more frequent experimentation. We also hypothesized that there might be an optimal post-diapause incubation temperature.

3. Chapter I.

Missing temporal effects of soil insecticides and entomopathogenic nematodes in reducing the maize pest *Diabrotica virgifera virgifera*

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3.1 Abstract and Introduction

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ORIGINAL PAPER

Missing temporal effects of soil insecticides and entomopathogenic nematodes in reducing the maize pest *Diabrotica virgifera virgifera*

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Abstract
Control methods for the larvae of the maize pest *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) are known to produce inconsistent results under field conditions. To better understand the effects of plant protection products on the root-feeding larvae, we looked for changes in efficacy of the granular soil insecticides chlorpyrifos, cypermethrin, and tefluthrin during a cropping season, as well as a fluid-applied entomopathogenic nematode *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae). Twelve field-scale experiments carried out in Hungary between 2010 and 2018 revealed that treatments, whether chemical or biological, are able to reduce *D. v. virgifera*. However, results were variable with failures in about a quarter of the experiments. Unexpectedly, our findings indicated only limited effect of time on treatments, meaning all products appeared capable of continuously reducing larvae during their time in the soil. Only chlorpyrifos seemed to slightly lose and tefluthrin to slightly increase efficacy over time. Nevertheless, there is no major evidence that failure of treatments is due to temporal effects. Other factors may play a larger role and merit investigation under field conditions.

Keywords Western corn rootworm · *Zea mays* · Chlorpyrifos · Cypermethrin · Tefluthrin · *Heterorhabditis bacteriophora* · Pest control

Key message

- The western corn rootworm is a maize pest in North America and Europe.
- Chemical and biological control of its root-feeding larvae is often variable.
- We hypothesized that soil insecticides lose and beneficial nematodes increase their efficacy with time.
- We analysed temporal effects of treatments in 12 Hungarian fields over 7 years.
- Treatments led to variable results with failures in about a quarter of the experiments.
- Findings indicated only limited effect of time on treatments.
- Other factors may play a larger role and merit investigation under field conditions.

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Introduction

Maize is one of the three major carbohydrate providers to humans next to rice and wheat. In the European Union, maize ranks second after wheat with around 65 million tonnes grain maize and maize-cob-mix harvested in 2017 (Cook 2018). In the USA, maize ranks second after soybean with around 400 million tons grain maize harvested in 2018 (USDA 2018). The reliance of humans on maize increases the impact of crop pests.

One such maize pest is the chrysomelid beetle, *Diabrotica v. virgifera* LeConte (western corn rootworm). This pest species is hypothesized to have originated from Mexico or

Central America (Branson and Krysan 1981). It invaded large areas of North America and Canada (Gray et al. 2009) as well as of Europe (Miller et al. 2005; Szalai et al. 2011). It is a univoltine species with eggs that overwinter in the soil (Krysan and Miller 1986). After maize has germinated, the eggs soon hatch, and its three larval instars feed almost exclusively on maize roots (Moeser and Hibbard 2005). This often causes plant lodging (Levine and Oloumi-Sadeghi 1991). Efficient larval control appears difficult as the larval population hatches over a period of at least a month and is found feeding on maize roots in the soil for at least 2 months (Toepfer and Kuhlmann 2006). Corn rootworms cause approximately 1 billion dollars of crop losses and pest management costs in the USA annually (Krysan and Miller 1986; Rice 2004). In Europe, maize losses were estimated to account for 472 million euros, when no control measures would be implemented (Wesseler and Fall 2010).

The main pest management approaches include (1) synthetic insecticides such as granular or fluid soil insecticides as well as seed coatings against larvae, or foliar sprays against adults; (2) entomopathogenic nematodes against the larvae; (3) cultural control of the larvae through crop rotation; or (4) transgenic maize through expressing insecticidal proteins in the roots (Levine and Oloumi-Sadeghi 1991; van Rozen and Ester 2010).

Particularly soil applications of synthetic insecticides into the furrow at sowing have been reported to reduce larval damage to roots and to prevent plant lodging (Sutter et al. 1989, 1990), such as for tefluthrin, chlorpyrifos ethyl, clothianidin, and λ-cyhalothrin (Blandino et al. 2016). However, soil as well as foliar insecticides occasionally fail in sufficiently reducing populations of this pest. As for adult control, pesticide inconsistency in efficacy has been often attributed to insecticide resistance. Such resistances are indeed known for *D. v. virgifera* adults, such as against some chlorinated hydrocarbons since the 1960s (Ball and Weekman 1962; Ciosi et al. 2009), or methyl-parathion and carbaryl since the mid-1990s (Meinke et al. 1998). Another problem is that *D. v. virgifera* beetles are mobile and can immigrate from untreated into treated fields (Levine and Oloumi-Sadeghi 1991; Gray et al. 1992). As for the larvae, less is known on their resistance to pesticides. However, adult resistance to pesticides is suggested to be inherited to the larvae and to reduce their susceptibility to certain pesticides (Wright et al. 2000). This was, for example, shown for larvae originating from adults in North America with resistance to methyl-parathion, terbufos, chlorpyrifos, carbofuran, and tefluthrin (Wright et al. 2000), and to bifenthrin (Pereira et al. 2015).

Nevertheless, even in areas where resistance has not yet been observed, soil pesticides have been reported to lead to inconsistent levels of root protection (Sutter et al. 1989; Furlan et al. 2006) and *D. v. virgifera* reduction (Gray et al. 1992; Boetel et al. 2003). As all larval instars of *D. v. virgifera* are susceptible to the pesticides, other factors may influence control efficacies. The chemical properties of insecticides (e.g. water solubility, evaporation) (Devare et al. 2004; Whiting et al. 2014), biodegradation levels through microbial activity (Chapman and Harris 1990), and the effect of environmental factors (soil properties, rainfall, temperature) on both may play roles in changing efficacies of treatments. Nevertheless, some soil pesticides such as chlorpyrifos, terbufos, or fonofos seem to well persist in the soil for up to 5 months being able to kill *D. v. virgifera* larvae and not being much influenced by rainfall and depletion. Other pesticides seem to be occasionally washed out or degrade as a result of rainfall, such as carbofuran or isofenphos (Sutter et al. 1989). In contrast, lack of soil moisture may generally lead to insufficient vertical and horizontal movement of an insecticide in the root zone and to less contact with the larvae (Sutter et al. 1991). Unfortunately, for some currently used insecticides, no such information is available from field conditions.

Any decrease or increase in efficacies of soil pesticides over the long period of 2 to 3 months of the larval population being in the soil should be also reflected in changed temporal emergence patterns of the adults from the soil. Female larvae seem to hatch from eggs on average at least 3 days later than the male larvae and need at least 2 days longer for their development to adults (Branson 1987). This may have implications on the efficacy of treatments. For example, Boetel et al. (2003) reported from a 3-year study that tefluthrin delayed the emergence of female *D. barberi* adults in at least two seasons and terbufos delayed the emergence of female *D. v. virgifera* adults in one season. Although results were not consistent in their studies, this may indicate that late female larvae may have been less reached by those pesticides in those years. Similarly, Sutter et al. (1991) reported from a 4-year study that ethoprop and chlorpyrifos delayed the 50% emergence of *D. v. virgifera* adults in one of four seasons, and carbofuran did so in two of four seasons. Otherwise, there is limited published information on temporal changes of soil pesticides in larval control and subsequent adult emergence patterns under field conditions, and if available reasons remain often unclear.

Due to the occasional failure and due to bans of some insecticides in maize, a biological control product had been developed based on the entomopathogenic nematode *Heterorhabditis bacteriophora* Poinar (Nematoda: Rhabditida) (Babendreier et al. 2006; Kergunteuil et al. 2016). It has recently reached the market in a number of European countries, such as Germany, Italy, Austria, or Hungary (Ehlers 2003; Toepfer et al. 2008). Nematodes can effectively kill all three larval instars of *D. v. virgifera*. Pilz et al. (2009) found in a 2-year field study that *H. bacteriophora* achieved, similar to tefluthrin, around 60% efficacy at reducing *D. v.*

virgifera, while clothianidin seed coating achieved around 70%. This suggests comparable efficacies of entomopathogenic nematodes to conventional insecticides. Unfortunately, also entomopathogenic nematodes seem variable in their efficacy at reducing *D. v. virgifera* and in preventing root damage when applied under field conditions (Toepfer et al. 2010a, c). In some cases, they may even entirely fail to control this pest (Rauch et al. 2017). Reasons behind inconsistencies and failures of nematodes under field conditions are still not fully understood. They likely include inappropriate handling of the living nematodes during storage, transport and mixing (Toepfer et al. 2010b), or suboptimal application, such as onto-soil sprays instead of into-soil applications of the moisture-requiring nematodes (Toepfer et al. 2010c). However, once successfully applied into the soil, nematodes seem well protected as the seed placement area is usually sufficiently moist. Moreover, nematodes can vertically move up and down in soil depending on moisture. Therefore, a positive effect of rainfall or the amount of water used for nematode application on the success of entomopathogenic nematodes is rarely found in the case of *D. v. virgifera* in maize fields (Toepfer et al. 2010a). Nematodes also seem to persist long enough in the soil to attack larvae and even propagate in them, an obvious advantage over pesticides. Thus, similar to soil pesticides, reasons behind inconsistency in efficacy results of nematodes under field conditions are little understood.

Therefore, we tried to better understand the occasionally suboptimal control efficacies of soil pesticides and entomopathogenic nematodes using existing data from a large number of field experiments (Ehlers et al. 2008; Toepfer et al. 2010a, c). Our hypotheses were that (a) soil insecticides may rather kill the early than late hatching larvae due to depletion or degradation of the active ingredients with time, and (b) entomopathogenic nematodes may increasingly reduce larvae with time due to propagation in the pest. Our analyses were based on the idea that such temporal changes in the efficacy of the applied control methods on the larvae should be also reflected in a change in temporal adult emergence patterns later in the cropping season (Sutter et al. 1991; Boetel et al. 2003). This could be reflected in shifts in time periods elapsed between emergence start and attaining linear adult emergence compared to untreated controls, in shifts in emergence peaks, or in changing emergence rates over time. We therefore applied such analyses to data from 12 different field-scale experiments from southern Hungary between 2010 and 2018 (Ehlers et al. 2008; Toepfer et al. 2010a, c).

Results may explain some of the reasons behind successes and failures of chemical and biological control methods. This may allow adaptations or further developments with the ultimate aim to provide growers with more effective and more diverse pest management tools.

Methods

Field sites

This study was carried out on 12 conventionally managed maize fields in southern Hungary between 2010 and 2018 (Table 1). All fields had been ploughed in autumn after the end of the previous cropping season and then tilled and harrowed in early to mid-April prior sowing maize. All sowing dates (Table 1) were within the southern Hungarian standard period for maize sowing, which is from mid-April to first week of May. Individual maize seeds were sowed every 16 to 18 cm in rows 75 cm apart, leading to 72–87,000 plants per ha using a 4-row or 6-row planter. All seeds had been coated with standard fungicides.

Target organism

The target organism was *Diabrotica virgifera* ssp. *virgifera* LeConte (Coleoptera: Chrysomelidae). Study fields had hardly any natural population of *D. v. virgifera* because non-maize crops had been planted the previous season or 2 years before (except fourth year maize in field P, Table 1). Therefore, the life cycle of the maize-restricted *D. v. virgifera* larvae was disrupted. Instead, plants were artificially infested with *D. v. virgifera* eggs to simulate well-established, but homogenously distributed pest populations. Eggs were obtained from a laboratory culture of field-collected beetles in southern Hungary in August and September the previous year (for procedures, see Singh and Moore 1999). Artificial infestations are known to lead to similar larval development and adult emergence as natural populations (Fisher 1984). *Diabrotica v. virgifera* eggs were overwintered for 7 months at 6 to 8 °C in moist sand and 60 to 70% of eggs successfully overwintered. Diapause was broken during third or fourth week of April the following year by transferring eggs to 22 to 24 °C.

Two sets of six or seven subsequent maize plants of each experimental plot were infested with viable and ready-to-hatch eggs per plant when the plants were at the first to fourth leaf stage (for egg densities and dates, see Table 1). Eggs were applied in 0.15% aqueous agar using a standard pipette (5 ml, Eppendorf company, Hamburg, Germany) in two to four portions of eggs (in about 1 to 2 ml water–agar each) into 100- to 140-mm-deep holes at a distance of 110 to 190 mm from both sides of the maize plant early May (Table 1).

A portion of eggs was transferred onto moist filter paper in Petri dishes and incubated at 20 to 25 °C in the laboratory to monitor time of first hatch as well as hatching rate of the larvae. In the laboratory, *D. v. virgifera* larvae started to hatch around 1 week after egg application date and hatching

Table 1 Characteristics of experimental field sites and treatments against *Diabrotica v. virgifera* larvae in southern Hungary

Experiment	U	S	T	Q	P	N	K	M	F	G	B	A	n
Year	2018	2017	2017	2016	2015	2015	2014	2014	2013	2013	2010	2010	7
Location	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	East of Mako	West of Kiszombor	East of Mako	West of Kiszombor	East of Mako	North of Szeged	South of Szeged	12
Coordinates	N 46° 44′ 57.426″	N 46° 44′ 58.88″	N 46° 45′ 02.89″	N 46° 44′ 29.775″	N 46° 44′ 8.036″	N 46° 14′ 36.012″	N 46° 11′ 05.8″	N 46° 14′ 29.1″	N 46° 11′ 05.4″	N 46° 14′ 34.8″	N 46° 17 47.0	N 46° 13.590	
	E 20° 49′38.406″	E 20° 48′ 56.11″	E 20° 48′ 59.40″	E 20° 48′ 39.764″	E 20° 48′ 57.193″	E 20° 30′ 52.109″	E 20° 24′ 33.2″	E 20° 31′ 02.0″	E 20° 24′ 31.1″	E 20° 30′ 49.5″	E 20° 07 18.6	E 20° 09.035	
Trial size (ha)	0.37	0.25	0.25	0.29	0.29	0.43	0.29	0.29	0.25	0.25	0.3	0.3	
Plots size (m)	6 rows 4.5 m×20 m	6 rows 4.5 m×20 m	6 rows 4.5 m×20 m	6 rows 4.5 m×20 m	6 rows 4.5 m×20 m	4 rows 3 m×30 m	4 rows 3 m×30 m	4 rows 3 m×30 m	4 rows 3 m×30 m	4 rows 3 m×30 m	4 rows 3 m×30 m	4 rows 3 m×30 m	4 to 5 plots/treatment/field
Pre-crop	Sunflower	Triticale	Triticale	Sunflower	Maize	Winter wheat	Soybean	Winter wheat	Maize	Winter wheat	Non maize	Non maize	
Maize hybrid	PR37F73 (Pioneer)	N01 (Pioneer)	N01 (Pioneer)	P9903 (Pioneer)	P0216 (Pioneer)	NK Kansas (Syngenta)	Pactol (Syngenta)	Mika (Syngenta)	Gavott (KWS)	Gavott (KWS)	Boglar (GK)	Boglar (GK)	
Sowing date	25-Apr	25-Apr	25-Apr	18-Apr	20-Apr	21-Apr	23-Apr	23-Apr	23-Apr	23-Apr	23-Apr	22-Apr	
Plants/ha	74000	72000	72000	72000	74000	80000	80000	80000	80000	80000	87000	87000	
D. v. virgifera eggs/plant	500	500*	500*	300	High native pop.	200, 300, 500	300	300	200, 300, 500*	200, 300, 500	300	300	
Egg infestation date	04-May	08-May	08-May	01-May	–	04-May	05-May	05-May	06-May	06-May	2 & 25 May	2 & 25 May	
Treatments													
Treatment date	25-Apr	25-Apr	25-Apr	18-Apr	20-Apr	21-Apr	23-Apr	23-Apr	23-Apr	23-Apr	23-Apr	22-Apr	
H. bacteriophora (billion/ha)	1	2	2	2	1.6; 2	2	2	2	2	2	1.5	1.5	42 plots (12 fields)
Cypermethrin 0.8% (kg/ha)	12	12	12	12	12	12	12	12					16 plots (8 fields)
Chlorpyrifos 5% (kg/ha)					10	10	10	10					16 plots (4 fields)
Tefluthrin 1.5% (kg/ha)					13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	31 plots (8 fields)

*Small native population present due to highly infested neighbouring fields the previous season

lasted until late May. An average hatching rate of 86 ± 13% was determined. In the field, larvae were expected to emerge between the middle and end of May and second-instar larvae were expected early June (Toepfer and Kuhlmann 2006).

Experimental design and treatments

The temporal effect of a fluid formulation based on an entomopathogenic nematode as well as of granule formulations of three synthetic insecticides was studied on *D. v. virgifera* populations in 12 field-scale experiments (Table 1). All experiments were conducted according to the efficacy evaluation standards PP 1/212 and PP 1/152 of EPPO (Anonymous 1999, 2007). In each field, four to five plots of four to six maize rows (3 to 4.5 m × 20 to 30 m plots) per treatment and control were systematically arranged with changing orders between the different fields and years. No field location was used twice. For replicate numbers of each treatment, see Table 1.

All agents were applied at sowing into sowing row behind seed placement at about 80 to 110 mm depth as the maize was sown. All treatments were conducted over entire plots. Treatment and sowing dates were about one to 3 weeks prior egg hatch in the field.

In each plot, twice six to seven successive maize plants (≈ 1.2 m) were randomly chosen among the two middle rows of each of the four- to six-row wide plots for artificial infestation with ready-to-hatch *D. v. virgifera* eggs about a week later as described above, and for data assessments from June to August as described below.

Heterorhabditis bacteriophora fluid

In all 12 experimental fields (Table 1), mostly about 100,000 to 150,000 infective juveniles (*ij*) of a commercial hybrid of European and the USA strains of *H. bacteriophora* (Rhabditida: Heterorhabditidae; Dianem Wurzelbohrer™, e-nema company, Schwentinental, Germany) were applied per row meter. This was the equivalent of 1.5 to 2 billion nematodes per hectare. They were provided in a formulation of light inert powder (Formulation: Water soluble powder SP according to GIFAP code, 15% a.i. *H. bacteriophora*, 20% water, 65% inert ingredients mainly diatomaceous earth).

Approximately 1 to 2 h before application, the infective juveniles were diluted, together with the carrier material, in cool tap water to the required doses. Prior mixing, the living status of nematodes in the product batch had been determined under stereomicroscope, assuring an at least 70% survival rate. No adjuvants were used.

In fields A to N, a four-row self-made fluid applicator on a Pneumasem sowing machine (Nodet Gugis, Lacaille SA, France) was used applying the nematode fluid via gravity through tubes without nozzles behind the seed placement, and before the soil closing wheels (450 to 550 litres per ha). No seed pressing wheels were on the sowing machine.

In fields P to U, a six-row self-made fluid applicator or a commercial fluid applicator (LIQ-Inject M1, Cult-tec GbR, Freiburg, Germany) were used on a Monosem NG sowing machine. Filters in the spraying system had been taken out. Tubes with core nozzles (Streamjet h1/4u –ss0010, TeeJet Spraying Systems Co., Wheaton, IL, USA) applied the nematode fluid after the seed placement, and before the soil closing wheels (200 litres per ha). No seed pressing wheels were on the sowing machine.

During application, subsamples of about 2 ml nematode solution were taken from below the nozzles per treatment and field to determine the quality of nematodes arriving into the soil. Laboratory quality control bioassays with larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were used (Toepfer et al. 2008). If a mortality of 35 to 65% of *T. molitor* was found after 1 week, and 75 to 95% after 2 weeks, the applied nematodes were considered of sufficient level of virulence according to the nematode producer e-nema, which requires at least 50% mortality. This was the case for all presented experiments.

Chlorpyrifos fine granules

In four experimental fields (Table 1), about 0.75 g of fine granules (1 to 2 mm diameter, Formulation: Fine granule, FG of GIFAP code) of the soil insecticide chlorpyrifos, i.e. the organophosphate with the active substance O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate (Kentaur™ 5 G, 5% Chlorpyrifos, Cheminova, Budapest, Hungary), were applied per row meter. This was the equivalent of the recommended dose of 10 kg granules per hectare. They were applied by a seeder-mounted fine granule applicator (Galdept-10 of Galenika Fitofarmacija, Srem Karlovci, Serbia; or MicroSem of Certis, UK), into seeding rows at about 80 to 110 mm depth into the soil just after seed placement.

Cypermethrin micro-granules

In eight experimental fields (Table 1), about 0.9 g of fine granules (0.8 to 1 mm diameter, Formulation: Microgranule, MG of GIFAP code) of the soil insecticide cypermethrin, i.e. the pyrethroid with the active substance [Cyano-(3-phenoxyphenyl)methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate (Belem™ 0.8 MG, 0.8% Cypermethrin, Spiess-Urania, Hamburg, Germany), were applied per row meter. This was the equivalent of 12 kg granules per hectare. However, due to its too fine granule size, the product had been diluted by ½ using river sand of similar particle size and then applied by a seeder-mounted micro-granule applicator and applied as described above.

Tefluthrin fine granules

In eight experimental fields (Table 1), 1 g of fine granules (1 to 2 mm diameter, Formulation: Fine granule, FG of GIFAP code) of the soil insecticide tefluthrin, i.e. the pyrethroid with the active substance 2,3,5,6-Tetrafluoro-4-methylbenzyl(Z)-(1RS,3RS)-3-(2-chloro- 3,3,3-trifluoro-1-propenyl-2,2-dimethylcyclopropanecarboxylate (Force™ 1.5 G, Syngenta, Budapest, Hungary), were applied per row meter. This was the equivalent of 13.3 kg per ha. They were applied by micro-granule applicators as described above.

Untreated control

Untreated *D. v. virgifera* egg-infested plots served as negative controls.

Assessment of *D. v. virgifera* dynamics and reduction

For each treatment and control, four to five sets of six to seven infested consecutive maize plants were cut to a height of about 100 cm and covered with gauze cages (inner size, 125 cm × 40 cm × 150 cm high). They were placed mid-June, i.e. prior the predicted start of adult emergence (Toepfer and Kuhlmann 2006). To assess the temporal emergence patterns of *D. v. virgifera* adults, we counted the emerged beetles on a weekly basis following the procedures outlined in the EPPO standards (Anonymous 1999, 2007; Toepfer et al. 2008). Counted beetles were sexed according to antenna length (Gloyna, K. 2008 pers. comm.; Hammack and French 2007) and removed from the cages at each check. Adult emergence data were standardised to 100 eggs per plant per week (except for field P that hosted a native pest population). The weekly cumulative emergence was calculated per time step for all experiments. The efficacy of each treatment was calculated as the *D. v. virgifera* emergence relative to the control (corrected efficacy % = 100 × (beetles in control plots − beetles in treated plots)/maximum (beetles in control or treated plots). Root damage changes over weekly time steps were not assessed as roots would have been destroyed and therefore interrupted adult emergence.

Data analyses

Temporal dynamics of pooled sex, male and female adult emergence as well as their cumulative emergence were plotted over weeks using loess smoothed (local polynomial regression) lines (Figs. 1, 2, 3, 5).

The cumulative adult emergence curves (= local polynomial regression lines) were used to estimate the date of start and end of adult emergence (Table 2). Differences between male and female emergence start, their 25, 50, and 75% emergence, and their peak emergence were analysed for each treatment and controls using paired t tests (Table 3). Differences in male and female cumulative emergence patterns (time shift and steepness) were analysed by comparing their regression curves using GLMs (Fig. 3).

Linear models were used to identify and compare control effects of treatments as well as to investigate the influence of treatments, sex, and their interaction on efficacies with additionally performed basic diagnostic plots for assumption of residual normality and homoscedasticity, as well as plots of Cook's distance for detecting influential data points (Faraway 2004).

To assess the temporal effects of treatments on the reduction of *D. v. virgifera*, we assumed that temporal shifts in adult emergence patterns reflect potential shifts in larval control by the treatments. The cumulative emergence of the adults in cages of each treatment and experiment was standardised and plotted as a percentage of the corresponding total emergence (Figs. 1, 5). This is (a) to standardise the different absolute emergence levels between treatments and (b) to standardise different *D. v. virgifera* densities across experiments. To identify temporal effects of treatments on *D. v. virgifera,* the deviances of the following quasi-binominal GLMs with logit link functions were compared using an F-test: one model with the explanatory variables time (days after emergence start), treatment (pairs of a particular treatment and control), and their interaction versus the second model with the single explanatory variable time. Those steps were performed separately for pooled sex, male, and female data, each treatment totalling in 12 comparisons of model deviances. Where a temporal effect was identified, the explanatory variables were tested separately using t tests to clarify whether the treatment (shift in time) or the interaction of treatment x time (steepness of the curve) or both influenced the temporal pattern of *D. v. virgifera* adult emergence. P values were corrected for false discovery rates using Benjamini and Hochberg method (Benjamini and Hochberg 1995).

R statistical software was used for all analyses (version: 3.5.2., R Development Core Team 2018)).

Results

Temporal population dynamics

Per maize plant, 1.5 ± 1.8 (SD) *Diabrotica v. virgifera* adults emerged on average per 100 infested eggs; that is, 0.8 ± 1 males and 0.7 ± 0.9 females ($n = 11$ fields) which is around some of the economic thresholds used in the USA (see 1.6 adults per plant, Godfrey and Turpin 1983). *Diabrotica v. virgifera* adults emerged in southern Hungary during a period of about 6 weeks between mid-June to early July and end July to mid-August, i.e. during 45 ± 5 days (min.:

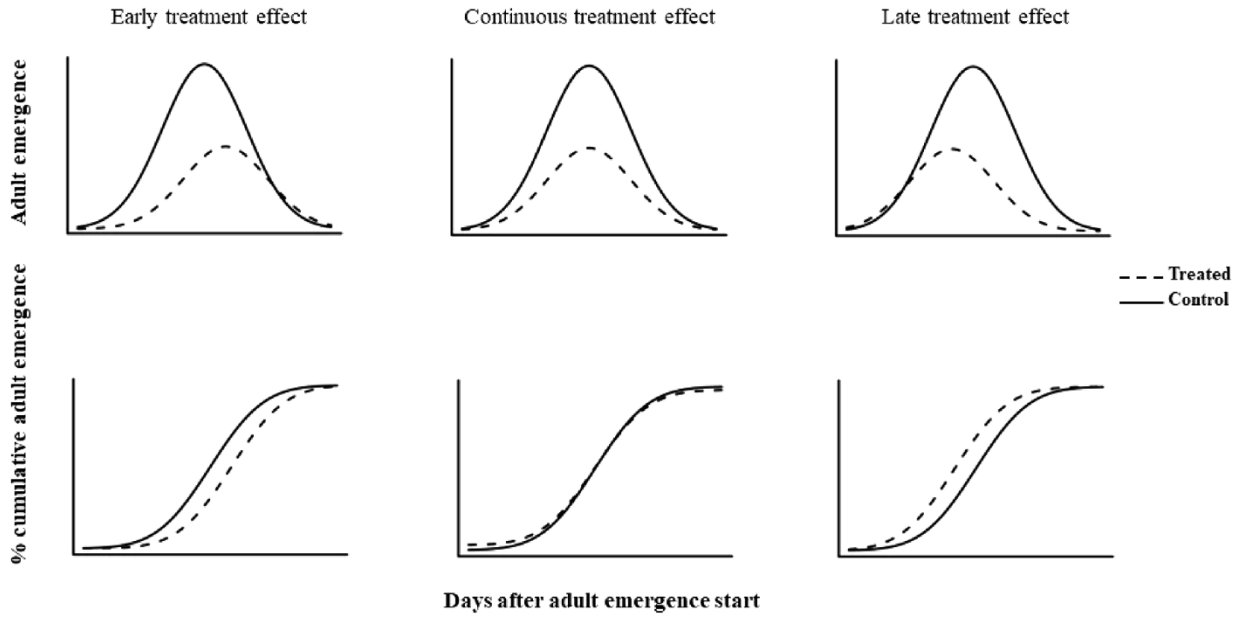

Fig. 1 Hypothetical temporal effects of treatments on *Diabrotica v. virgifera* reflected in the emergence patterns of adults over time and standardised as per cent cumulative emergence of the corresponding total emergence in a season. Smoothed trend lines plotted

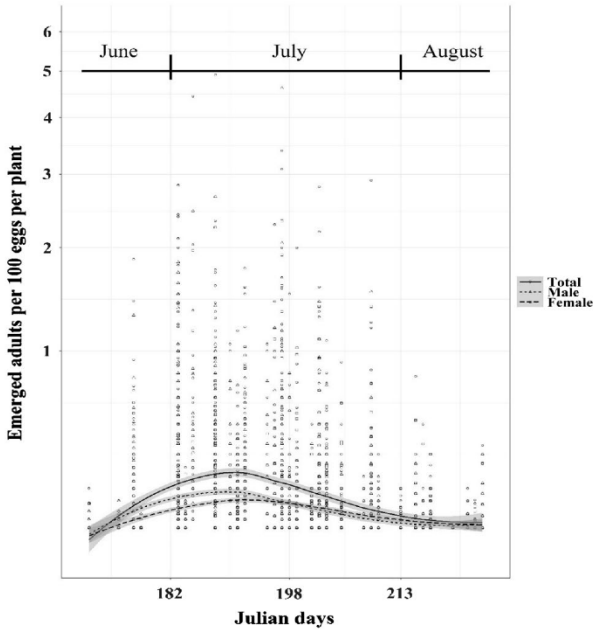

Fig. 2 Temporal dynamics of adult emergence of *Diabrotica virgifera virgifera* standardised per 100 eggs per plant in 11 artificially infested maize fields in Hungary between 2010 and 2018 (data from untreated control plots, smoothed trend lines)

36 days; max.: 53 days) (Fig. 2, Table 2). The start of emergence largely varied between years with an early start around 14 June in 2018 and a late start around 7 July in 2010. Half of the adults usually emerged until 10 July ± 8 days (192 Julian days), and this is about 16 days after emergence start. During the same date, the peak emergence of adults was ongoing. Adult stopped emerging around 9 August ± 7 days (Table 2).

Male and female beetles emerged over a period of 45 ± 5 and 43 ± 6 days, respectively (Table 2, Fig. 2) with comparable emergence curves (GLM: time × sex interaction: comparable curve steepness, $p = 0.64$). Male populations emerged earlier than female populations (time shift in GLM regression curves, $p < 0.001$, Table 1, Fig. 3). The first record of males in the emergence gauze cages was about 1 day before the female first record. Half of the male population had emerged already 5 ± 8 days before the female population (50% at 7 July ± 9 days vs. 12 July ± 8 days; paired *t* test: $p = 0.02$, $CI_{95\%} = 1, 9.5$ days, Table 2, Fig. 2, 3). Males reached their peak emergence around 8 ± 11 days earlier than females (8 July ± 9 days versus 16 July ± 7 days; $p = 0.03$, $CI_{95\%} = 0.69, 14.47$, Table 2, Fig. 2, 3).

Efficacy at reducing *D. v. virgifera*

All tested products regardless of chemical or biological were able to reduce male and/or female *Diabrotica v. virgifera*. Variability in efficacy appeared high (see SEMs in Fig. 4). All treatments occasionally failed to sufficiently reduce pest populations, i.e. no statistical difference was detected between adult numbers in treated and untreated plots. Chlorpyrifos failed in one of four experiments (25%),

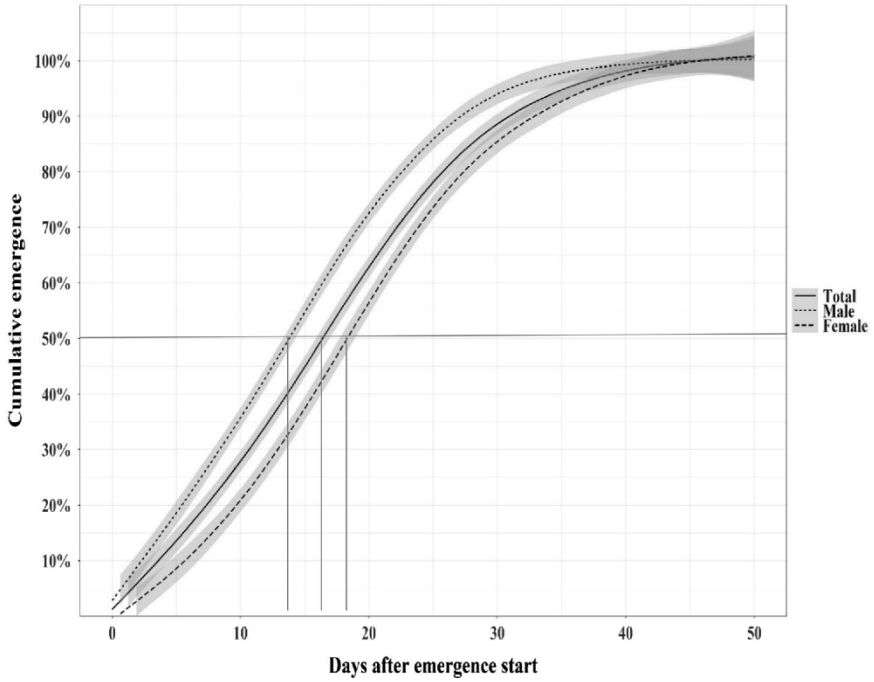

Fig. 3 Protandry in *Diabrotica virgifera virgifera* adult emergence presented as cumulative emergence over time in 12 maize fields in southern Hungary between 2010 and 2018 (data from untreated control plots, smoothed trend lines)

cypermethrin and tefluthrin in two of eight (25%), and *H. bacteriophora* in three of 12 experiments (25%).

Considering both sexes together, cypermethrin, tefluthrin, and *H. bacteriophora* reduced *D. v. virgifera* with comparable efficacies (Fig. 4, $p = 0.94$ of GLM for explanatory variable treatment). In detail, cypermethrin reduced 48 ± 33 (SD) of *D. v. virgifera* on average across fields and years (GLM, fdr-corrected $p = 0.004$); tefluthrin reduced 39 ± 43% ($p = 0.002$), and *H. bacteriophora* reduced 34 ± 37% ($p = 0.002$). However, such control efficacies were statistically not detected for chlorpyrifos ($p = 0.09$).

When considering the reduction of males and females separately, a similar picture was found for the male control efficacies as for the pooled-sex efficacy described above (GLM, explanatory variable sex, $p = 0.83$; treatment x sex, $p = 0.82$). Most treatments reduced males, i.e. cypermethrin (fdr-corrected $p = 0.005$), tefluthrin ($p = 0.005$), *H. bacteriophora* ($p = 0.004$), but chlorpyrifos did not ($p = 0.2$). As for females, all treatments including chlorpyrifos ($p = 0.02$) were able to reduce *Diabrotica v. virgifera* (cypermethrin: $p = 0.02$, tefluthrin: $p < 0.001$, *H. bacteriophora*: $p < 0.001$).

Temporal effects of treatments

All treatments regardless of chemical or biological had no major efficacy changes in reducing *D. v. virgifera* over time (Fig. 5, Table 3). The time needed to reach 25, 50, peak, or 75% adult emergence did not differ between treatments and the untreated control (Table 3).

In detail, *Heterorhabditis bacteriophora* and cypermethrin continuously reduced *D. v. virgifera* larvae over time, regardless of insect sex (Fig. 5). This is because there was no temporal effect of those treatments found on the adults' standardised cumulative emergence curve compared to the standardised emergence curve in the untreated control. In other words, no difference was found between logistic GLMs with factor treatment (levels of a certain treatment and control) x time versus GLMs with time only (Fig. 5).

Chlorpyrifos slightly better controlled early than late female larvae (Fig. 5). This is because there was no temporal effect of chlorpyrifos detected on the standardised male or pooled-sex cumulative emergence curve compared to the control curve, but a slight temporal effect on the female curve (difference between logistic GLMs with factor treatment × time versus GLMs with time only, $p = 0.03$, Fig. 5). This was reflected in a time shift (delay) in the adult female emergence curve according to the analyses of deviance of the emergence curve (logistic GLM treatment × time vs. emergence, $p = 0.03$), but not due to a change in curve steepness ($p = 0.11$) (see curves in Fig. 5).

Tefluthrin continuously reduced male *D. v. virgifera* larvae over time, but slightly improved efficacy later on. This is reflected in a slightly better control of late than early female larvae. In other words, there was no temporal effect of tefluthrin detected on the standardised male adult emergence curve compared to the untreated control curve, but on the female and pooled-sex curves. This is reflected in both a time shift (earlier) in the emergence pattern and in a change

Table 2 Temporal emergence patterns of adult *Diabrotica virgifera virgifera* in southern Hungary between 2010 and 2018 ($n = 4$ to 5 adult emergence gauze cages over 6 to 7 plants each, placed onto 4 to 5 untreated control plots per each of 12 maize fields)

Year	Experiment	Estimated start+	First recorded adults	Adult emergence		Last recorded adults	Estimated end+	Estimated duration (days)[1]
				50% emergence	Peak emergence			
Pooled sexes								
2018	U	14/06/2018*	20/06/2018*	03/07/2018	20/07/2018	20/07/2018*	20/07/2018	36
2017	S	20/06/2017	26/06/2017	04/07/2017	09/07/2017	11/08/2017	11/08/2017	50
2017	T	20/06/2017	26/06/2017	02/07/2017*	04/07/2017	01/08/2017	11/08/2017***	50
2016	Q	06/07/2016	02/07/2016	23/07/2016**	15/07/2016	09/08/2016	12/08/2016	45
2015	N	24/06/2015	24/06/2015	06/07/2015	07/07/2015	05/08/2015	05/08/2015	42
2015	P++	25/06/2015	25/06/2015	12/07/2015	16/07/2015	10/08/2015	10/08/2015	46
2014	M	19/06/2014	02/07/2014	09/07/2014	02/07/2014*	05/08/2014	07/08/2014	49
2014	K	19/06/2014	02/07/2014	06/07/2014	02/07/2014*	05/08/2014	11/08/2014	53
2013	G	25/06/2013	27/06/2013	03/07/2013	08/07/2013	01/08/2013	08/08/2013***	45
2013	F	27/06/2013	27/06/2013	17/07/2013	17/07/2013	01/08/2013	08/08/2013***	50
2010	A	07/07/2010	07/07/2010**	22/07/2010	15/07/2010	12/08/2010**	18/08/2010	43
2010	B	07/07/2010**	07/07/2010**	19/07/2010	22/07/2010**	12/08/2010**	18/08/2010	42
Mean (date)		24 June	28 June	10 July	11 July	6 August	8 August	
Mean (Julian days) ± SD		176 ± 7	180 ± 5	192 ± 8	193 ± 7	219 ± 6	221 ± 7	45 ± 5
Males								
2018	U	14/06/2018*	20/06/2018*	21/06/2018*	20/06/2018**	20/07/2018*	20/07/2018	36
2017	S	19/06/2017	26/06/2017	02/07/2017	09/07/2017	11/08/2017	11/08/2017	50
2017	T	20/06/2017	26/06/2017	03/07/2017	04/07/2017	01/08/2017	11/08/2017***	50
2016	Q	05/07/2016	02/07/2016	18/07/2016	15/07/2016	09/08/2016	15/08/2016	48
2015	N	24/06/2015	24/06/2015	05/07/2015	07/07/2015	05/08/2015	05/08/2015	42
2015	P++	25/06/2015	25/06/2015	12/07/2015	16/07/2015	10/08/2015	10/08/2015	46
2014	M	19/06/2014	02/07/2014	05/07/2014	02/07/2014*	05/08/2014	05/08/2014	47
2014	K	19/06/2014	02/07/2014	05/07/2014	02/07/2014*	05/08/2014	08/08/2014	50
2013	G	25/06/2013	27/06/2013	03/07/2013	08/07/2013	01/08/2013	08/08/2013***	45
2013	F	27/06/2013	27/06/2013	15/07/2013	17/07/2013	01/08/2013	08/08/2013***	50
2010	A	07/07/2010	07/07/2010*	22/07/2010**	16/07/2010	12/08/2010**	06/07/2010	42
2010	B	07/07/2010**	07/07/2010 *	17/07/2010	20/07/2010 **	12/08/2010 **	18/08/2010	42
Mean (date)		24 June	28 June	7 July	8 July	6 August	8 August	
Mean (Julian days) ± SD		176 ± 7	180 ± 5	189 ± 9	190 ± 9	219 ± 6	221 ± 8	45 ± 5
Females								
2018	U	20/06/2018 *	20/06/2018*	07/07/2018	20/07/2018	20/07/2018 *	20/07/2018	36
2017	S	23/06/2017	26/06/2017	05/07/2017	11/07/2017	11/08/2017	11/08/2017	50
2017	T	22/06/2017	26/06/2017	03/07/2017 *	05/07/2017 *	01/08/2017	11/08/2017	50
2016	Q	06/07/2016	02/07/2016	26/07/2016 **	23/07/2016	09/08/2016	11/08/2016	44
2015	N	24/06/2015	02/07/2015	08/07/2015	10/07/2015	05/08/2015	05/08/2015	42
2015	P++	25/06/2015	25/06/2015	13/07/2015	16/07/2015	10/08/2015	10/08/2015	46
2014	M	19/06/2014	02/07/2014	18/07/2014	28/07/2014**	05/08/2014	06/08/2014	48
2014	K	19/06/2014	02/07/2014	09/07/2014	20/07/2014	05/08/2014	06/08/2014	48
2013	G	26/06/2013	02/07/2013	04/07/2013	09/07/2013	01/08/2013	08/08/2013***	45
2013	F	25/06/2013	02/07/2013	17/07/2013	17/07/2013	01/08/2013	08/08/2013***	50
2010	A	04/07/2010	07/07/2010**	21/07/2010	16/07/2010	12/08/2010**	12/08/2010	37
2010	B	07/07/2010**	07/07/2010**	21/07/2010	22/07/2010	12/08/2010**	17/08/2010	41
Mean (date)		25 June	29 June	12 July	16 July	6 August	7 August	
Mean (Julian days) ± SD		177 ± 6	181 ± 5	194 ± 8	198 ± 7	219 ± 6	220 ± 7	43 ± 6

+Local polynomial regression lines of cumulative emergence used to estimate the earliest/latest date of emergence in cases where beetles had been found in the emergence cages at the first or last check

++Field P was the only field with heavy natural *D. v.virgifera* population, but their emergence did not deviate from average emergence patterns across experiments

*Earliest among experiments

**Latest among experiments

***Not estimated end-data, but real data, i.e. no beetles were found any more in emergence cages

3.4 Discussion

Table 3 Temporal difference of cumulative *Diabrotica v. virgifera* adult emergence between treatments and the untreated control (days ± SD) in 4 to 12 maize fields in Hungary between 2010 and 2018. Days reaching a certain percentage of cumulative emergence in a treatment subtracted from the days of the corresponding emergence in the untreated control with *p* values according paired *t* test (*p* values in brackets are fdr-corrected using the Benjamini and Hochberg method)

	Cumulative adult emergence							
	25%		50%		Peak		75%	
Treatments	Day difference to control	*p* values	Day difference to control	*p* values	Day difference to control	*p* values	Day difference to control	*p* values
Pooled sexes								
Chlorpyrifos	1.8 ± 2.2	0.21 (0.79)	0 ± 2.5	0.12 (0.79)	1.5 ± 5.1	0.09 (0.79)	−0.8 ± 4.2	0.75 (0.87)
Cypermethrin	0.1 ± 4.9	0.95 (0.98)	−1.4 ± 1.9	0.49 (0.79)	2 ± 6.8	0.14 (0.79)	−1.8 ± 3.7	0.22 (0.79)
H. bacteriophora	−0.4 ± 3.4	0.55 (0.79)	−0.5 ± 2.8	0.54 (0.79)	−0.2 ± 6.4	0.78 (0.87)	−0.6 ± 2.5	0.42 (0.79)
Tefluthrin	0.5 ± 3.5	0.7 (0.79)	−0.5 ± 4.3	0.75 (0.87)	4.6 ± 8.8	0.15 (0.79)	−1.8 ± 4.5	0.23 (0.79)
Male								
Chlorpyrifos	0.5 ± 1.3	0.5 (0.79)	−2.2 ± 2.8	0.53 (0.79)	0.1 ± 4.6	0.4 (0.79)	1.8 ± 2.8	0.31 (0.79)
Cypermethrin	0.6 ± 3	0.58 (0.79)	−1.9 ± 3.8	0.48 (0.79)	−3.2 ± 7	1 (1)	1.4 ± 5.8	0.53 (0.79)
H. bacteriophora	−0.1 ± 2.9	0.09 (0.79)	0.5 ± 3.4	0.36 (0.79)	−0.4 ± 7.2	0.36 (0.79)	0.4 ± 3.2	0.48 (0.79)
Tefluthrin	−0.8 ± 3.3	0.54 (0.79)	0.5 ± 4.1	0.24 (0.79)	0.3 ± 4.4	0.29 (0.79)	−3.8 ± 5.1	0.84 (0.91)
Female								
Chlorpyrifos	1.8 ± 3.6	0.5 (0.79)	−0.2 ± 2.6	0.5 (0.79)	−4.3 ± 13.3	0.42 (0.79)	−0.5 ± 3.3	0.78 (0.87)
Cypermethrin	0.8 ± 1.9	0.30 (0.79)	−2 ± 3.2	0.59 (0.79)	−0.6 ± 7	0.73 (0.87)	−1.3 ± 2.4	0.2 (0.79)
H. bacteriophora	0 ± 3.1	1 (1)	0 ± 2.2	0.89 (0.95)	−2 ± 4.8	0.29 (0.79)	0.7 ± 3.5	0.51 (0.79)
Tefluthrin	0.8 ± 3.8	0.6 (0.79)	−0.7 ± 4	0.42 (0.79)	0 ± 4.6	0.77 (0.87)	−2 ± 4.5	0.25 (0.79)

in steepness of the curve (logistic GLM treatment × time vs. emergence, pooled-sex shift $p = 0.033$; steepness $p = 0.006$; females $p = 0.025$; $p < 0.001$).

When adjusting the *p* values of the pairwise curve comparisons between treatment and control adult emergence for false discovery, no temporal treatment effects were found for *Heterorhabditis bacteriophora*, cypermethrin, and chlorpyrifos (see *p* values in brackets in Fig. 5). The only remaining effect was tefluthrin's increasing efficacy over time in controlling females (fdr-corrected $p = 0.002$).

Discussion

Larvae of the root-feeding maize pest *D. v. virgifera* are difficult to control due to their relatively long egg-hatching period (Toepfer and Kuhlmann 2006), and an at least 2-month long period of a population of its three larval instars feeding on and inside the roots (Levine and Oloumi-Sadeghi 1991). However, our multiple-location, multiple-year field study showed that common soil insecticides such as the pyrethroids tefluthrin and cypermethrin or the organophosphate chlorpyrifos can, with few exceptions, successfully reduce *D. v. virgifera* larvae over a relatively long period. The same was true for the applied entomopathogenic nematode. Nematodes are known to be able to propagate in *D. v. virgifera* larvae and can subsequently attack new larvae and therefore persist in field soils for several months (Pilz et al. 2014).

To better understand whether pesticides' control efficacies may decrease over time, and nematode efficacies may increase due to propagation, we investigated possible changes in the efficacies of such treatments during their control of the pest larvae in the soil.

First, we time-plotted the adult emergence from the untreated plots of field-scale experiments from different locations and years to get a general picture about the adult emergence dynamics under field conditions. The average adult emergence started between mid to end of June in southern Hungary (Figs. 2, 3, Table 1), which is comparable to previous studies from Hungary (Toepfer and Kuhlmann 2006) and Croatia (Bazok 2001). In the US Corn Belt, adult emergence may begin in late June to early July with peak emergence often occurring during July (Darnell et al. 2000; Nowatzki et al. 2002; Meinke et al. 2009). In our study in Hungary, adults emerged during around 45 days across locations and years, which is comparable to the USA. In Iowa, duration of emergence from 78 continuous maize fields over a 6-year study averaged 33 days for males and 51 days for females (Meinke et al. 2009). Our data also confirmed that male *D. v. virgifera* adults emerge earlier than the females and reach their 50% emergence around five days before the

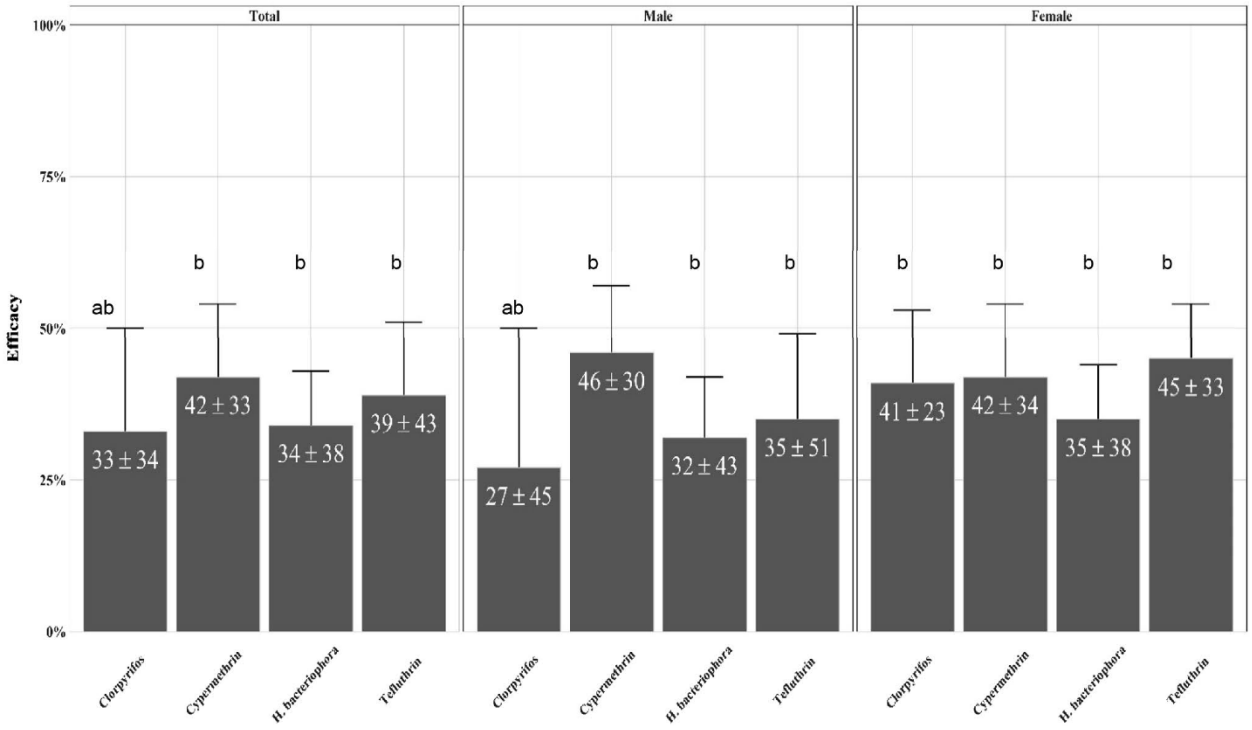

Fig. 4 Efficacy of chemical and biological treatments at reducing *Diabrotica v. virgifera* in 12 maize fields in Hungary between 2010 and 2018. 4 to 5 plots with 6 to 7 assessed plants per treatment per field. Efficacy is the adult emergence in a treatment compared to the untreated infested control; error bars = SEM; ± values on data labels = SD; letters indicate significant differences to untreated control **a** and between treatments as per Tukey Post hoc multiple comparison tests after GLM

females (Fig. 3), as it has been already observed in many other studies (Ruppel et al. 1978; Branson 1987; Nowatzki et al. 2002; Meinke et al. 2009). The apparently low adult emergence rate of less than 5% of the initial egg infestation is normal for this species due to high natural mortality on its larval stages (Toepfer et al. 2006).

On average across the diverse field and year situations of our study, all treatments, regardless of chemical or biological, comparably reduced around 33 to 46% of *D. v. virgifera* adult emergence (overall mean 38%). Those efficacies seem lower than those reported from several single field trials (Rozen and Ester 2010; Pilz et al. 2014), but correspond well to the ranges of pesticide efficacies found in larger field studies (Sutter et al. 1991; Gray et al. 1992). This is also true for larger field studies with entomopathogenic nematodes against *D. v. virgifera* (Toepfer et al. 2010a, c). We also confirmed that treatment effects against this pest are variable and may sometimes fail as it had been reported by Sutter et al. (1991); Gray et al. (1992); Furlan et al. (2006); Rauch et al. (2017); and others. For example, chlorpyrifos efficacies appeared so variable that statistical differences were difficult to detect, in fact only possible on female *D. v. virgifera* (Fig. 4). Failures of chlorpyrifos have been reported, such as by Sutter et al. (1991) from the USA, or by Furlan et al. (2006) from Italy. The confirmed high variability in the efficacy of the tested products in our study was then addressed with regard to temporal changes in efficacy.

Therefore, we compared the temporal patterns of *D. v. virgifera* from treated plots of the 12 different experiments from 7 years to the temporal patterns from the untreated control plots. Interestingly, we found that all treatments continuously reduced *D. v. virgifera* without larger changes in their efficacies over time.

Only the temporal patterns of chlorpyrifos in our study suggest a slightly decreasing control efficacy with time, reflected in a slightly better control of early than late female larvae, although differences appeared small (see curve patterns in Fig. 5). Despite that, no such temporal differences were found when comparing the accumulative 25%, 50%, 75%, and peak emergence of adults compared to the patterns in the control (Table 3). Normally, chlorpyrifos is known to persist well in soils. Sutter et al. (1989) showed that chlorpyrifos in soil samples taken 158 days after application still caused 100% mortality of third-instar *D. v. virgifera* larvae. In Hungary, soil is usually moist during young vegetative maize stages, thus in the earlier period of larval feeding. During that period, chlorpyrifos may, despite its low solubility (2 mg/l water), still be dissolved enough to permit

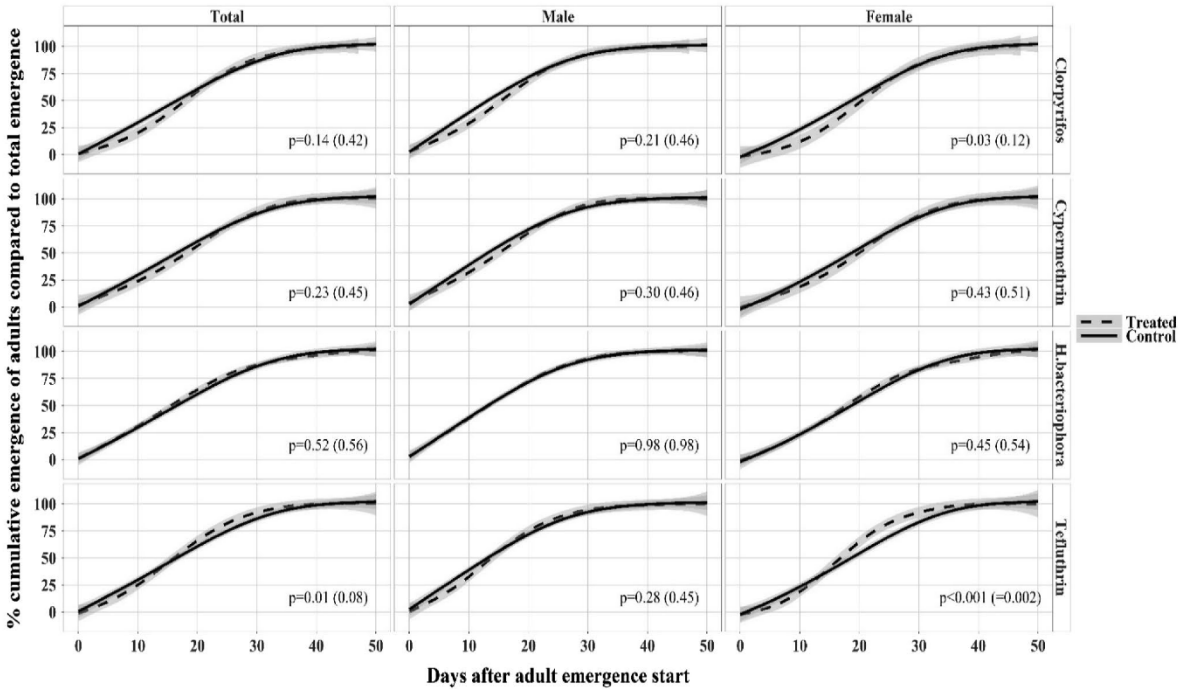

Fig. 5 Per cent cumulative emergence of *Diabrotica v. virgifera* adults over time in treatments and control, standardised as a proportion of their corresponding total cumulative emergence over a season in 4 to 12 maize fields in Hungary between 2010 and 2018. Loess smoothed trend lines plotted with 95% confidence levels; *p* values at <0.05 represent a difference between the quasi-binomial GLM fit of a certain treatment × time and the control × time (*p* values in brackets are fdr-corrected using the Benjamini and Hochberg method)

some vertical and horizontal movement of the insecticide in the root zone (Royal Society of Chemistry 1986; Sutter et al. 1989; Racke 1993). This may explain why chlorpyrifos was able to reduce early female larvae (Fig. 5). Later in the season, soil may become drier; thus, this pesticide may hydrolyse (Racke et al. 1996), and therefore, late female larvae may be comparatively less reached. Also, Sutter et al. (1991) argued that adequate soil moisture in the upper soil layers favours pesticide efficacy, and drought later in the season may be disadvantageous. Therefore, Sutter et al. (1991) reported occasionally delayed adult emergence due to chlorpyrifos, ethoprop, and carbofuran. Those patterns were suggested to be likely due to high efficacies in reducing early larvae soon after treatments, something not obviously reflected in our study results. As for other organophosphates, Boetel et al. (2003) reported occasionally delayed and flattened accumulative adult emergence slopes of *D. v. virgifera* and *D. barberi* in terbufos-treated plots. Chlorethoxyfos decreased the inflection point and slope of the adult emergence curve of male *D. v. virgifera* and delayed the maximum emergence period of females *D. v. virgifera.* (Time period elapsed before attaining the linear beetle emergence period.) Nevertheless, all those reported temporal effects of organophosphates as well as the slight effects found in our study are in their magnitude minor and may be of little relevance for the overall control efficacy of *D. v. virgifera* under field conditions.

Our study also included two common pyrethroid insecticides (cypermethrin and tefluthrin), but only tefluthrin appeared to have a slight temporal effect in pest control. It constantly reduced the male *D. v. virgifera* larvae. Then, interestingly, tefluthrin's comparative control efficacy slightly improved with time, reflected in a comparatively better control of late than early female larvae. This was also reflected in a time shift and change in steepness of the female as well as pooled-sex emergence curves (Fig. 5). As for the above-mentioned chlorpyrifos, tefluthrin's temporal effects seem so small that they are not reflected in changes in the 25, 50, 75%, and peak cumulative adult emergence compared to the controls. Nevertheless, tefluthrin seems to be present and effective over a relatively long period in the soil under field conditions. Chapman et al. (1993) and Whiting et al. (2014) argued that the applied concentrations should remain, despite ongoing degradation, high enough for killing the pest larvae over time. Reasons for comparatively increasing control effects against female larvae with time remain hypothetical. But they may be due to exposure of female larvae to low dosage tefluthrin, and therefore sublethal and subsequently

delayed lethal effects, as it had been reported for *D. barberi* (Michaelides et al. 1997). Branson (1987) showed a prolongated pre-hatch and longer post-hatch development time for females, resulting in an about 5 days later female than male adult emergence, the latter also been shown in our study for *D. v. virgifera,* and by Boetel et al. 2003 for female *Diabrotica barberi*.

Interestingly, no such time patterns were found for the other tested pyrethroid: cypermethrin. Also, cypermethrin appeared variable in controlling *D. v. virgifera* and failed in few experiments, as did tefluthrin. To our knowledge, there are no field studies addressing failures of cypermethrin in controlling *D. v. virgifera* larvae, and any reasoning would be highly speculative.

Heterorhabditis bacteriophora, as expected, successfully reduced larvae over time, regardless of sex. It is known that applied nematodes in maize fields persist due to propagation for several months (Kurtz et al. 2007; Pilz et al. 2014), although not as long as in crops with better vegetation cover (Pilz et al. 2014). However, our hypotheses that the propagation of the nematodes in the larvae as reported by Kurtz et al. (2009) would lead to a largely increasing comparative efficacy of this agent over time were not reflected in the temporal data of *D. v. virgifera* emergence under field conditions. It is known that nematodes can effectively kill first-instar larvae, but may have difficulties to reproduce in them due to the small size of those larvae (Kurtz et al. 2009). Propagation is better in the larger-, second-, and third-instar larvae potentially leading to an increasing control of pest populations in the soil as time passes. Indeed, in our study, nematodes were able to continuously reduce the pest larvae over time, even at their suspected larval population peak in the soil, indicating successful propagation. However, this propagation of nematodes seemed not to have been high enough to have a detectable additional positive effect in controlling larvae later in the season. Reason behind the lack of such a pattern remains hypothetical. They may be found in less successful propagation under field conditions than in the laboratory (Pilz et al. 2014), because e.g. saprophytes are decomposing the killed, although somewhat symbiotic-bacteria protected, larvae before nematodes can propagate, or that the offspring of the commercial mass-produced nematodes undergoes trait changes that slightly reduce their host inding and/or virulence under field conditions (Bilgrami et al. 2006). Nonetheless, it is encouraging that nematodes can well reduce the early *D. v. virgifera* larvae and continue to do so for the late larvae under field conditions.

In conclusion, our results suggest that commonly used pesticides as well as nematode-based novel biocontrol products can, in general, control *D. v. virgifera* larvae over their relatively long presence in the soil. The exception that chlorpyrifos slightly better reaches early female than late female larvae, and that the comparative relative efficacies of tefluthrin increased with time, is in their absolute differences hardly detectable. Therefore, the often observed inconsistent and fluctuating levels of efficacies of treatments may be due to other reasons, such as locally varying abiotic and environmental factors. This merits further investigations leading to larger data sets from diverse field situations.

Author contributions

ST and JK developed the projects. ST designed the trials. ST, SZT, and collaborators implemented the studies. SZT and MSZ conducted the analysis. SZT wrote the manuscript with support from all authors.

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Compliance with ethical standards

Conflict of interest All authors declared that they have no conflict of interest. There is no financial or other dependency between authors and any of the companies considered.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants or vertebrates performed by any of the authors.

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4. Chapter II.

Limited influence of abiotic and biotic factors on the efficacies of soil insecticides and entomopathogenic nematodes against *Diabrotica v. virgifera* adult emergence and root damage prevention under field conditions

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Limited influence of abiotic and biotic factors on the efficacy of soil insecticides and entomopathogenic nematodes when managing the maize pest *Diabrotica v. virgifera* (Coleoptera: Chrysomelidae)

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Abstract: *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) is a serious pest that infects maize. Insecticides or entomopathogenic nematodes are used to control the root-damaging larvae. However, such treatments are reportedly inconsistent in terms of efficacy under farming conditions. To better understand the reasons behind these inconsistencies, we studied the control efficacy of seed coatings, such as clothianidin; granular soil insecticides, such as cypermethrin and tefluthrin; and fluid-applied entomopathogenic nematodes, such as *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae). We assessed the influence of 12 biotic and 20 abiotic factors on the reduction of *Diabrotica v. virgifera* populations and on the prevention of root damage in 20 field-scale experiments in Hungary between 2010 and 2020. Results confirmed that all treatment types are able to control pest populations and prevent root damage, but with high variability. Our analyses showed that most investigated factors, for example, air temperature, most soil parameters, and pest infestation levels, did not influence the efficacy of the treatments. The efficacy of clothianidin in preventing root damage decreased slightly with increasing soil bulk density but improved with late maize sowing, and therefore late treatment, as well as with increasing soil moisture in July. The efficacy of cypermethrin in preventing damage improved slightly with increasing clay content in the soil. Tefluthrin was slightly less effective in reducing *D. v. virgifera* with increasing soil moisture in June. However, all these factorial influences were minor in their absolute effects. Surprisingly, none of the investigated factors seemed to influence the efficacy of *H. bacteriophora*. In conclusion, the efficacy of chemical and biological treatments against this soil pest remains difficult to predict under farming conditions.

Keywords: western corn rootworm; integrated pest management; environmental factors

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1. Introduction

Rice, wheat, and maize are the top three food providers for humans, accounting for an estimated 42% of the food calories consumed across the world [1]. In the latest FAO survey, the sowing area of maize worldwide is estimated to be 197 million ha, producing 1.14 million tons of maize per year [1]. In the developed economies, maize yield is mainly used for feeding livestock (~75%). In sub-Saharan Africa, Latin America, and some of the Asian countries, about 20% of the harvest is consumed by humans directly [2] [3]. However, numerous threats endanger the optimal level of maize cultivation all around

the globe, including climate change [4], for example, in the form of drought or heat stress [5], and new invasive species [6]. One such problematic invasive maize pest is *Diabrotica v. virgifera* LeConte (Coleoptera: Chrysomelidae) or western corn rootworm. It has spread through large parts of the north American continent and successfully invaded maize growing regions of Europe since the late 1980s [7].

Diabrotica virgifera ssp. *virgifera* probably originated from Central America, likely around Mexico. From there, it had invaded North America by the early 19th century [8] [9] [10]. *D. v. virgifera* was accidentally introduced from North America into Europe on at least five separate occasions [11]. The first of these successful introductions was probably in Serbia in the 1980s, but it took until 1992 when the damage it caused (and, thus, the species) was detected [12]. Over the last three decades, *D. v. virgifera* has invaded most maize growing areas of Central Europe, parts of Eastern Europe, parts of the Balkans, as well as Italy [7]. Altogether, it has invaded 32 European countries until now [13].

The pest is an univoltine species with eggs overwintering in the soil [8]. After maize germination, eggs hatch and larvae appear on the maize roots [12]. After pupation, adult beetles emerge from the soil during late vegetative and flowering stages of maize [14]. The larvae exclusively feed on maize roots, lowering nutrient and water intake of the plants and causing plant lodging [14] [15]. Adult beetles can also cause some damage in maize by feeding on pollen and silk, hindering fertilization [16]. In areas with high pest densities, yield loss can be significant [17] and become particularly serious when combined with adverse weather conditions [18] [19] [20]. To manage this pest and to prevent damage and yield losses, several control options are available to farmers.

In short, the most important control methods are the following: (a) crop rotation, (b) chemical insecticides such as granular formulations, fluids, or seed treatments against the larvae, (c) foliar insecticides against the adults, (d) transgenic hybrids against the larvae, and (e) biological control agents against the larvae. Historically, four groups of insecticides have been used widely and abundantly against the rootworms: pyrethroids (e.g., tefluthrin, bifenthrin, cypermethrin, and deltamethrin), organophosphates (e.g., terbufos, chlorpyriphos, and methyl parathion), carbamates (e.g., carbofuran, carbaryl), and neonicotinoids (e.g., clothianidin, thiamethoxam, and imidacloprid). All these can be formulated as granules or liquids and applied into the furrow, usually at sowing time; and some of these are also formulated as seed coating [21] [22] [23]. However, there is a lot of variability in the efficacy of the soil insecticides or seed coatings, probably due to technical failures such as incorrect calibration of machines or other human errors, On the other hand, two other important causes can be considered, one is the probability of insecticide resistance, or the influence of abiotic and biotic factors of the environment [24].

The first confirmed instance of insecticide resistance shown by *D. v. virgifera* was against two organochlorine substances, aldrin and heptachlor, by field-collected beetles (in 1960-1961) in Nebraska, where the LD_{50} was about 80-fold greater for heptachlor and 50-fold greater for aldrin compared to a susceptible population [25]. Later, in the 1990s, bioassays with field-collected populations confirmed carbamate (e.g., carbaryl) and organophosphate (e.g., methyl parathion) resistance [26]. Moreover, pyrethroids are not untouched by this phenomenon, as recently reported [27].

In addition to insecticide resistance, biotic factors such as the degradation of an insecticide by microbials can hinder effective rootworm control. For example, Felsot et al. [28] [29] have investigated why repeated carbofuran treatments started to fail to control rootworms after some years. Different *Pseudomonas* and *Actinomycetes* bacteria started to use the chemical themselves, and the insecticide rapidly disappeared from the soil. Similar observations were made for the organophosphate isophenos [30]. Different abiotic factors also explain the insufficient performance of some soil insecticides [31], such as the type, texture, and organic content of the soil; soil moisture; and soil temperature. For example, organochlorines and organophosphates are believed to be more effective in soils with a high organic matter content [32]. Moreover, increasing soil moisture may elevate their toxicity [31]. With some exceptions, it seems that increasing soil tempera-

tures increases the toxicity of soil insecticides [33] [34] [35]. Cultural control practices, for example, late sowing of maize or late treatments closer to the hatching of the larvae, may increase insecticide efficacy [36] [37].

Despite some insecticide resistance [20] [38], bans on active ingredients and sometimes entire chemical groups [39] under new agri-policies have reduced the diversity of modes of actions of chemical pesticides [40]. Nevertheless, soil insecticides still seem an essential element in the toolkit of farmers for soil insect control. For example, in Hungary, an EU member state, 5000 tons of the granular soil insecticide tefluthrin was sold in 2019, which was 46% of all insecticides sold [41]. However, alternatives are needed due to environmental concerns and because more ingredients will likely be taken off the market sooner or later.

One such alternative option is the use of entomopathogenic nematodes, tiny soil-living worms living together with symbiont bacteria in their gut, jointly killing insects and using their resources for reproducing inside them [42]. One of its representatives, the *Heterorhabditis bacteriophora* has been proven effective against *D. v. virgifera* larvae and commercial products have been developed [43] [44] [45] [46]. *H. bacteriophora* can persist in the soil of maize fields for up to 6 weeks after treatment [47]. However, its efficacy against larvae can, as for insecticides, be variable under field conditions [48]. Similar to soil insecticides, several studies aimed at understanding which environmental factors may influence the performance of entomopathogenic nematodes [49]. For example, infectivity and persistence of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* modestly increased with increasing loam and organic matter content of soils compared with sandy soils [49] [50]. Less soil moisture of soils is occasionally reported to negatively affect nematode infectivity, survival, and pathogenicity [51] [52], but other studies could not find such effects [53]. However, soil characteristics and moisture are likely not the only factors influencing nematode efficacy.

Our work was aimed at better understanding the effects of abiotic and biotic factors on the efficacy of control agents under farming conditions. We built our analyses on a larger set of field trials from Hungary, i.e., on 20 field experiments on farmer fields over 10 years. We attempted to detect relationships between various abiotic and biotic factors and the efficacy of insecticides as well as entomopathogenic nematodes in managing the larval populations of and preventing root damage by *D. v. virgifera*. We hypothesized that some of the abiotic and/or biotic factors may negatively or positively affect treatment efficacy and thus may explain their variability under field conditions. The findings are expected to contribute to a better understanding of why certain treatments may sometimes fail or may not be satisfactory. This, in turn, could lead to new developments and actions for improving plant protection products or strategies against soil insect pests, such as the invasive alien *D. v. virgifera*.

2. Materials and Methods

2.1. Study fields

Experiments were carried out on 20 grain maize fields in Hungary between 2010 and 2020 (Supplementary data, Table S1). Experimental fields were ploughed at the end of each season and then tilled and harrowed before maize was sowed. Maize was sowed between mid-April and early May (Table S1). Individual maize seeds were sowed every 16 to 18 cm in rows 75 cm apart, using a 4-row or a 6-row planter, leading to 72000 - 87000 plants per ha. Except for the seeds treated with insecticide clothianidin, the rest had been coated with standard fungicides only.

2.2. Target pest

4.2 Materials and Methods

The subject of the study was the western corn rootworm, *Diabrotica virgifera* ssp. *virgifera* LeConte (Coleoptera: Chrysomelidae). The majority of the study fields had no natural pest population because the pre-crop had not been maize (Table S1). Only in a few study fields (Fields T, S, P, O, and H) had maize been sown for the second year in a row, and in one (Field L), maize had been sown for the third year in a row. Maize plants were artificially infested with eggs of *D. v. virgifera* to simulate homogenously distributed pest populations. Eggs were obtained from laboratory-reared beetles that had been collected from fields in southern Hungary the previous year. For rearing and overwintering procedures, see [54] [55]. In April, the eggs were transferred to a 24-degree-Celsius environment to stop diapause. Parallel with the artificial infestation in the field, some eggs were transferred onto moistened filter paper to check their overwintering survival and hatching success. The average hatching ratio was always between 50 and 80%, with a relatively high variability, and hatching patterns were comparable to reports from the literature [55].

In the laboratory, *D. v. virgifera* larvae started to hatch around 1 week after the egg application date (more than 2 weeks after diapause stopped), and hatching lasted until late May. In the field, larvae likely emerged slightly later [56].

Two series of six or seven maize plants in each plot were infested with ready-to-hatch eggs at the 1st to 4th leaf stage (Table S1 presents the dates and egg densities). The eggs in 1 to 2 ml aqueous agar were applied using a 5 ml pipette into two to four holes at both sides of a maize plant [54].

2.3. Experimental design and treatments

Experiments were implemented following the EPPO standards PP 1/212 and PP 1/152 [57] [58]. Four or five plots of four to six rows of maize were systematically arranged per treatment and control (Table S1).

Soil insecticides, insecticide-coated seeds, and entomopathogenic nematodes were applied into the sowing row. The entire plots were treated. Treatment and maize sowing were carried out about 1 to 3 weeks prior to the expected hatching of eggs. About a week later, two to three series of six to seven maize plants were randomly chosen from among the middle rows of each plot to check for infestation with *D. v. virgifera* eggs as described above and, therefore, also to assess the data from June to August (described below).

2.3.1. *Heterorhabditis bacteriophora* fluid

About 0.1 to 0.15 million infective juveniles (ij) of the entomopathogenic nematode *H. bacteriophora* (Rhabditida: Heterorhabditidae) were applied per row meter (hybrid of European and US strains, Dianem™, e-nema GmbH, Germany), which equals 1.5 to 2 billion ij per hectare. The formulation was a water-soluble inert powder (SP GIFAP code). Prior to application, the quality of nematodes had been checked under a stereomicroscope to ensure a survival rate of at least 70%. The formulated nematodes were then diluted in water and injected into the seeding row behind seed placement (200 up to 550 liters of water per hectare). Different applicators were used as described in [55].

2.3.2. Clothianidin seed coating

We used maize seeds coated with clothianidin, i.e., the nicotinoid of the active substance (E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidin (Poncho™ FS 600, Bayer Crop Science Hungaria KFT, Budapest, Hungary; formulation type: Solution for seed treatment LS according to GIFAP code). About 0.006 ml of clothianidin was coated on a seed.

2.3.3. Cypermethrin microgranules

About 12 kg of the soil insecticide cypermethrin, equaling 0.9 per row meter, was applied per hectare with seeder-mounted micro-granule applicators (Belem™ 0.8 MG,

0.8% a.i., Spiess-Urania, Germany). Cypermethrin is a pyrethroid of the chemical compound [Cyano-(3-phenoxyphenyl) methyl] 3-(2,2-dichloro-ethenyl)-2,2-dimethyl-cyclopropane-1-carboxylate (IRAC 3A).

2.3.4. Tefluthrin fine granules

About 13.3 kg of the soil insecticide tefluthrin, equaling 1 g per row meter, was applied per hectare (Force™ 1.5 G, Syngenta, Hungary). Tefluthrin is a pyrethroid of the chemical compound 2,3,5,6-tetrafluoro-4-methylbenzyl(Z)-(1RS,3RS)-3-(2-chloro-3,3,3-trifluoro-1-propenyl-2,2-dimethyl-cyclopropanecarboxylate.

2.3.5. Untreated control

Infested but untreated plots served as negative control.

2.4. Assessment of abiotic and biotic factors

The characteristics of the assessed abiotic and biotic factors are presented in Table S1 and Table 1.

The quality of entomopathogenic nematodes and their application was assessed by counting surviving nematodes before and after the application of the following [44]. Virulence of the nematodes was assessed with subsamples from the delivered product as well as with subsamples post-application using quality control bioassays with *Tenebrio molitor* larvae (Coleoptera: Tenebrionidae) [44].

From each experimental site, from an area of 5 to 30 cm, 1 L of five to six mixed soil samples was taken. These samples were sent to the Soil Conservation Service, Szolnok, Hungary, to analyze for clay, loam, sand, $CaCO_3$, and humus content, as well as the pH of the soils. Soil moisture and soil bulk density were measured according to [57] on a monthly basis from April to August. Temperature and rainfall data were recorded hourly through a weather station (Davis Instruments).

2.5. Assessment of pest populations and root damage

Four to five series of six to seven infested maize plants were assessed per treatment and control per field. Plants were covered with gauze cages (0.125 × 0.4 × 1.5 m high). They were placed prior to the expected start of adult emergence, which was usually in mid-June [44]. The adult beetles of *D. v. virgifera* that emerged were counted on a weekly basis and removed from cages at each assessment [48]. Weekly emergence data of adults were standardized to 100 eggs per plant. The efficacy of each treatment was calculated as the reduced emergence of beetles relative to the emergence of beetles from untreated control (corrected efficacy % = 100 × (beetles in control plots − beetles in treated plots) / maximum (beetles in the control or treated plots).

To assess the root damage, the roots of 24 to 30 maize plants per treatment were dug out from each field every year from early-July to the beginning of August. Firstly, the soil was removed by shaking the roots. We were careful not to break off any of the primary roots. Secondly, the remaining soil was removed from the root system using a high-pressure water sprayer after soaking the roots for a few hours. Damage was rated using two scales: (i) the 1.0 to 6.0 Iowa scale for the general root damage [58], which is the most commonly used scale despite the fact that it may overestimate the importance of minor damage (such as feeding scars) and (ii) the 0.00–3.00 node-injury scale for heavy root damage [59], which is a linear and decimal scale that measures only totally destroyed roots or nodes. The corrected efficacy of root damage was calculated as 100 × (damage in control plots – damage in treated plots) / maximum (damage in control or treated plots).

2.6. Data analysis

We used ordinary least squares linear models to detect the overall effect of the treatments on adult emergence and root damage. This was followed by ANOVA tests with a post-hoc Tukey test to detect the differences between the treatments.

Due to the high multicollinearity between the factors, which otherwise would not be allowed in regression modeling, we preselected the most relevant factors by calculating Pearson's correlation coefficients (r coefficient) between the different factors and between the efficacies of treatments in controlling adult emergence as well as in preventing general and heavy root damage. Factors appearing to have r coefficients of at least -0.4 =< r coefficient >= +0.4 with a p-value < 0.05 were chosen for developing regression models. This means that factors with small r coefficients were dropped from further analysis.

Simple or multiple linear regressions were performed for correlated factors to identify relationships between factors and control efficacies (Table 1). To avoid any misinterpretation of the models, non-linearity of the response–predictor pairs, correlation of the error terms, non-constant variance of error terms, outliers, high-leverage points, and multicollinearity were investigated before model execution [60].

R v.4.0.2 (R Core Team, 2020) was used for data visualization and statistical analysis.

Table 1. Characteristics of abiotic and biotic factors analyzed for their influence on the efficacy of soil insecticides and entomopathogenic nematodes in reducing *Diabrotica v. virgifera* larvae and protecting the roots against damage in maize (at 20 sites over 10 years in Hungary).

Factor	Unit	Mean	Standard deviation	Minimum	Maximum	Range	Shapiro–Wilk normality test		Sample size	Levels of a factor
							W	p	n	Unique values
Biotic factors										
Eggs per plant		406	221	200	1100	900	0.7	<0.001	482	6
Billion nematodes per ha injected		1.7	0.2	1.5	1.9	0.4	0.7	<0.001	282	3
Water per ha injected with nematodes	Liters	328.7	158.4	133	558	425	0.6	<0.001	499	8
Nematode mortality before application	%	15	8	2.9	25	22.1	0.9	<0.001	107	9
Nematode mortality after application	%	23	15	4	57	53	0.8	<0.001	91	14
Nematode virulence before application (1-week bioassay)	%	41	27	8	90	82	0.9	<0.001	136	14
Nematode virulence after application (1-week bioassay)	%	38	18	10	76	66	0.9	<0.001	105	9
Maize sowing date	Julian days	112	3	108	122	14	0.8	<0.001	530	9
Egg infection date	Julian days	125	3	122	134	12	0.8	<0.001	451	8

Maize density	Plants per ha	78000	53760	72000	87000	15000	0.8	<0.001	530	5
Elevation	m	90	15	80	150	70	0.6	<0.001	530	11
Natural mortality of adult *D. v. virgifera*	%	98.6	1.1	96.2	99.8	3.6	0.8	<0.001	83	25
Abiotic factors										
Clay content	% m/m	34	7	22	54	32	0.9	<0.001	509	16
Loam content	% m/m	29	6	9	39	30	0.8	<0.001	509	16
Sand content	% m/m	37	8	24	51	27	0.8	<0.001	509	16
Soil bulk density	g/cm³	1	0.1	0.9	1.34	0.45	0.9	<0.001	522	11
CaCO3	% m/m	5	3	1	12	11	0.9	<0.001	509	16
Soil pH		7.9	0.2	7.4	8.2	0.8	0.8	<0.001	509	13
Humus content	% m/m	2.7	0.7	1.63	3.9	2.27	0.9	<0.001	509	17
Soil moisture in April	w% = grav. %	16	3	11.1	21	9.9	0.9	<0.001	476	17
Soil moisture in May	w% = grav. %	21.3	7	9.9	32	22.1	0.9	<0.001	522	19
Soil moisture in June	w% = grav. %	16.2	6.8	8	29.5	21.5	0.8	<0.001	420	16
Soil moisture in July	w% = grav. %	12.1	4.1	7	22.9	15.9	0.9	<0.001	522	18
Air temperature in April	°C	13.3	1.5	11	16.8	5.8	0.9	<0.001	522	9
Air temperature in May	°C	16.8	1.7	13.8	20.2	6.4	0.9	<0.001	522	9
Air temperature in June	°C	21.3	0.7	20	23	3	0.9	<0.001	522	9
Air temperature in July	°C	23.3	1.1	21.6	25	3.4	0.9	<0.001	490	10
Cumulative rainfall in April	mm	17.6	17.6	1.4	56	54.6	0.8	<0.001	522	11
Cumulative rainfall in May	mm	66	31.4	20.3	134	113.7	0.9	<0.001	522	11
Cumulative rainfall in June	mm	36.4	30	3.3	93	89.7	0.8	<0.001	522	9
Cumulative rainfall in July	mm	45.5	32.8	14	127	113	0.8	<0.001	490	10
Rain around sowing and treatment (±1 day)	mm	1	0.9	0	1.9	1.9	0.7	<0.001	393	6

3. Results

3.1. Treatment efficacies

All four studied treatment types applied at the sowing of maize, i.e., clothianidin seed coating, cypermethrin granular soil insecticide, tefluthrin granular soil insecticide, and the biocontrol agent *H. bacteriophora*, were able to control *D. v. virgifera* larvae (reduced adult emergence: LM, $R^2 = 0.3$, $p < 0.001$). Achieved control efficacies were comparable among the treatments (ANOVA, $R^2 = 0.08$, $p = 0.11$).

In detail, across fields and years, clothianidin reduced the pest population by 69% ± 8% (SD) on average (median 71%, LM, $p_{adj} < 0.001$, 95% CI [41, 98]; Figure 1), cypermethrin reduced the pest population by 31% ± 34% (median 30%, LM, $p_{adj} < 0.001$, 95% CI [12, 50]), tefluthrin reduced the pest population by 44% ± 41% (median 63%, LM, $p_{adj} < 0.001$, 95% CI [21, 47]), and the entomopathogenic *H. bacteriophora* reduced the pest population by 34% ± 34% (median 46%, LM, $p_{adj} < 0.001$, 95% CI [27, 60]). Achieved efficacies of treatments in pest reduction were highly variable, as reflected in the large SDs (numbers after ± above the boxplots) and the wide spread of the data points in Figure 1. Most treatment types occasionally failed. Although clothianidin reached control efficacies in all experiments and years, cypermethrin was successful in only 70% of the experiments, tefluthrin in 79% of the experiments, and *H. bacteriophora* in 80% of the experiments (data points at or below 0 in Figure 1).

All four treatment types, i.e., the clothianidin seed coating, cypermethrin and the tefluthrin granular soil insecticide as well as the *H. bacteriophora* were able to prevent general root damage from *D. v. virgifera* larvae (1.0 to 6.0 modified Iowa scale: LM, $R^2 = 0.3$, $p < 0.001$). Control efficacies were different between the treatments (ANOVA, $R^2 = 0.4$, $p < 0.001$), the efficacy of clothianidin and tefluthrin were more than 4x higher compared to cypermethrin and *H. bacteriophora*.

Clothianidin prevented 19% ± 13% (median 20%, LM, $p_{adj} < 0.05$, 95% CI [14, 23]) of general root damage. The cypermethrin prevented 4% ± 16% (median 5%, LM, $p_{adj} < 0.05$, 95% CI [0.1, 8]) of the root damage. Tefluthrin prevented 20% ± 16% (median 20%, LM, $p_{adj} < 0.05$, 95% CI [17.1, 23]) of the root damage. The entomopathogenic *H. bacteriophora* prevented 3% ± 16% (median 3%, LM, $p_{adj} < 0.05$, 95% CI [0.07, 5]) general root damage. Achieved efficacies of treatments in prevention of the general root damage were highly variable, as reflected in the large SDs (numbers after ± above the boxplots) and the wide spread of the data points in Figure 1. All treatment types occasionally failed. In other words, clothianidin prevented general root damage in 94% of the experiments and years, whereas cypermethrin was successful in 65% of the experiments, tefluthrin in 81% of the experiments, and *H. bacteriophora* in 59% of the experiments.

All treatments were also able to prevent heavy root damage by *D. v. virgifera* larvae (1.00 to 3.00 node-injury scale: LM, $R^2 = 0.3$, $p < 0.001$), but to different levels (ANOVA, $R^2 = 0.62$, $p < 0.001$). The efficacy of clothianidin and tefluthrin was about double that of cypermethrin and *H. bacteriophora*.

Here, clothianidin prevented 83% ± 17% (median 84%, LM, $p_{adj} < 0.05$, 95% CI [67, 98]) of heavy root damage. Cypermethrin prevented 50% ± 65% (median 100%, LM, $p_{adj} < 0.05$, 95% CI [37, 63]) of the root damage. Tefluthrin prevented 83% ± 31% (median 100%, LM, $p_{adj} < 0.05$, 95% CI [73, 92]) of the root damage. *H. bacteriophora* prevented 47% ± 62% (median 72%, LM, $p_{adj} < 0.05$, 95% CI [38, 92]). Although clothianidin was able to prevent heavy root damage across all experiments and years, cypermethrin was successful only in 75% of the experiments, tefluthrin in 95% of the experiments, and *H. bacteriophora* in 79% of the experiments.

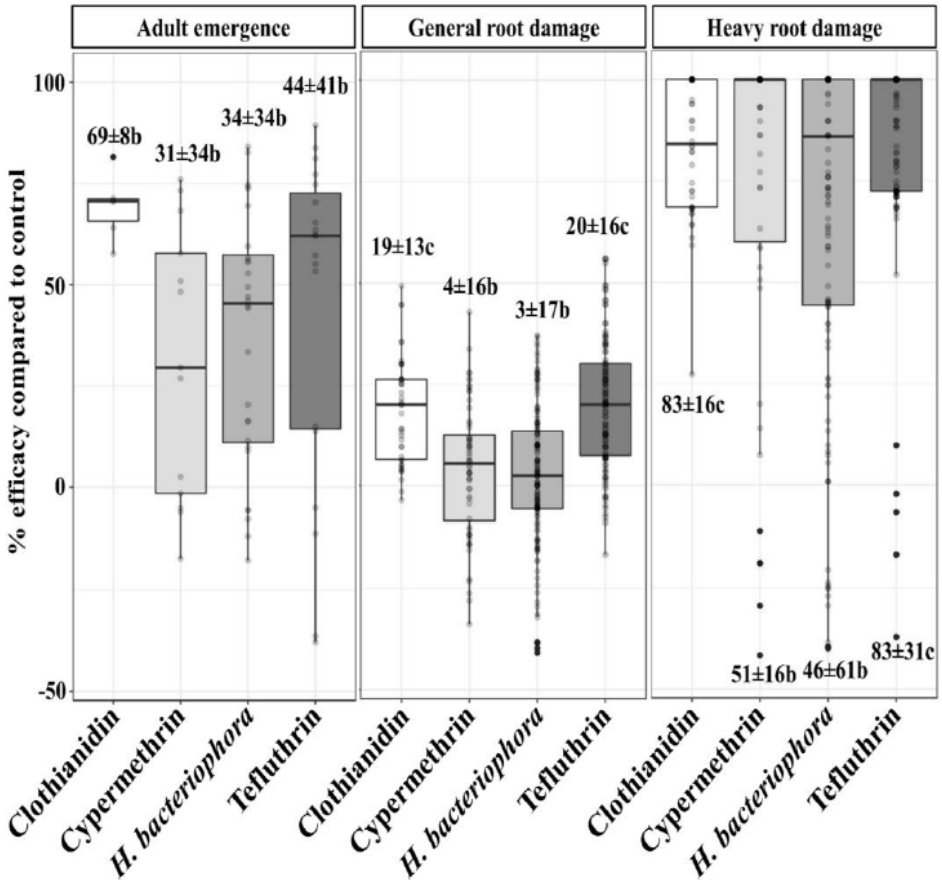

Figure 1. Efficacy of different treatments in controlling *Diabrotica v. virgifera* larvae and in preventing root damage in maize fields compared to the untreated, infested control. General root damage assessed via the 1.0 to 6.0 modified Iowa scale, and heavy root damage assessed via 0.00 to 3.00 Oleson's node-injury scale. Pest population assessed by counting adults emerging from the soil in gauze cages. The spread of the data points represents the mean number of adults per field and the means of the general and heavy root damage per plot. The median is presented as a full line inside box plots; the mean ± the SD is presented as numbers above the boxes. Different letters on box plots indicate the significant difference as per post-hoc Tukey HSD test at $p < 0.05$ following ANOVA; untreated, infested control significance letter is a, n = 20 sites, and the study was conducted across 10 years in southern Hungary.

3.2. Abiotic and biotic factors influencing treatment efficacies

The efficacy of *Heterorhabditis bacteriophora* in controlling *D. v. virgifera* and preventing root damage was hardly influenced by any of the 12 assessed biotic and 20 assessed abiotic factors. In fact, none of the factors had a notable impact on efficacies (see the narrow distributions of Pearson's r values around 0 in Figure 2 and the lack of influencing factors in models, presented in Table 2).

The efficacy of clothianidin in controlling *D. v. virgifera* and preventing root damage was hardly influenced by any of the 6 assessed biotic and 20 assessed abiotic factors.

Only two soil components appeared negatively correlated with the efficacy of clothianidin seed coatings in suppressing the pest population: $CaCO_3$ soil content (Pearson's r coefficient = -0.82, p = 0.04) and humus content (Pearson's r coefficient = -0.85, p = 0.02). Regression model for both factors explained ~60% of the variance of efficacies, which was not enough to allow a reliable prediction (LM: R^2 adj = 0.63, df = 3, p = 0.1). This was also

true when separately analyzing $CaCO_3$ (β = -0.8, 95% CI [-4.5, 2.8], p = 0.5) and humus content (β = -8.8, 95% CI [-33.2, 15.5], p = 0.3) (Table 2).

Only one soil component appeared to be negatively correlated with the efficacy of clothianidin in preventing general root damage as assessed by the 1.0 to 6.0 Iowa scale, namely the soil bulk density (Pearson's r = -0.51, p < 0.001). The fitted regression model was able to predict a slight decrease in efficacy general root damage prevention with increasing soil bulk density and explained ~30 of the variance (R^2_{adj} = 0.28, df = 40, p < 0.001).

Prevention of heavy root damage (assessed through the 1.00 to 3.00 node-injury scale) increased with late sowing and late treatment dates (Pearson's r = 0.71, p < 0.001; LM: β = 1.9, 95% CI [1.1, 2.8], p < 0.001) as well as with increasing soil moisture in July (Pearson's r = 0.57, p < 0.001; LM: β = 1.2, 95% CI [0.2, 2.2], p = 0.02). The fitted linear model with these two factors explained about half of the variance (R^2_{adj} = 0.55, df = 39, p < 0.001).

Cypermethrin's efficacy in controlling *D. v. virgifera* was not influenced by any of the 6 assessed biotic and the 20 assessed abiotic factors. As for its efficacy in preventing root damage, we identified a few factors that influenced it.

Four factors correlated to the efficacy of cypermethrin in preventing general root damage according to the 1.0 to 6.0 Iowa scale. Two were correlated to it positively and moderately: the clay content of the soil (Pearson's r = 0.51, p < 0.001) and the air temperature in July (Pearson's r = 0.54, p < 0.001). The other two factors were negatively and moderately correlated with it, namely the pH of the soil (Pearson's r = -0.51, p < 0.001) and the eggs applied per plant number (Pearson's r = -0.43, p < 0.001). The regression model with all factors explained less than half of the variance in efficacies, but its predictive values were reliable (R^2_{adj} = 0.39, df =35, p < 0.001). However, within the model, separate factor effects were non-significant.

The prevention of heavy root damage (assessed with the 1.00 to 3.00 node-injury scale) increased with increasing clay content of the soil (Pearson's r = 0.41, p < 0.001; LM: β = 4, 95% CI [0.6, 7.5], p = 0.02). The fitted linear model that contains this factor had explained about 1/3 of the variance in the efficacies (R^2_{adj} = 0.36, df = 44, p < 0.001).

The efficacy of tefluthrin in controlling *D. v. virgifera* and preventing root damage was hardly influenced by any of the 6 assessed biotic and 20 assessed abiotic factors.

Two factors were moderately and positively correlated with the efficacy of the tefluthrin granules in suppressing the pest population, the air temperature in June (Pearson's r = 0.46, p = 0.04) and the cumulative rainfall in July (Pearson's r = 0.52, p = 0.02), but one factor was highly and negatively correlated with it, namely the soil moisture in June (Pearson's r = -0.82, p < 0.001). The regression model with these factors has a high predictive value, explaining almost 80% of the variance in tefluthrin's efficacies (R^2_{adj} = 0.77, df = 9, p < 0.001). Tefluthrin's efficacy slightly decreased with increasing soil moisture in June (β = -2.3, 95% CI [-4.6, -0.04], p = 0.047) but increased with higher rainfall in July (β= 0.4, 95% CI [0.1, 0.8], p = 0.02).

We were not able to detect any factors that are correlated with tefluthrin's efficacy in preventing general root damage.

Prevention of heavy root damage slightly decreased with increased sand content of the soil (Pearson's r = -0.4, p < 0.001; LM: β = -1.4, 95% CI [-2, -0.8], p < 0.001). The regression model explained only a small fraction, of around 1/10, of the variance (R^2_{adj} = 0.15, df =109, p < 0.001).

Table 2. Correlations and regressions between abiotic or biotic factors and the efficacy of soil insecticides and entomopathogenic nematodes in reducing *Diabrotcia v. virgifera* larvae and protecting against root damage in maize; only factors that had an influence are presented; n = 20 sites or fields in southern Hungary.

		Pearson correlation		Ordinary least squares regression model						
Treatment	**Factors**	**r**	**p**	**Model no.**	**Adjusted R^2**	**Df**	**p**	**β coefficient**	**p**	**95% CI**
Pest infestation (Adults that emerged per plant)										
Clothianidin	$CaCO_3$ soil content	-0.82	0.04	1	0.63	3	0.1	-0.8	0.5	-4.5, 2.8
	Humus content	-0.85	0.02					-8.8	0.3	-33.2, 15.5
Tefluthrin	Soil moisture in June	-0.82	<0.001	2	0.77	9	<0.001	-2.3	0.047	-4.6, 0.04
	Air temperature in June	0.46	0.04					15.2	0.13	-5.2, 35.7
	Rainfall in July	0.52	0.02					0.4	0.02	0.1, 0.8
Cypermethrin	No factor found	NA	NA	NA	NA	NA	NA	NA	NA	NA
H. bacteriophora	No factor found	NA	NA	NA	NA	NA	NA	NA	NA	NA
General root damage (1.0 to 6.0 modified Iowa root damage scale)										
Clothianidin	Soil bulk density	-0.51	<0.001	3	0.28	40	<0.001	-47.2	<0.001	-70.2, -24.3
Tefluthrin	No factor found	NA	NA	NA	NA	NA	NA	NA	NA	NA

Cypermethrin	Clay content	0.51	<0.001	4	0.39	35	<0.001	1.2	0.2	-0.8, 3.3
	Soil pH	-0.51	<0.001					-15.6	0.2	-39, 8
	Air temperature in July	0.54	<0.001					4.7	0.4	-5.9, 15.3
	Pest eggs per plant	-0.43	0.001					0.02	0.5	-0.03, 0.06
H. bacteriophora	No factor found	NA	NA	NA	NA	NA	NA	NA	NA	NA
Heavy root damage (0.00 to 3.00 Oleson node-injury scale)										
Clothianidin	Maize sowing date	0.71	<0.001	5	0.55	39	<0.001	1.9	<0.001	1.1, 2.8
	Soil moisture in July	0.57	<0.001					1.1	0.02	0.2, 2.2
Tefluthrin	Sand content	-0.4	<0.001	6	0.15	109	<0.001	-1.4	<0.001	-2, -0.8
Cypermethrin	Clay content	0.41	0.001	7	0.36	44	<0.001	4	0.02	0.6, 7.5
	Soil pH	-0.5	<0.001					-66.7	0.2	-173, 40
	Air temperature in June	0.47	<0.001					14.7	0.6	-41, 70.6
H. bacteriophora	No factor found	NA	NA	NA	NA	NA	NA	NA	NA	NA

4.4 Discussion and Conclusions

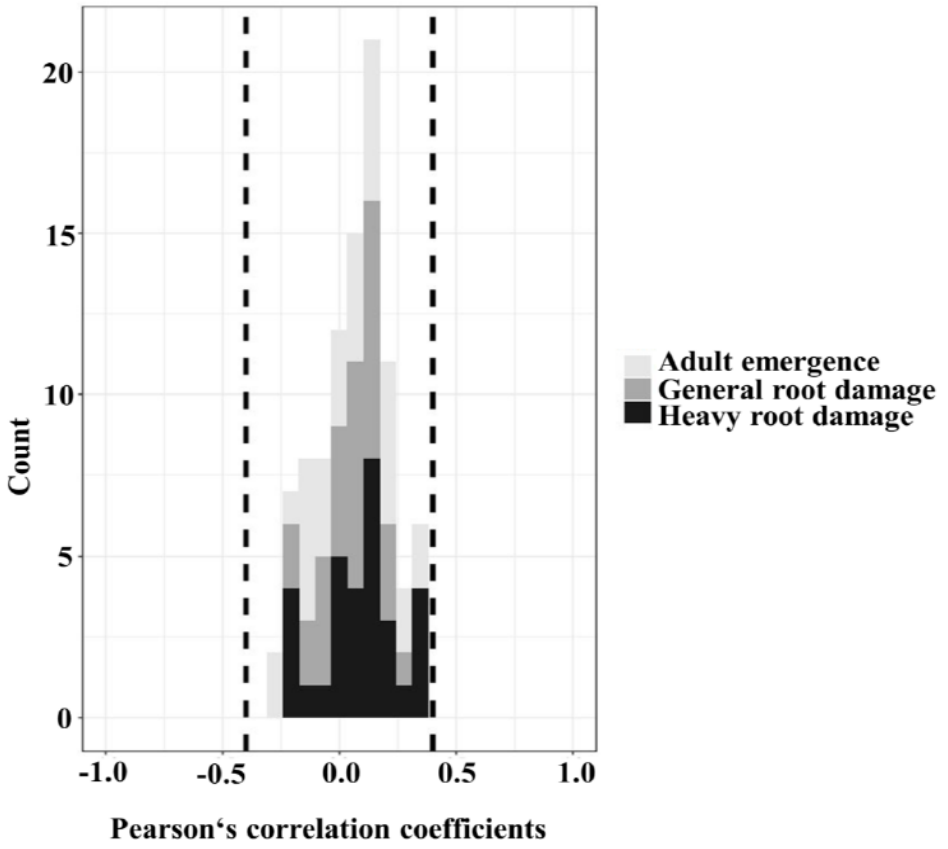

Figure 2. Histogram of the distribution of Pearson's correlation coefficients between the 32 investigated abiotic and biotic factors and the efficacies of the entomopathogenic nematode *Heterorhabditis bacteriophora* in reducing *Diabrotica v. virgifera* population and protecting against heavy and general root damage in maize at 20 sites across 10 years in southern Hungary. Dashed lines represent the border of -0.4 and 0.4 for the r coefficient values that were set to be the threshold for factor selection for regression modeling.

4. Discussion

In general, the applied soil insecticides, the insecticide seed coating, and the entomopathogenic nematodes reduced the *D. v. virgifera* population by 31 to 69% across the fields and years of this study, and this at comparable levels. The found efficacies of the chemical insecticides were comparable with results from others studies, as was the observation that efficacies recorded from different fields trials or years can vary considerably [61] [62] [63] [22]. Such variability in pest reduction seems also true for entomopathogenic nematodes [64] [59]. Moreover, there was high variability among the treatments in our study in terms of root damage, such as a 3 to 20% efficacy in preventing general root damage as assessed by the 1.0 to 6.0 Iowa damage rating scale. This also holds true for the prevention of heavy root damage as assessed by the 0.00 to 3.00 node-injury scale, where we found variable efficacies of 46 to 82%. This issue had been also been observed by [65] and other authors over a long period. Therefore, we wanted to find out how abiotic and biotic factors may influence the efficacies of chemical insecticides or entomopathogenic nematodes in reducing *D. v. virgifera* populations and preventing root damage in maize.

We analyzed relationships between the efficacies of the above-mentioned control methods and a large number of abiotic and biotic factors (32) across 10 years in 20 fields in Hungary. However, we found only a few indications that some of the studied factors may influence the efficacy of the agents in our study. Neonicotinoids seed coatings, such as clothianidin, have been widely used for the control of rootworms and other soil pests. In our study, clothianidin reduced *D. v. virgifera* populations by 69% ± 8% and prevented general root damage by 19% ± 13% and heavy root damage by 83% ± 17% in maize (Figure 1). However, variability in efficacy was relatively high. Schwarz et al. [66], who tested clothianidin seed treatment effects on four *Diabrotica* species, found that it reduced root damage to a similar extent as chlorpyriphos, tefluthrin, and fipronil. However, clothianidin sometimes failed to prevent heavy root damage (i.e., in 26% of cases). In another study, clothianidin reduced larvae in four out of five different locations and protected the roots from heavy damage in only two locations [67]. In a 2-year study in Austria, Rauch et al. [46] reported the failure of clothianidin in pest reduction. Others too have reported occasional inconsistent results of *D. v. virgifera* larval control when clothianidin is used over several years [68] [69] [23]. Some argue that clothianidin seed treatment is recommended only when the population is low to moderate and it is more a protective tool than a control solution against root damage under high *Diabrotica* pressure [70]. On the contrary, in our experiments, the performance of clothianidin in controlling the pest population and in protecting the roots slightly exceeded that of all other tested treatments and it failed in only a few cases.

There is not much published information on factors that directly affect clothianidin toxicity in the soil or may cause its degradation. We know that clothianidin and another neonicotinoid thiamethoxam are both considered hydrolytically stable, but with high solubility properties: 0.327 g/L at 20°C and 4.1 g/L at 25°C [71]. More knowledge has been accumulated over the years about other neonicotinoids. Mahapatra et al. [72] studied the effects of abiotic factors on the degradation of imidacloprid. They found that the rate of dissipation of imidacloprid from the soil samples was faster under submerged conditions compared to when the soil was left in its field capacity and soil samples were air-dried. Imidacloprid dissipated non-significantly between sterile and non-sterile soils. Similarly, under submergence, the dissipation of imidacloprid was up to 66% and 80% of the initial concentration in sterile and non-sterile soils, respectively. Imidacloprid was more stable in acidic and neutral water than in alkalic conditions. Similar results were found for thiamethoxam persistence [73]. Longer persistence was observed under dryer conditions than under submerged conditions. In a leaching experiment in a soil column with water equivalent to 65 cm rainfall, 66–79% of the applied thiamethoxam was recovered from leachate and no residues remained in the soil. Some studies have addressed the biodegradation of neonicotinoids [74]. For example, Parte and Kharat [75] showed that *Pseudomonas stutzeri* can degrade 62% of clothianidin within 2 weeks at 30°C under aerobic conditions.

In our study, there were few indications of possible slight factorial effects on clothianidin. For example, clothianidin was slightly less effective in preventing damage at a high soil bulk density; but late maize sowing and therefore late treatment as well as high soil moisture in July slightly improved the prevention of heavy root damage. Others also achieved similar results, for example, organochloride dieldrin volatilization is highest at low soil bulk density (here 0.75 g/cm^3) but lowest at high density (1.25 g/cm^3) [76]. This is comparable to the results of our studied fields, with a bulk density from 0.89 to 1.3 g/cm^3. Therefore, in all probability, clothianidin volatilization, i.e., free movement in the soil, may have been hindered around the root system of the maize plants by the high bulk density of the soil. As mentioned above, elevated soil moisture in July, i.e., during the flowering stage of maize in Europe, seems to slightly aid clothiandin's efficacy in protecting maize against heavy root damage. A handful of studies have shown this result, that is, that higher soil moisture elevates the toxicity of different soil insecticides [77] [78] [79]. However, in our case, this is something hard to explain because many larvae are

already either pupae or have emerged from the soil as adults by mid-July in Hungary. One reason might be that high soil moisture in July helps roots to recover from damage, and damage then becomes less obvious and more difficult to assess. In our study, late sowing seemed to lead to a slightly better protective role of clothianidin seed treatment against *D. v. virgifera* damage than early sowing. A smaller time window between the treatment and hatching of the first larvae may have favored the treatment efficacy. Alford and Krupke [80] detected that after sowing maize, there is only a low amount of clothianidin in the plants (maximum 1.34% from the plant tissues and 0.26% from root tissues). This is surprising because in treated seeds, clothianidin is considered a highly translocatable substance. They also found the amounts of plant-bound active ingredients to rapidly decrease after 20 days, putting a question mark on its long-term protective role throughout the cropping seasons. We found something similar in the case of the protective role of clothianidin against heavy root damage.

Pyrethroid insecticides such as tefluthrin have been used against a number of soil-dwelling insect pests since the 1980s [26]. A number of studies have demonstrated that tefluthrin (usually put as granules into furrows) can reduce *D. v. virgifera* larvae infestation, leading to less root damage, fewer lodged plants, and increased yield [81] [82] [83] [67]. This is confirmed in our study, wherein tefluthrin reduced the pest population by 44% ± 41%, prevented the general root damage by 20% ± 16%, and heavy root damage by 83% ± 31%. However, inconsistent results, especially in controlling *D. v. virgifera* populations, have been reported [48] and are confirmed here. Insect resistance could be one of the explanations for the observed inconsistencies. Pyrethroid resistance has been reported in the *D. v. virgifera* populations found in some maize fields in the USA [27]. However, in Europe, resistance of *D. v. virgifera* against pyrethroids has not been reported or maybe not even studied. Therefore, the inconsistent performance of tefluthrin could be caused by environmental factors.

In our analysis, soil moisture in June, i.e., during the vegetative stages of maize in Hungary, had a slight negatively effect on the efficacy of tefluthrin against *D. v. virgifera* populations. This is hard to explain, considering that dry soils are known to absorb insecticides whereas soil moisture favors their release from the formulation [84]. An explanation could be that moisture accelerates chemical [85] or biological degradation of the insecticide [86] and therefore the majority of the molecules are not in the soil fraction anymore [87]. On the contrary, higher cumulative rainfall in July seemed to have a positive influence on tefluthrin's efficacy against *D. v. virgifera* population in our study. This too is somewhat unusual to see because, normally, many larvae in the soil have emerged by mid-July in Hungary. However, in years when larvae hatching and development are extended due to cool weather, it might be possible that bigger rain quantities wash the tefluthrin into lower or wider layers of soil, allowing the tefluthrin to better reach late instars that have moved due to competition or for pupation [88]. According to Sutter et al. [65], a low density of larvae around roots caused less competition; thus, larvae could live outside of the treated band and avoid contact with soil insecticides. We have also found that soil with a higher sand content may negatively influence tefluthrin's efficacy in preventing heavy root damage. Higher sand or quartz content of the soil is known to absorb some insecticides and may, therefore, reduce their effects on insect pests [84] [31].

In our study, another pyrethroid, cypermethrin, reduced *D. v. virgifera* by 31% ± 34%, prevented the general root damage by 4% ± 16, and heavy root damage by 50% ± 65%. Although these values are lower than those found for tefluthrin or clothianidin, we could not detect any major influence of the studied biotic and abiotic factors. The only thing we found was that elevated clay content of the soil may slightly aid cypermethrin's efficacy in protecting maize against heavy root damage. This is contrary to the above-discussed role of sandy soils and to the observation that a higher clay content may lower the toxicity of insecticides in the soil [31]. The pH of the soil may have also played an additional role. For example, Bhat et al. [89] showed that biological degradation of pyrethroid molecules is mediated by different hydrolases. These hydrolases remove ester groups from

the molecules and thus they will be inactive. However, the activity of these hydrolases depends on the changes in the pH of the environment. In general, it remains unclear which factors cause a high variability in cypermethrin efficacy and may lead to the occasional low efficacy or even failure.

For biological control agents, such as the entomopathogenic nematodes in our study, variability in efficacies is a well-known phenomenon. There are also numerous indications from both laboratory and field studies that abiotic factors can influence the mortality and infectivity of entomopathogenic nematodes. For example, *H. bacteriophora* infectivity against *Popillia japonica* was the highest in potting mix soils, did not differ between loamy sand and other types of loamy soils, and was the lowest in acidic sand [90]. Toepfer et al. [91] investigated the influence of soil types on the efficacy of *H. bacteriophora*, *H. megidis*, and *S. feltiae* against *D. v. virgifera* larvae under field conditions. They found that all nematodes were able to control *D. v. virgifera* larvae in most soils. However, their efficacy was slightly higher in heavy clay or silty soils than in sandy soils. At high soil moistures, the rehydration effect favors many nematode species [51] [52] [92], and this can also be true for elevated temperatures (5°C vs. 15-30°C) [52]. Soil salinity seems to play a role. For example, elevated levels of $CaCl_2$ and KCl had no effect on *H. bacteriophora* survival, penetration efficiency, or movement through a soil column, but moderate concentrations of these salts enhanced *H. bacteriophora* virulence [93]. Survival of infective juveniles of *Steinernema carpocapsae* and *S. glaseri* gradually declined over 16 weeks when pH decreased from pH 8 to pH 4 [94]. However, despite the findings of others on the importance of soil moisture in the pathogenicity of entomopathogenic nematodes [49] [51] [52], we found no such relationship in our study under field conditions.

In our study, we detected a high variability in the efficacy of the applied *H. bacteriophora* in controlling the pest population and preventing root damage. Therefore, it came as a surprise that we found no relationship between nematode efficacy and the more than 30 tested abiotic or biotic factors. This means that factors other than those studied here may influence the performance of entomopathogenic nematodes in the control of soil insect pests. An increasing number of studies are highlighting the importance of the below-ground interactions between the entomopathogenic nematodes and the fauna and microbiological communities of the soil. For example, predation or infection of the nematodes [95] [96] or competition with other organisms [97] [98] may play a role. This interaction could influence nematode efficacy, but these were not examined in our study. What is clear is that nematode product quality (measured as virulence and mortality before and after field application) had no influence on their efficacy, indicating that is easy to handle and apply nematodes. This could be crucial in view of farmers who are often less experienced in working with living organism than with pesticides. Another positive aspect is that regardless of the nematodes being applied into the furrow with 100 liters of water or more than 500 liters, there efficacy remained comparable. Voros et al. [54] made similar observations and argued that the amount of water used for application has little importance when nematodes are applied into the seeding furrow, which is usually already moist and provides a protected environment to nematodes. So, farmers have to carry less water to the fields for application.

5. Conclusions

In conclusion, our work clearly showed that only a few factors may influence the efficacy of the chemical and biological control options used against *D. v. virgifera* in maize fields. The efficacy of chemical and biological treatments against this serious soil insect pests remains difficult to predict under real-time farming conditions. Other biological factors could influence their performance, such as the degradation of the insecticide by the microbiota and the effect of the microfauna and microbiological communities on the entomopathogenic nematodes, something that merits more investigation. Nevertheless, it

is positive that many types of treatments help manage *D. v. virgifera* in most cases under diverse conditions.

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Data Availability Statement: The analysis, with the data alongside, is openly available in Github at https://github.com/DiabroticaHULab/EnvFactInfluDvv.

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Supplementary Data

Table S1. Properties of the experimental maize fields and treatments against *D. v. virgifera* larvae in Hungary between 2010 and 2020

Field site code	T	S	R	Q	P	O	N	M	L	K	J	I	H	G	F	E	D	C	B	A	n
Study year	2020	2020	2018	2018	2017	2017	2016	2016	2015	2015	2014	2014	2013	2013	2012	2012	2011	2011	2010	2010	9
Location	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	Southeast Kondoros	East of Mako	West of Kiszombor	East of Mako	West of Kiszombor	East of Mako	West of Kiszombor	East of Mako	South of Szeged (Szeged GK2)	North of Szeged (Nagyfekete)	North of Szeged (Feherto)	South of Szeged	20
Coordinates	N 46° 44` 27.70`` E 20° 49`39.00``	N 46° 44` 30.00`` E 20° 49`29.00`	N 46° 44` 49.152`` / 46.750742 E 20° 49`22.986`` / 20.816259	N 46° 44` 57.426`` / 46.749710 E 20° 49`38.406`` / 20.827370	N 46° 44` 58.88`` E 20° 48` 56.11``	N 46° 45` 02.89`` E 20° 48` 59.40``	N 46° 44` 20.715`` E 20° 49` 7.430``	N 46° 44` 29.775`` E 20° 48` 39.764``	N 46° 44` 8.036`` E 20° 48` 57.193``	N 46° 14` 36.012`` E 20° 30` 52.109``	N 46° 11` 05.8`` E 20° 24` 33.2``	N 46° 14` 29.1`` E 20° 31` 02.0``	N 46° 11` 05.4`` E 20° 24` 31.1``	N 46° 14` 34.8`` E 20° 30` 49.5``	N 46° 11` 01.60`` E 20° 30.99``	N 46° 14` 39.31`` E 20° 31` 07.38``	N 46° 13.590 E 20° 09.035	N 46° 18` 45.7`` E 20° 06` 49.2``	N 46° 17 47.0 E 20° 07 18.6	N 46° 13.590 E 20° 09.035	
Elevation (m)	82	82	82	86	85	84	123	123	122	90	89	89	89	90	83	80	80	82	82	80	
Field size	ca. 10	ca. 10	ca. 2	ca. 2	ca. 1.5	ca. 2	ca. 6	ca. 15	ca. 0.4	ca. 0.45	ca. 0.4	ca. 0.4	0.36	0.36	0.5	0.3	0.6	15	10	0.5	

Field site code	T	S	R	Q	P	O	N	M	L	K	J	I	H	G	F	E	D	C	B	A	n
Study year	2020	2020	2018	2018	2017	2017	2016	2016	2015	2015	2014	2014	2013	2013	2012	2012	2011	2011	2010	2010	9
Trial size (ha)	0.6 ha (25 x 240 m)	0.6 ha (25 x 240 m)	0.37 (27 x 140 m)	0.37 (27 x 140 m)	0.25 (18 x 140 m)	0.25 (18 x 140 m)	0.29 (18 x 160 m)	0.29 (18 x 160 m)	0.29 (36 x 80 m)	0.43 (36 x 120 m)	0.29 (24 x 120 m)	0.29 (24 x 120 m)	0.25 (21 x 120 m)	0.25 (21 x 120 m)	0.24 (20 x 120 m)	0.24 (20 x 120 m)	0.3 (24 x 120 m)	0.3 (24 x 120 m)	0.3 (24 x 120 m)	0.3 (24 x 120 m)	3 to 4 plots / treatment/ field
Plots size (m)	6 rows (4.5 m) x 30 m	6 rows (4.5 m) x 30 m	6 rows (4.5 m) x 20 m	6 rows (4.5 m) x 20 m	6 rows (4.5 m) x 20 m	6 rows (4.5 m) x 20 m	6 rows (4.5 m) x 20 m	6 rows (4.5 m) x 20 m	6 rows (4.5 m) x 20 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	4 rows (3m) x 30 m	
Pre-crop	Maize	Maize	Sunflower	Sunflower	Triticale	Triticale	Peas	Sunflower	Maize	Winter wheat	Soybean	Winter wheat	Maize	Winter wheat	Winter wheat	Winter wheat	Maize	Winter wheat	None maize	None maize	
Maize hybrid	P9241(Pioneer)	P9241(Pioneer)	PR37F73 (Pioneer)	PR37F73 (Pioneer)	N01 (Pioneer)	N01 (Pioneer)	P9903 (Pioneer)	P9903 (Pioneer)	P0216 (Pioneer)	NK Kansas (Syngenta)	Pactol (Syngenta)	Mika (Syngenta)	Gavott (KWS)	Gavott (KWS)	NK Kansas (Syngenta)	NK Kansas (Syngenta)	NK Kansas (Syngenta)	NK Kansas (Syngenta)	Boglar (GK)	Boglar (GK)	
Sowing date	24-Apr-20 (JD 114)	24-Apr-20 (JD 114)	25-Apr-18 (JD 115)	25-Apr-18 (JD 115)	25-Apr-17 (JD 114)	25-Apr-17 (JD 114)	18-Apr-16 (JD 109)	18-Apr-16 (JD 109)	20-Apr-15 (JD 115)	21-Apr-15 (JD 116)	23-Apr-14 (JD 113)	23-Apr-14 (JD 113)	23-Apr-13 (JD 113)	23-Apr-13 (JD 113)	2 and 7 - May -12 (JD 123 & 128)	2 and 4 - May -12 (JD 123 & 125)	18-Apr-11 (JD 108)	19-Apr-19 (JD 109)	23-Apr-10 (JD 113)	22-Apr-10 (JD 114)	
Plants / ha	74000	74000	74000	74000	72000	72000	72000	72000	74000	80000	80000	80000	80000	80000	87000	87000	73000	73000	87000	87000	
D. v. virgifera eggs / plant	300 *	300 *	500	500	500 *	500 *	300	300	high native population	200, 300, 500	300	300	200, 300, 500 *	200, 300, 500	200, 300, 500	300	200, 500	200, 500	300	300	
Egg infestation date	08-May-20 (JD 129)	08-May-20 (JD 129)	04-May-18 (JD 124)	04-May-18 (JD 124)	08-May-17 (JD 128)	08-May-17 (JD 128)	01-May-16 (JD 122)	01-May-16 (JD 122)	-	04-May-15 (JD 124)	05-May-14 (JD 125)	05-May-14 (JD 126)	06-May-13 (JD 127)	06-May-13 (JD 127)	16-May-12 (JD 137)	14-May-12 (JD 125)	2 & 3 May 2011 (JD 122 & 123)	2 & 3 May 2011 (JD 122 & 123)	2 & 25 May 2010 (JD 122 & 145)	2 & 25 May 2010 (JD 122 & 145)	

Field site code	T	S	R	Q	P	O	N	M	L	K	J	I	H	G	F	E	D	C	B	A	n
Study year	2020	2020	2018	2018	2017	2017	2016	2016	2015	2015	2014	2014	2013	2013	2012	2012	2011	2011	2010	2010	9
Treatments																					
Treatment date	24-Apr-20 (JD 114)	24-Apr-20 (JD 113)	25-Apr-18 (JD 115)	25-Apr-18 (JD 115)	25-Apr-17 (JD 114)	25-Apr-17 (JD 114)	18-Apr-16 (JD 109)	18-Apr-16 (JD 109)	20-Apr-15 (JD 115)	21-Apr-15 (JD 116)	23-Apr-14 (JD 113)	23-Apr-14 (JD 113)	23-Apr-13 (JD 113)	23-Apr-13 (JD 113)	2 and 7 - May -12 (JD 123 & 128)	2 and 4 - May -12 (JD 123 & 128)	18-Apr-11 (JD 108)	19-Apr-19 (JD 109)	23-Apr-10 (JD 113)	22-Apr-10 (JD 114)	
H.bacteriophora (billion / ha)	2	2	2	2	2	2	2	2	2 & 1.6	1.5	2	2	2	2	2	2	2	2	1.5	1.5	74 (20 fields)
0.006 ml Clothianidin /seed															522 ml clothianidin / 87000 seeds /ha	516 ml clothianidin / 87000 seeds /ha	438 ml clothianidin / 73000 seeds /ha	438 ml clothianidin / 73000 seeds /ha	522 ml clothianidin / 87000 seeds /ha	522 ml clothianidin / 87000 seeds/ha	22 (6 fields)
Cypermethrin 0.8% (kg /ha)	12	12	12	12	12	12	12	12	12	12	12	12									44 (12 fields)
Tefluthrin 1.5% (kg /ha)									13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	46 (12 fields)

*Background infestation from natural infestation is present on the fields

5. Chapter III.

Can the botanical azadirachtin replace phased-out soil insecticides in supressing the soil insect pest *Diabrotica virgifera virgifera?*

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5.1 Abstract and Introduction

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Can the botanical azadirachtin replace phased-out soil insecticides in suppressing the soil insect pest *Diabrotica virgifera virgifera*?

Stefan Toepfer[1,2*], Szabolcs Toth[1,2] and Mark Szalai[2]

Abstract

Background: Due to recent bans on the use of several soil insecticides and insecticidal seed coatings, soil-dwelling insect pests are increasingly difficult to manage. One example is the western corn rootworm (*Diabrotica virgifera virgifera*, Coleoptera: Chrysomelidae), a serious root-feeder of maize (*Zea mays*). We investigated whether the less problematic botanical azadirachtin, widely used against above-ground insects, could become an option for the control of this soil insect pest.

Methods: Artificial diet-based bioassays were implemented under standard laboratory conditions to establish dose response curves for the pest larvae. Then, potted-plant experiments were implemented in greenhouse to assess feasibility and efficacy of a novel granular formulation of azadirachtin under more natural conditions and in relation to standard insecticides.

Results: Bioassays in three repetitions revealed a 3-day LD_{50} of 22.3 µg azadirachtin/ml which corresponded to 0.45 µg/neonate of *D. v. virgifera* and a 5-day LD_{50} of 19.3 µg/ml or 0.39 µg/first to second instar larva. No sublethal effects were observed. The three greenhouse experiments revealed that the currently proposed standard dose of a granular formulation of 38 g azadirachtin/hectare for in-furrow application at sowing is not enough to control *D. v. virgifera* or to prevent root damage. At 10× standard-dose total pest control was achieved as well as the prevention of most root damage. This was better than the efficacy achieved by cypermethrin-based granules and comparable to tefluthrin-granules, or thiamethoxam seed coatings. The ED_{50} for suppressing larval populations were estimated at 92 g azadirachtin/ha, for preventing heavy root damage 52 g/ha and for preventing general root damage 220 g/ha.

Conclusions: There seems clear potential for the development of neem-based botanical soil insecticides for arable crops such as maize. They might become, if doses are increased and more soil insecticides phased out, a promising, safer solution as part of the integrated pest management toolkit against soil insects.

Keywords: Integrated pest management, Western corn rootworm, Azadirachtin, *Zea mays*, Biopesticide, Soil insecticide, Biological control

Introduction

Corn rootworms are, next to wireworms, grubs and cutworms, serious soil-dwelling insect pests of maize (*Zea mays*). One of the rootworms, the western corn rootworm (*Diabrotica virgifera virgifera*, Coleoptera: Chrysomelidae) is one of the most problematic (Krysan and Miller 1986). Its three larval instars feed almost exclusively on the roots of maize, which becomes apparent when plants lodge (Chiang 1973). *Diabrotica v. virgifera* causes yield losses to large maize production areas of the USA and southern Canada (Kim and Sappington 2005) as well as in Central Europe (Miller et al. 2005).

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Affected growers attempt to manage the pest mainly through rotating their fields, thereby interrupting the life cycle of *D. v. virgifera*. Many growers apply granular or fluid soil insecticides, mainly pyrethroids or organophosphates, or use neonicotinoid-coated maize seeds to target the root feeding larvae. North American growers also use transgenic maize expressing different *Bacillus thuringiensis* proteins which are toxic to rootworms (Levine and Oloumi-Sadeghi 1991; Domínguez-Arrizabalaga et al. 2020), but their efficacies under field conditions are variable (Clair et al. 2020; Gassmann et al. 2020). Additionally, broad-spectrum foliar insecticides are occasionally sprayed against the adults using high clearance spraying machinery (Rozen and Ester 2010). Foliar insecticides are often broad spectrum and knock-down contact-pesticides with considerable non-target effects. Also, many soil insecticides and seed coatings are problematic due to their human toxicity and/or serious non-target or other environmental effects.

This has resulted in public concerns and in a ban on the use of neonicotinoid seed-coatings in field crops (Georgiadis et al. 2011), and restrictions in the use chlorpyrifos- and tefluthrin-based soil insecticides in many countries. Only few countries remain, that still have such ingredients on the soil pesticide market, such as the USA. In Hungary, tefluthrin is only registered under special emergency registration. However, tefluthrin will definitely disappear from the European pesticide market (European-Commission 2011) and likely from the entire global market due to its high acute toxicity (World Health Organization 2009). Therefore, options for growers to control *D. v. virgifera*, and also other soil insect pests such as wireworms, grubs and cutworms will be limited, particularly in Europe. Novel pest management solutions and agents are urgently needed, particularly less environmentally-disruptive ones.

For example, neem preparations with their active ingredients of different azadirachtins are widely-used botanical insecticides (Saxena 1989; Scmutterer 1995; Dougoud et al. 2019). Although its modes of action are still somewhat uncertain, they are known to have broad spectrum insecticidal activity as well as some nematocidal, isopodicidal, fungicidal and plant promoter activity (CRC 1989; Doshi et al. 2018, 2020). There are numerous products available in most world regions, mainly against above-ground, soft-bodied insect pests (Dougoud et al. 2019). In some regions, growers also prepare self-made homebrews from leaves or seeds of the tree *Azadirachta indica,* which is of south Asian origin but nowadays widely distributed in the tropics and sub-tropics of many regions (Dougoud et al. 2019). The advantage of neem is that it has a low acute or chronic toxicity to humans and breaks down relatively quickly in the environment (Boeke et al. 2004). Another advantage is that neem is systemic, translaminar as well as of contact mode of activity (Stark and Rangus 1994; Dougoud et al. 2019), allowing its diverse usage. It can directly cause mortality to insects, can inhibit growth and moulting, and even can cause chronic effects such as on insect fertility (Ladd Jr et al. 1984; Al-Sharook et al. 1991; Stark and Rangus 1994; Mehaoua et al. 2013; Merabti et al. 2017).

Astonishingly, there are still limited studies and limited use of neem products against below-ground insect pests (Bhagat 2005). This is surprising because neem leaves, grinded seeds or leftovers from seed processing are occasionally used as a soil amendment against plant parasitic nematodes (Dougoud et al. 2019) as well as a biofertilizer, even in maize (Vageesh et al. 2016). Neem seed extracts also has some fungicidal properties when used as coating of maize seeds (Sowley et al. 2017). Consequently, there exist some experience for in-soil applications of neem, such as leaves or commercial granules (Balaji 2014). Nevertheless, only few products are available for soil application, and knowledge on their effects against soil insects is limited due to their concealment below-ground in or on the roots of crops.

This is particularly true for rootworms (Diabroticina), several of them, as stated above, being serious root-feeding pests of maize. Azadirachtin is known to be toxic to rootworm larvae, such as against *D. speciosa* (Boiça Júnior et al. 2017) or the here-studied *D. v. virgifera* (Xie et al. 1991), and repellent to larvae of cucumber beetles such as *Diabrotica undecimpunctata howardi* (Landis and Gould 1989) or to adults of the closely related *Acalymma vittatum* (Reed et al. 1982). However, further experimentation on the use of azadirachtin against rootworms seem scarce. Only Estes et al.(2018) attempted to examine liquid and granular formulations of neem against *D. v. virgifera* larvae in Illinois, USA, but with inconclusive results. There seems a knowledge gap on how to effectively use neem against rootworms or other soil insect pests, a gap we try to close with the here-presented study.

We aimed at better understanding the pest control potential of azadirachtin using a novel granular neem-based soil insecticide. First, bioassays were conducted to establish the LD_{50} of azadirachtin on the neonates of *D. v. virgifera*. Second, the suggested standard dose of neem granules was compared with lower and higher dosages in relation to standard insecticides in potted-maize plant trials under semi-natural conditions. This set of experimental steps was hoped to allow conclusions on the efficacy and potential feasibility of neem-based granule applications for corn rootworm control. Ultimately, this would help to diversify the currently limited integrated

pest management toolbox against rootworms and potentially other soil insect pests.

Material and methods

Target pest *Diabrotica v. virgifera*

Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae, western corn rootworm, EPPO code DIABVI) were mass-reared following procedures of George and Ortman (1965), Branson et al. (1975) and Singh and Moore (1999). A non-diapause colony (USDA ARS, Bookings, USA) was used to infest artificial diet-based bioassay with neonates and potted—maize plants in greenhouse experiments with ready-to-hatch eggs (see procedures below). The population is considered susceptible to most insecticides or novel agents as it had not been exposed to any of those, and therefore resistance is considered unlikely (Wright et al. 2000; Magalhaes et al. 2007).

Test agents

An azadirachtin-based granular soil insecticide was tested against *D. v. virgifera* larvae in comparison to a set of commonly used contact and/or systemic insecticides (positive controls) as well as to untreated controls (Tables 1, 2).

In artificial-diet based bioassays against neonates of *D. v. virgifera* in the laboratory, azadirachtin-granules were dissolved in water and 20 µl applied/well/larva (see details below). Those dissolved azadirachtin granules were compared with a commercial fluid azadirachtin formulations and the insecticides tefluthrin, cypermethrin, imidacloprid as well as with untreated control (Table 1).

In potted-maize experiments under greenhouse conditions, azadirachtin-granules were manually applied when the maize seeds were placed into a sowing furrow across the pots (see details below). The azadirachtin-based granular soil insecticide was compared with the soil insecticides tefluthrin and cypermethrin and to thiamethoxam-coated seeds as well as with untreated infested and un-infested control (Table 2).

Azadirachtin was tested as per Tables 1 and 2. The main test product was a granule formulation provided by Coromandel International Ltd, India (NeemAzal™ 0.15G similar to Avana™ by Parry America). It contained 0.165% azadirachtin (mainly A with some B, chromatogram-verified, batch 2001-10) as well as 0.35% other neem compounds (Aza-F, Aza-H, Aza-I, salanin,

Table 1 Treatment characteristics in artificial diet-based bioassay against neonates of *Diabrotica v. virgifera* under standardised semi-sterile laboratory conditions

Treatment	Formulation	Dose/ml (a.i.)	Dose/cm² (a.i.)	Dose/20 µl/0.34 cm² well/larva (a.i.)	Experiment number (# plates; # wells)
Test agent					
Azadirachtin 0.15% (Neem Azaal 0.15G)[a]	Granule	0.067 mg (0.1 µg)	0.0038 mg (0.006 µg)	0.0013 mg (0.002 µg)	3 (6; 48)
		0.67 mg (1 µg)	0.038 mg (0.06 µg)	0.013 mg (0.02 µg)	3 (6; 48)
		6.7 mg (10 µg)	0.38 mg (0.6 µg)	0.13 mg (0.2 µg)	3 (6; 48)
		66.7 mg (100 µg)	38 mg (6 µg)	1.33 mg (2 µg)	3 (6; 48)
Azadirachtin 1% (NeemAzal-T/S 10EC)	Fluid	0.01 µl (0.1 µg)	0.0006 µl (0.006 µg)	0.0002 µl (0.002 µg)	3 (6; 48)
		0.1 µl (1 µg)	0.006 µl (0.06 µg)	0.002 µl (0.02 µg)	3 (6; 48)
		1 µl (10 µg)	0.06 µl (0.6 µg)	0.02 µl (0.2 µg)	1 (3; 24), 2 (6; 48), 3 (6; 48)
		3 µl (30 µg)	0.18 µl (1.8 µg)	0.06 µl (0.6 µg)	1 (3; 24)
		5 µl (50 µg)	0.3 µl (3 µg)	0.1 µl (1 µg)	1 (3; 24)
		10 µl (100 µg)	0.6 µl (6 µg)	0.2 µl (2 µg)	1 (3; 24), 2 (6; 48), 3 (6; 48)
		100 µl (1000 µg)	6 µl (60 µg)	2 µl (20 µg)	1 (3; 24), 2 (6; 48)
		500 µl (5000 µg)	30 µl (300 µg)	10 µl (100 µg)	2 (6; 48)
		1000 µl (10,000 µg)	60 µl (600 µg)	20 µl (200 µg)	2 (6; 48)
Positive controls					
Imidacloprid 20% (Confidor 200 SL)	Fluid	0.01 µl (2 µg)	0.0006 µl (0.12 µg)	0.0002 µl (0.04 µg)	1 (3; 24), 2 (6; 48), 3 (6; 48)
Cypermethrin 0.8% (Belem 0.8 MG)	Micro granule	12.5 mg (100 µg)	0.73 mg (5.9 µg)	0.25 mg (2 µg)	3 (6; 48)
Tefluthrin 1.5% (Force 1.5G)	Fine granule	6.7 mg (100 µg)	0.38 mg (5.9 µg)	0.13 mg (2 µg)	3 (6; 48)
Negative controls					
Untreated-infested	–	–	–	–	1 (3; 24), 2 (6; 48), 3 (6; 48)
Untreated un-infested	–	–	–	–	1 (3; 24), 2 (6; 48), 3 (6; 48)

Each diet-filled well infested with one neonate larva

[a] Similar to Avana™ by Parry America

Table 2 Treatment characteristics in potted-maize plant experiments against larvae of *Diabrotica v. virgifera* under greenhouse conditions

Treatment	Formulation	Dose/ha (a.i.)	Dose/meter furrow (a.i.)	Dose/10 cm furrow/plant/pot (a.i.)	Dose/cm^2 (a.i.)	Experiment number (# blocks; # plants)
Test agent						
Azadirachtin 0.15% (Neem Azaal 0.15G)[a]	Granule	18 kg (27 g)	1.3 g (2 mg)	130 mg (0.2 mg)	26 mg (40 µg)	3 (3; 15)
		25 kg (38 g) [5]	1.9 g (2.8 mg)	190 mg (0.28 mg)	38 mg (56 µg)	1 (3; 15), 2 (4; 20), 3 (3; 15)
(limoid)		135 kg (200 g)	10.2 g (15 mg)	1020 mg (1.5 mg)	204 mg (300 µg)	3 (3; 15)
		250 kg (370 g)	19 g (28 mg)	1900 mg (2.8 mg)	380 mg (560 µg)	3 (3; 15)
		2500 kg (3700 g)	190 g (280 mg)	19,000 mg (28 mg)	3800 mg (5600 µg)	3 (3; 15)
Positive controls						
Cypermethrin 0.8% (Belem 0.8 MG) (pyrethroid)	Micro granule	12 kg (96 g) [5]	0.9 g (7.2 mg)	90 mg (0.72 mg)	18 mg (0.14 µg)	1 (3; 15), 2 (4; 20)
		25 kg (200 g)	1.9 g (15 mg)	188 mg (1.5 mg)	38 mg (0.3 µg)	3 (3; 15)
Tefluthrin 1.5% (Force 1.5G) (pyrethroid)	Fine granule	13.3 kg (200 g) [5]	1 g (15 mg)	100 mg (1.5 mg)	20 mg (0.3 µg)	1 (3; 15), 2 (4; 20), 3 (3; 15)
Thiamethoxam 30% (Cruiser 350FS) (neonicotinoid)	Seed coating	180 ml (63 g/50,000 seeds) [5]	18 µl (6.25 mg/5 seeds)	3.6 µl (1.25 mg/ seed)	3.6 µl (1.25 mg/ seed)	1 (3; 15), 2 (4; 20)
Negative controls						
Untreated-infested	–	–	–	–	–	1 (3; 15), 2 (4; 20), 3 (3; 15)
Untreated uninfested	–	–	–	–	–	1 (3; 15), 2 (4; 20), 3 (3; 15)

About 2 × 10 cm treatment strips along soil surface in the pots extrapolated to hectare doses of products for 13,300 row meters. 1.5 l soil in pots with maize plants used. Plants of experiment 1 and 3 infested with 50 ready-to-hatch eggs each, and of experiment 2 with 100 eggs. Block numbers reflect the within experiment replicates. Plants numbers reflect the sample size per treatment per experiment

[5] Standard doses

[a] Similar to Avana™ by Parry Americaa

nimbin and fatty acids). The granules were of 2 to 3 mm size (Formulation G of GIFAP code) and of slow release.

As a comparison, a common fluid formulation (NeemAzal™ T/S, Trifolio-M, Germany) was used in artificial diet-based bioassays. It contained 1% azadirachtin A originating from 4% neem seed extract (*Azadirachta indica* tree), but with unknown concentration of other neem compounds.

Tefluthrin served as a positive control (Tables 1 and 2). The product Force™ 1.5G (Syngenta, Hungary) are fine granules (1 to 2 mm diameter, Formulation FG of GIFAP code) with 1.5% active ingredient.

Cypermethrin served as a positive control (Tables 1 and 2). The product Belem™ 0.8MG (Certis, SBM Development SAS, France) are micro granules (0.8 to 1 mm diameter, Formulation MG of GIFAP code) with 0.8% active ingredient..

Imidacloprid served as a positive control in the artificial diet-based bioassays in the laboratory (Table 1). The product Confidor™ 200SL (17.8% w/w imidacloprid, Bayer Crop Science, Germany) is a soluble concentrate (SL of GIFAP code) with about 20% active ingredient.

Thiamethoxam served as a positive control in the potted-plant greenhouse experiments (Table 2). The product Cruiser™ 350FS (25 to 30% w/w thiamethoxam, Syngenta, Hungary) is a suspension concentrate for seed treatments (FS of GIFAP code) with about 30% active ingredient.

Artificial diet-based laboratory bioassays

Experimental setup

To assess lethal doses of azadirachtin on neonates of *D. v. virgifera*, artificial diet-based bioassays with different dosage were conducted in three replicates under controlled semi-sterile conditions (Table 1). Azadirachtin from a common fluid formulation was compared with a novel granular formulation. The insecticides cypermethrin, tefluthrin and imidacloprid served as positive control. Sterilised tap water or no treatment at all served as negative controls. Each bioassay consisted

of 3 to 6 polystyrene plates of 96 wells each (07-6096 of Biologix Ltd., USA, or Costar 3917 of Corning Inc., USA). Each well was of 330 µl volume with 5 mm in diameter and 10 mm height, and had a 0.34 cm^2 surface. Each treatment was applied to 8 wells of each plate per bioassay. Each treatment-dose combination was tested in at least in two true replicates.

The larval diet for a bioassay had been prepared 1 day before treatment and infestation. The diet was prepared under semi-sterile conditions following methods of Sutter et al. (1971); Pleau et al. (2002), Moar et al. (2017), Clark and Boland (2016, Genective, pers. comm.). A commercial southern corn rootworm diet was used (Frontier #F9800B, Frontier Scientific Ltd., USA), but maize roots and food colour were added. This diet consists of D(+) sucrose, vitamin-free casein, cellulose, Wesson's salt mix, methyl paraben fungicide, sorbic acid, cholesterol, raw wheat germ, Vanderzant's vitamin mix, raw linseed oil, streptomycin sulphate antibiotic, and chlortetracycline antibiotic. For 100 ml of diet, 13.8 g of the #F9800B diet was grinded and added to 88 ml fluid 60 to 70 °C agar (1.5 g agar CAS 9002-18-0, Chejeter, Japan in deionized water). After blending and cooling to 55 to 60 °C, 0.75 g grinded lyophilized maize roots were added (GLH5939 Pioneer, USA, or Phileaxx RAGT, Hungary) as well as 0.1 g green food colour for better larvae observation (Les Artistes, France). Thereafter, 1.7 to 1.8 ml 10% w/v KOH were added to reach a pH between 6.2 and 6.5. This mix was blended again, and then stirred at 50 to 55 °C. Then, 190 µl diet was pipetted into each 330 µl well filling each to around 2/3rd (repeater pipette P-8, Topscien Co., Ltd, China). Plates with diet were allowed to dry in a laminar flow cabinet during 45 min, and then stored at 3 to 5 °C overnight. The following day, treatments were applied. This is, 20 µl of a treatment were applied to the 0.34 cm^2 diet surface in each well (10 to 100 µl pipette, Biohit Proline, Finland). Order of treatments were shifted every other plate to avoid edge effects. Plates were dried for 1 to 1.5 h, and then cooled for 1 h in a 3 to 5 °C fridge.

Two weeks prior the bioassays, soil dishes with freshly laid eggs had been removed from *D. v. virgifera* adult rearing cages to allow sufficient incubation time until egg hatch. Eggs were washed with cool tap water with < 0.01% NaOCl through a 300 µm mesh sieve. Around 5000 eggs were transferred to sterilised, slightly moist river sand (< 200 µm grains) in Petri dishes. They were incubated at 24 ± 2 °C in darkness for 8 to 12 days until hatching started. One day before a bioassay, the ready-to-hatch eggs were again washed and sieved. Eggs were then again mixed into sterile moist sand and placed onto slightly moist tissue paper into a dish to allow clean hatching conditions of new neonates and their use for bioassays.

One neonate larva was placed per well using a fine artist brush. A fast-moving, healthy-looking larva was chosen, and lifted from the end of abdomen with the brush, moved towards a well surface, and allowed to crawl off the brush onto the diet. Larvae were not placed in treatment column order but rectangular to avoid systemic errors. After every 12th larva, the brush was cleaned in 70% ethanol followed by sterile tap water. The filled plate was closed with an optically clear adhesive qPCR seal sheet (#AB-1170, Thermo Scientific, USA or #BS3017000, Bioleader, USA) allowing data assessments without opening the plate. Four to five holes were made with flamed 00-insect pins into the seal per well to allow aeration.

The plates were incubated at 24 ± 2 °C and 50 to 70% r.h. in dark in a ventilated incubator (Friocell 22, MMM Medcenter, Munich, Germany) for 5 days.

Data assessments and analyses

After 3 and 5 days of incubation, larval mortality, stunting, feeding and contamination were visually assessed through the clear seals using a stereomicroscope (10× magnification, SMZ-B4, Optec, Chongqing, China). Data from a plate were only used when the natural mortality threshold in the untreated control had not been reached, i.e. no more than 3 dead per 8 larvae per column of wells (37.5% threshold). This is in contrast to common practices in bioassays with other insects where the quality acceptance is usually < 10% natural background mortality (Dulmage et al. 1990). However, this is rarely achievable with rootworm larvae as the artificial diets known to date remain suboptimal (Hibbard, University of Missouri, 2019, pers. comm.; Huynh et al. 2018).

Stunting was qualitatively assessed as an indicator for sublethal effects in comparison to the size and form of larvae in the untreated control. Feeding was assessed through observing food remains, frass, and diet in the larval gut to assure that diet and a treatment had been ingested. The coefficient of variation (CV) was determined in each bioassay as a measure of data precisions. A CV should ideally be < 0.2, and at a CV of > 2 further bioassays would be needed (Dulmage et al. 1990). In our experiments, the CVs of 1.2 for bioassay 1, 0.4 for bioassay 2, 0.7 for bioassay 3, and 0.8 for all bioassays, indicated good quality of data (Additional file 1: Table).

Larval data were compared between treatments within each experiment using Chi-Square statistics (because of nominal data type) with an fdr-correction of p-values (Benjamini and Hochberg 1995). To allow across-experiments comparisons, data were standardised to the untreated control data. Distributions of data were investigated using histograms, normal and detrended normal probability Q–Q plots and one-sample

Kolmogorov–Smirnov test (Kinnear and Gray 2000). Equality of variances was assessed using Levene's test. When data appeared normal distributed, influences of treatments were analysed through unifactorial Generalized Linear Model (GLM) and multiple comparisons were applied using Tukey HSD post hoc comparison of data of equal variances and Games Howell post hoc comparison for unequal variances. Logistic regression analyses were applied to assess the dose response of each treatment including lethal dose leading to 50% or 90% mortality ($LD_{50/90}$) (R package MASS) and McFadden pseudo R-square values (package DescTools, R Development Core Team 2020).

Potted-plant greenhouse experiments

Experimental setup

To assess the efficacy of azadirachtin granules against *D. v. virgifera* larvae under semi-natural conditions, three systematic controlled trials were conducted using infested potted—maize plants in a greenhouse. As positive control served tefluthrin fine granules, cypermethrin microgranules and thiamethoxam—seed coating (Table 2). As negative controls served untreated infested plants as well as untreated uninfested plants. Each treatment was applied into the soil of three to four systematically arranged blocks (=replicates) of five pots. This totalled 15 to 20 data points (=sample size) per treatment per experiment.

In detail, each pot (plastic garden pot, 15 cm inner diameter × 10 cm height, 2 l) was first filled with 1 l sterilised soil. Two maize seeds were added (hybrid Szegedi 386, GK Hungary in experiment 1 and 3, or Futurixx, RAGT, France in experiment 2). Thereafter, 200 ml water were applied to each pot. Treatments were applied either as granules along a 2 cm wide strip across the 10 cm diameter of the pots, or as seed coating. Finally, 1/2 l soil was added burying the treatment and seed 3 cm into the soil leading to a soil surface of 14 cm diameter. The used soil contained 77% sand, 8% loam, 15% clay, 2.8% humus, 1.7% $CaCO_3$, 0.1% salts, and had a Ph of 7.7 (analysed by Szolnoki Talajvedelmi Laboratorium, Hungary). It had a soil bulk density 0.9 to 1.1 g cm^{-3} and a 7 to 11% soil moisture (w%=grav.%). An average temperature of 20±5 °C and a relative humidity of 97±3% were recorded 5 cm deep in the soil in the pots as well as 24±4 °C and 44±13% in the air 1 m above the pots using climate data loggers (PeakTech 5185 data logger, Germany). Plants germinated between 4 and 12 days after sowing.

Maize pots were infested with 50 viable ready-to-hatch eggs per plant in experiment 1 and 3 or with 100 eggs in experiment 2. At this point in time, the majority of plants was at 3 leaf stage (height 15 to 20 cm). The eggs were applied in 0.15 to 0.2% aqueous agar with a standard pipette (1 to 5 ml, Eppendorf AG, Germany) in half-portions into two 50 mm deep holes 20 to 30 mm distant from both sides of the plant. A portion of eggs was incubated on moist filter paper at 20 °C in the laboratory to estimate emergence patterns (5 dishes with 10 to 20 eggs each/experiment). They revealed an emergence start 9±5 days after placement. Hatching duration was 10±4 days. Hatching rate was 47±30, 47±23% and 56±19, leading to 24 hatched larvae per pot in experiment 1, 47 larvae in experiment 2, and 28 larvae in experiment 3, respectively. This indicates a medium, but consistent egg quality across experiments, and is comparable to similar studies of Xie et al. (1991).

Data assessments and analyses

Selectivity of the test agents was assessed by recording germination rate, plant phenology and phytotoxicity. Leaf number, plant height and the BBCH growth stage were assessed weekly as well as phytotoxicity according to Anonymous (2009).

At the expected second and early third instar stage, numbers of surviving larvae, root damage and above-ground biomass were assessed. This was 52±18 days after planting and treatment, thus 40±7 days after infestation. Each maize plant was pulled out of the soil, and gently shaken to remove loosely adhering soil particles from roots. Each maize plant was cut 1 cm above roots, and fresh weight, leaf number and plant height were measured. Then, the soil and root of each pot was placed onto a plastic screen for drying out and letting surviving larvae exit and drop onto the wet tissue paper in a tray below following a Berlese approach (Dent and Walton 1998). Larvae and their instars were counted 1, 3, 5 and 7 days later.

The untreated control was aimed to have a minimum level of infestation with 2nd or 3rd instar larvae of 20% to validate the results on agent efficacies. In all experiments, more than 90% of pots of the infested untreated second control yielded larvae. The infested control lead to 6±5 s or third instar larvae/100 applied eggs.

One day after Berlese-placement, the dried roots were removed, gently shaken to remove remaining soil, soaked in water for 5 min, and then washed in 1% NaOCl and then water for 1 min to allow the assessment of root damage. Damage was rated using two scales recommended by EPPO (Anonymous 1999); this is, (1) the non-linear 1.0 to 6.0 traditional IOWA scale (Hills and Peters 1971) which slightly overestimates minor damage; and (2) the linear 0.00 to 3.00 node injury scale (Oleson et al. 2005) which measures only destroyed roots and therefore misses minor damage. To avoid subjective bias on these ratings, root damage was estimated independently by the experimenters, neither of whom knew whether the roots were from a treated or untreated pot.

Distributions of data were investigated using histograms as well as normal and detrended normal probability Q–Q plots (Kinnear and Gray 2000). Equality of variances was assessed using Levene's test. Influences of treatments on assessed factors were analysed through GLM analyses or through independent samples Kruskal–Wallis H test. Tukey HSD post hoc multiple comparison tests were applied following GLM in case of equal variances, and Games Howell test in case of unequal variances. Logistic regression analyses were applied to assess the dose response of each treatment including the effective dose leading to 50% suppression of the larval populations or root damage prevention (ED_{50}) The mean corrected efficacy of each treatment was calculated relative to the untreated control, this is *corrected efficacy* % = 100 × (larvae or damage in control plots − larvae or damage in treated plots)/maximum (larvae or damage in control or treated plots) (Toth et al. 2020). As the 1.0 to 6.0 IOWA root damage scale is a non-linear ordinal scale, and a value of 1 equals no damage, the damage data were converted to a 0.0 to 5.0 scale to estimate percent damage prevention across experiments. Results from azadirachtin treatments were validated in relation to the results from the corresponding positive controls of standard insecticides.

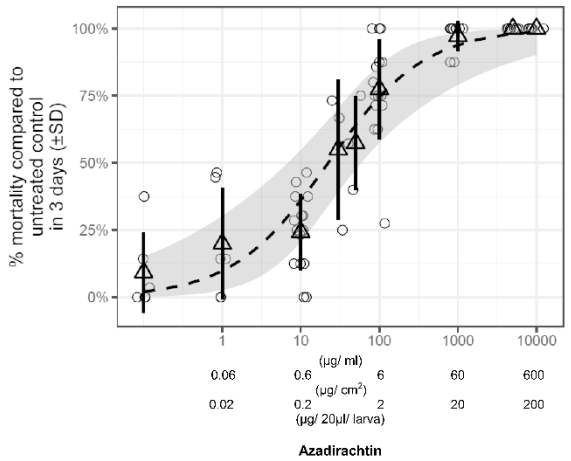

Fig. 1 Dose-efficacy response of azadirachtin in killing neonates of *Diabrotica v. virgifera* in artificial diet-based bioassays of 96-well plates under semi-sterile laboratory conditions. Corrected mortality shown, i.e. standardised for natural background mortality in the untreated control. Fluid neem product used (NeemAzal-T/S 10EC). 3 experiments as true replicates with Δ representing the mean values. 8 wells per treatment per each of 6 plates per each experiment. Trendline added of fitted logistic regression model: larval mortality (3d) = 1/(1 + exp(2.22 − 0.71 * ln(dose)) with confidence interval of the fitted line in grey

Results

Control of neonates in artificial-diet based laboratory bioassays

Azadirachtin appeared toxic to neonates of *D. v. virigifera* larvae (Figs. 1, 2, Additional file 1: Table). A clear dose-mortality response was observed. The corresponding fit of a logistic regression 3 days after treatment was: *larval mortality (3d) = 1/(1 + exp(2.22 − 0.71 * ln(dose))* (Chi-square test for *ln* of dose: $p < 0.0001$, df = 67; McFadden pseudo $R^2 = 0.70$, Fig. 1). Accordingly, the 3-day LD_{50} of azadirachtin was estimated 22.3 µg active ingredient (a.i.)/ml (CI $_{95\%}$ 8.8–56 µg a.i./ml). This corresponds to 1.34 µg a.i./cm^2 treated surface, and to 0.45 µg a.i./20 µl/larva. The 3-day LD_{90} was 480 µg a.i./ml (CI $_{95\%}$ 92–2742 µg).

The dose–response did not change much from day 3 until day 5. The 5-day LD_{50} was 19.3 µg a.i./ml (CI $_{95\%}$: 7.3–51.2 µg) according to the logistic regression fit: *larval mortality (5d) = 1/(1 + exp(1.99 − 0.67 * ln(dose))* (Chi-square test for *ln* of dose: $p < 0.0001$, McFadden pseudo $R^2 = 0.68$). This corresponds to 1.16 µg a.i./cm^2 treated surface, and to 0.39 µg a.i./20 µl/larva. The 5-day LD_{90} was 502 µg a.i./ml (CI $_{95\%}$ 94–2746 µg).

No sublethal effects of azadirachtin such as stunting of larvae were observed (ANOVA for logarithmic model: $F_{1;41} = 1$, $p = 0.31$, adjusted $R^2 = 0.001$).

Control of larvae and prevention of root damage in potted -plant greenhouse experiments

Azadirachtin treatments at increasing dose reduced the larval survival on the maize roots (relative to control, GLM, $F_{5;30} = 17.6$, $p < 0.0001$, adjusted $R^2 = 0.73$).

Multiple comparison tests revealed efficient control of *D. virgifera* larvae on maize roots by standard doses of tefluthrin and thiamethoxam, but not by standard doses of cypermethrin and azadirachtin (Figs. 3, 4). When increasing doses of azadirachtin, control of larvae became evident. Between 25 and 67% of larvae were killed by a 5× standard-dose (200 g a.i./ha), and 100% control efficacy was reached at a 10× standard-dose (380 g a.i./ha). Doubling the standard dose of cypermethrin did not improve its efficacy in reducing larvae numbers.

The corresponding logistic regression fit of efficacy to different doses of azadirachtin was: *efficacy in reducing larvae (%) = 1/(1 + exp(2.203 − 1.14 * ln(dose)* (Chi-square test for ln of dose: $p = 0.0027$, df = 19; McFadden pseudo $R^2 = 0.43$, Fig. 3). Accordingly, the ED_{50} of azadirachtin was 6.9 mg active ingredient/meter of maize furrow (CI $_{95\%}$ 2.6–18.5 mg). This corresponds to approximately 92 g azadirachtin/hectare. The ED_{90} was 47.7 mg active ingredient/meter of maize (CI $_{95\%}$ 5.2–430 mg).

Reduction of larvae through treatments was only partly reflected in the level of prevention in root damage (Figs. 5, 6). Azadirachtin treatments at increasing dose

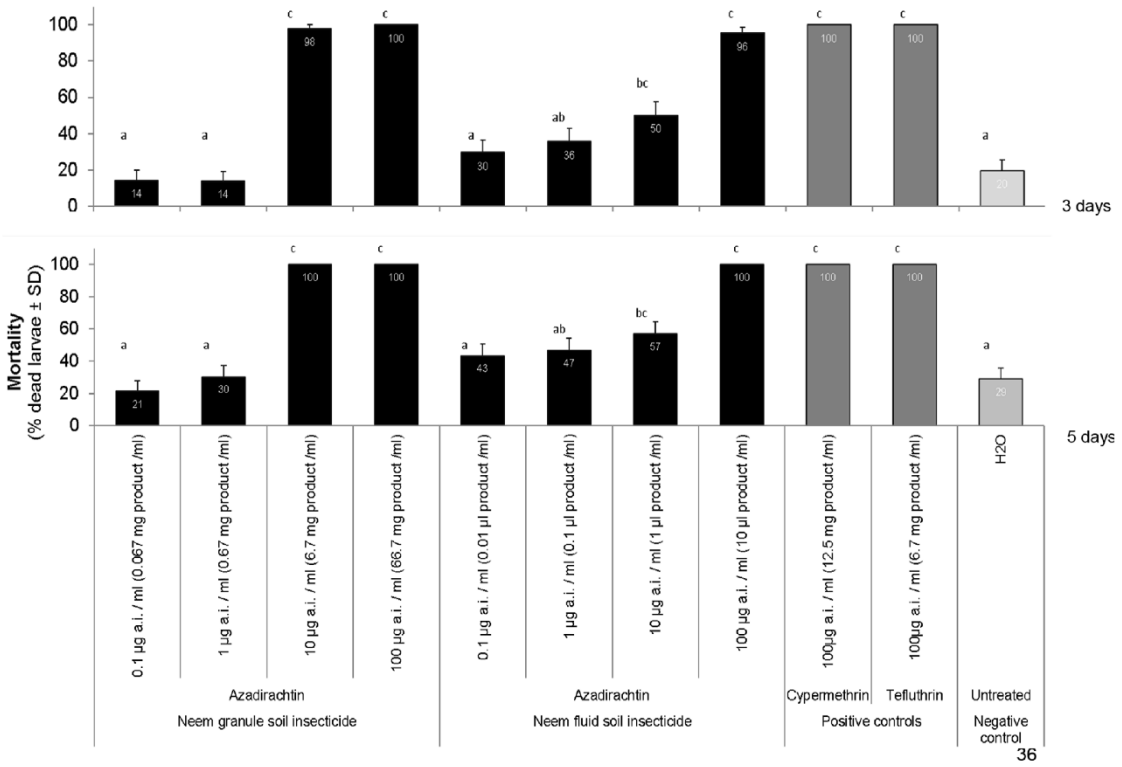

Fig. 2 Comparison of two different azadirachtin formulations and products in their dose effects at killing neonates of *Diabrotica v. virgifera* in artificial diet-based bioassays of 96-well plates under standardised semi-sterile laboratory conditions. 8 wells per treatment per each of 6 plates. Letter above bars indicate significant difference at $p < 0.05$ according to Games-Howell post hoc multiple comparison test

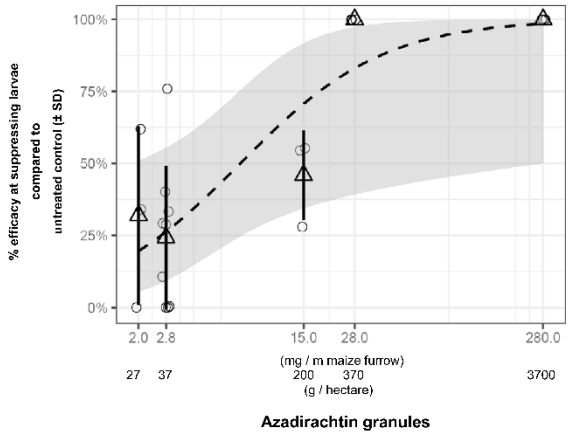

Fig. 3 Dose-efficacy response of azadirachtin granules in reducing larvae of *Diabrotica v. virgifera* in three potted-maize plant trials under greenhouse conditions in Hungary in 2020. Granular neem product for soil applications used (Neem Azaal 0.15G). Corrected efficacy shown, i.e. standardised to the untreated control data. Plant roots artificially infested with 50 to 100 eggs. Trendline added of fitted logistic regression model, with confidence interval of fitted line in grey

improved the level of prevention in root damage (relative to control, GLM, $F_{5;30} = 29.5$ for general root damage, 22.5 for heavy root damage, adjusted $R^2 = 0.83$ and 0.78; $p < 0.0001$).

Multiple comparison tests revealed that standard dose of tefluthrin and thiamethoxam consistently prevented the overall as well as heavy root damage (Fig. 4). The standard dose of cypermethrin only inconsistently prevented some of the root damage and the standard doses of azadirachtin was usually not sufficient. When increasing the doses of azadirachtin, prevention of root damage became evident. About 40% of the overall root damage and 67% of the heavy root damage were prevented by a 5× standard-dose (200 g a.i./ha). Root damage was nearly entirely prevented by a 10×-standard-dose (380 g a.i./ha).

The corresponding logistic regression fit of root damage prevention to different doses of azadirachtin was: *efficacy in preventing general root damage (%) = 1/(1 + e xp(2.39 − 0.85 * ln(dose))* (Chi-square test for *ln* of dose: $p = 0.0075$, $df = 19$; McFadden pseudo $R^2 = 0.55$, Fig. 3). Accordingly, the ED_{50} of azadirachtin was 16.5 mg active ingredient/meter of maize furrow ($CI_{95\%}$ 4.2–65.9 mg). This corresponds to approximately 220 g azadirachtin/

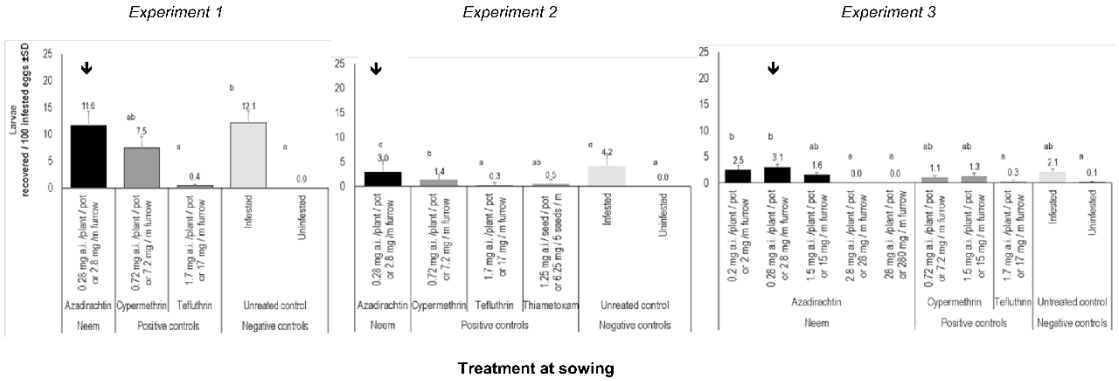

Fig. 4 Effects azadirachtin granules and standard insecticides in reducing larvae of *Diabrotica v. virgifera* in three potted-maize plant trials under greenhouse conditions in Hungary in 2020. ⬇ is the currently proposed standard dose of the granular azadirachtin test product for soil applications (Neem Azaal 0.15G). Experiment 1 and 3 with 5 plants per each of 3 blocks totalling 15 plants per treatment, experiments 2 with 4 such blocks totalling 20 plants per treatment. Plant roots artificially infested with 50 to 100 eggs and data standardised to 100 eggs. Games-Howell post hoc multiple comparison test at $p < 0.05$.

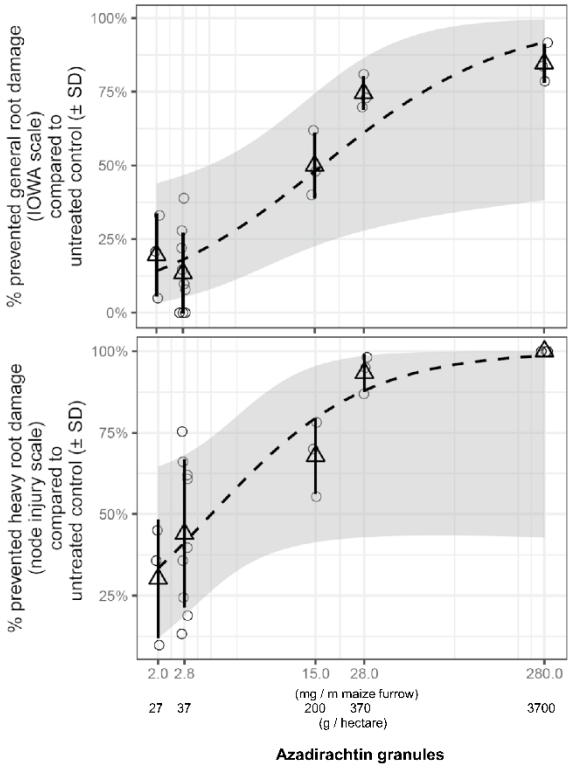

Fig. 5 Dose-efficacy response of azadirachtin granules in preventing root damage caused by *Diabrotica v. virgifera* in three potted -maize plant trials under greenhouse conditions in Hungary in 2020. Granular neem product for soil applications used (Neem Azaal 0.15G). General root damage assessed through 1.0 to 6.0 traditional IOWA scale (Hills and Peters 1971) and heavy root damage through 0.00 to 3.00 node- injury scale (Oleson et al. 2005). Corrected efficacy shown, i.e. standardised to the untreated control data. Plant roots artificially infested with 50 to 100 eggs. Trendline added of fitted logistic regression model with confidence interval of fitted line in grey

hectare. The ED_{90} was 218 mg a.i. /meter of maize furrow (CI $_{95\%}$ 9.–5277 mg).

The logistic regression fit of preventing heavy damage to different doses was: *efficacy in preventing heavy root damage (%) = 1/(1 + exp(1.397 -1.016* ln(dose))* (Chi-square test for *ln* of dose: $p = 0.011$, $df = 19$; McFadden pseudo $R^2 = 0.39$, Fig. 3). The corresponding ED_{50} of azadirachtin was 3.96 mg active ingredient/meter of maize furrow according to the logistic model fit (CI $_{95\%}$ 1.4–10.8 mg). This corresponds to approximately 52 g a.i./ha. The ED_{90} was 34.4 mg active ingredient/meter maize furrow (CI 95% 3–392 mg).

Preventing root damage through certain treatments was only little reflected in yield-related parameters. When differences were found between treatments and the untreated control, then their absolute differences were small.

Whilst the standard dose of azadirachtin and up to 2.8 mg a.i./plant/pot (28 mg/m furrow) did not improve biomass of 6 to 10 leaf stage maize, a high dose of 28 mg azadirachtin (280 mg/m) improved biomass (8.3 ± 2.7 g versus 3.9 ± 2 of infested control plants, GLM, $F_{5;30} = 16$, adjusted $R^2 = 0.72$; $p < 0.0001$). The standard dose of tefluthrin also improved biomass in one of three experiments, but no such improvements were detected by cypermethrin or thiamethoxam.

Whilst the standard dose of azadirachtin and up to 1.5 mg a.i./plant/pot (15 mg/m furrow) did not improve height of maize, a high dose of 2.8 mg azadirachtin (28 mg/m) or even 28 mg (280 mg) increased plant height (49 ± 11 cm or 50 ± 11 cm versus 36 ± 15 cm of infested control plants, GLM, $F_{5;30} = 3.9$, adjusted $R^2 = 0.33$; $p = 0.009$). The standard doses of tefluthrin,

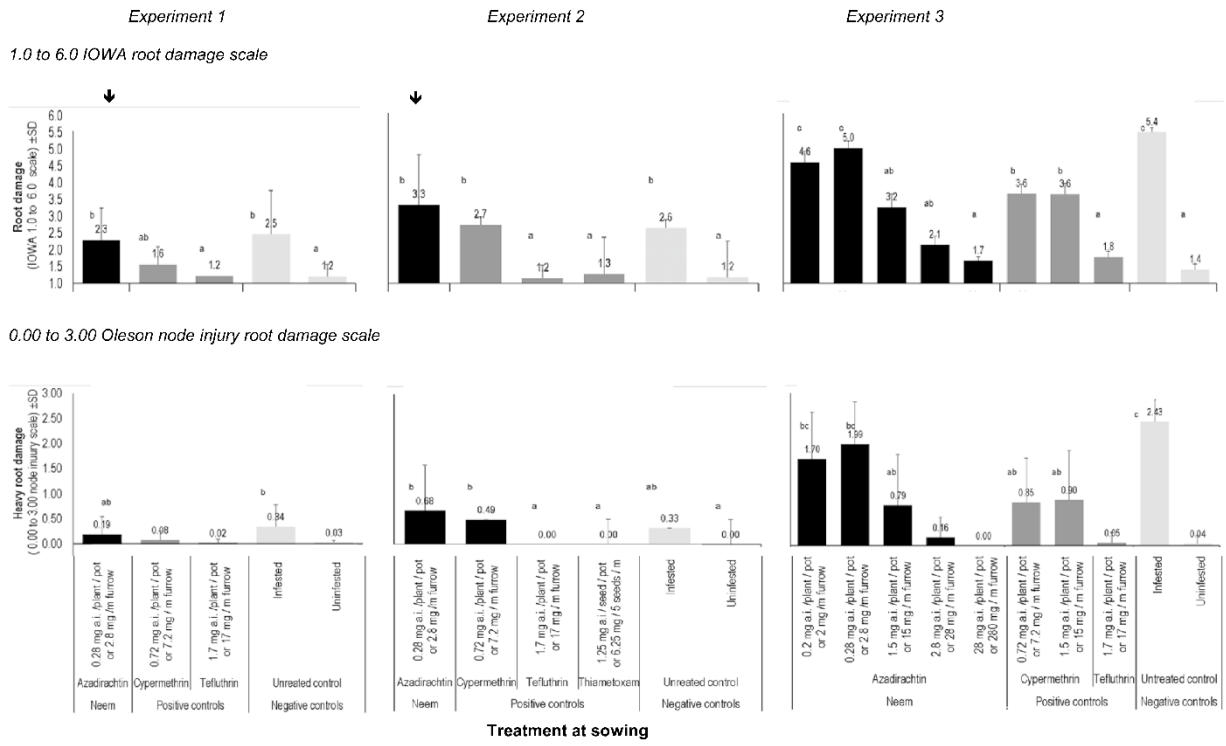

Fig. 6 Effects of azadirachtin granules and standard insecticides in preventing root damage caused by *Diabrotica v. virgifera* in three potted -maize plant trials under greenhouse conditions in Hungary in 2020. ↓ is the current standard dose of the granular azadirachtin test product for soil applications (Neem Azaal 0.15G). General root damage assessed through 1.0 to 6.0 traditional IOWA scale (Hills and Peters 1971) and heavy root damage through 0.00 to 3.00 node-injury scale (Oleson et al. 2005). Experiment 1 and 3 with 5 plants per each of 3 blocks totalling 15 plants per treatment, experiments 2 with 4 such blocks totalling 20 plants per treatment. Plant roots artificially infested with 50 to 100 eggs and data standardised to 100 eggs. Games-Howell post hoc multiple comparison test at $p < 0.05$

cypermethrin or thiamethoxam did not affect plant height in none of the experiments.

None of the treatments and applied doses did increase average leaf numbers of 6 to 10 leaf stage maize, except of a small positive effect of thiamethoxam in experiment 2 (9.3 ± 0.9 versus 8.5 ± 0.7 leaves of infested control plants).

Treatments did not affect germination rates (GLM, $F_{12;76} = 1.25$; $p = 0.27$) and did not lead to any delay in germination ($F = 0.8$; $p = 0.58$) regardless of azadirachtin or synthetic pesticides and regardless of the different doses being applied. Those treatments also did not cause any phytotoxic effects such as yellowing, chlorosis, necrosis or deformation of leaves or stunting of plants.

Discussion

Our sets of laboratory-bioassays as well as potted-plant experiments confirmed Xie et al. (1991) that the neem plant-derived azadirachtin is toxic to larvae of *Diabrotica v. virgifera,* one of the key pests among rootworms. This is not surprising as azadirachtins are of broad-spectrum activity (Dougoud et al. 2019). Positively, an application of this botanical insecticide as a granule into the sowing furrow can lead to a suppression of the later hatching larvae and to a significant prevention of root damage. Granule applications at sowing are preferred by many maize growers over fluid applications or applications later in the maize growing season (Toepfer et al. 2010). This should be of high interest to industry because of recent bans on the use of a number of soil insecticides and insecticidal seed-coatings due of their either high human toxicity and/or serious non-target effects or other environmental concerns (World Health Organization 2009; European-Commission 2011; Georgiadis et al. 2011).

Consequently, growers in numerous countries are left with few or no management options for soil insect pests in field crops. However, soil insects, such as corn rootworms (*Diabrotica* spp.), cutworms (*Agrotis* spp.), wireworms (*Agriotes* spp.) or grubs (Melolonthidae) account for a large proportion of below-ground damage to maize, and the latter two pest groups also to a number of other crops (Toepfer et al. 2014). In Hungary for example, 46% of all the 5000 tons of insecticides sold in 2019 were the granular soil insecticide tefluthrin (Demeter and Lazar

2020). This indicates the high importance of such insecticides for the control of soil insect. However, tefluthrin will probably be retreated from the pesticide markets due to its high toxicity being classified as a WHO-class Ib acutely hazardous ingredient (World Health Organization 2009). Similarly, neonicotinoids have already been phased-out in many countries due to their pollinator toxicity, accumulation in the soil and other environmental effects (Georgiadis et al. 2011). Agri-policies try to address the public concerns with regard to pesticides and try to promote alternative and safer pest management solutions. Examples of such attempts aare the directive on "Sustainable Pesticide Use" of the European Union (European Commission 2009) or the "Green Pest Control Policy" of China (Fan 2006; MoA 2011). However, such policies do not necessarily lead to new pest control options, as the discovery and development of novel plant protection agents with new modes of action and low enviormental impact is difficult and costly.

We have therefore investigated whether the safe and easily biodegradable botanical insecticide azadirachtin (Boeke et al. 2004), which is widely used against aboveground insects (Saxena 1989), might become an option for the control of soil insect pests. First, we confirmed through laboratory bioassays that the azadirachtin*s* in the here-tested granular formulation were similarly effective as the ones in commonly used fluid formulations. This was necessary because neem products can be variable in their contents of active ingredient(s) as well as in their efficacy for pest control (Stark and Walter 1995; Dougoud et al. 2019). Therefore, the provider of the neem granules had run gas chromatographic analyses prior our experimentation confirming 0.165% azadirachtin as labelled (mainly A with some B) as well as 0.35% other neem compounds (Aza-F, Aza-H, Aza-I, salanin, nimbin and fatty acids). This is important, because in many neem products, the ingredients are not well assessed and/or declared on the product label (Dougoud et al. 2019). In our bioassays, the dose–response curves of azadirachtin from the granular formulation did not differ from those in common fluid formulations (Fig. 2), indicating a good quality of the test products and correctness of label information.

Our artificial diet-based bioassays on neonates of *D. v. virgifera* larvae revealed a 3-day LD_{50} of 22.3 µg azadirachtin/ml which corresponds to 0.45 µg/neonate. Azadirachtin appeared of relatively fast mode of action on *D. v. virgifera* as the dose–response did not change much from day 3 to day 5. The 5-day LD_{50} was 19.3 µg/ml which corresponds to 0.39 µg/neonate up to early second instar larva. Also, Xie et al. (1991); Stark and Rangus (1994) and a number of other authors suggested that azadirachtin has contact activity on insects in addition to its widely reported systemic and chronic modes of action. There are a number of LD_{50} reported for immature stages of several insect groups, such as 77 µg/ml for first instar *Ectomyelois* spp. (Lepidoptera) over 5 days and 438 µg/ml over 1 day (Mehaoua et al. 2013), 7.6 to 7.7 µg/ml for fourth instars of two *Culex* spp. (Diptera: Culicidae) with not-reported exposure time (Merabti et al. 2017), and 2.8 µg/ml for young *Aphis* spp. (Hemiptera: Aphididae) over 7 days (Stark and Rangus 1994). Ladd et al. (1984) reported a low LD_{50} of only 0.1 µg of topically applied azadirachtin per third instar *Popillia japonica* (Coleoptera: Scarabaeidae), but over an exposure of 20 days. This suggests that our reported LD_{50} by 0.45 µg within 3 days or 0.39 µg within 5 days for the much smaller neonates of *D. v. virgifera* would be much lower when assessing mortality over longer periods. In general, it appears difficult to compare LD_{50} across studies and insect species due to different experimental setups particularly the involved insect food, exposure periods, different insect weights, and sometimes unclear compositions of azadirachtins and related compounds in the test agents. Despite the often reported LD_{50} as µg/ml, some of those studies do not report the amount of azadirachtin applied per larva or per insect weight. For example, Xie et al. (1991) reported a 3-day LD_{50} of only 3.9 (2.5 to 5.9) µg azadirachtin/ml when applying 1.7 ml of the 3.9 µg/ml-solution onto filter paper in a Petri dish with 10 neonates of *D. v. virgifera* and a maize seedling, thus effectively 6.6 µg. The reported effective dose seems low compared to our data, and reasons are difficult to explain. On one hand, the experimental arrangements of Xie et al. (1991) with filter paper assays in Petri dishes differs to our approach of exposing one larva to azadirachtin on artificial diet, on the other hand their sample size was low (5 Petri dishes only). Qadri and Narsaiah (1978) reported a 1-day LD_{50} of 1.5 mg azadirachtin/gram body mass of older nymphs of *Periplaneta* spp. (Blattodea), but there is no such information for other *Diabroticina*. Our tested *D. v. virgifera* larvae weighted about 0.42 ± 0.23 mg (across neonates and young second instars). This would roughly correspond to an LD_{50} of 0.9 to 1.1 mg azadirachtin/gram *D. v. virgifera* larva, being comparable with the LD_{50} on *Periplaneta*. Astonishingly, we did not detect any sublethal effects of azadirachtin to *D. v. virgifera* larvae such as stunting or moulting inhibition. Some authors reported growth inhibitor and deformation effects to insects, such as to *Culex* larvae (Al-Sharook et al. 1991) or *Aphis* nymphs (Stark and Rangus 1994). Landis and Gould (1989) reported anti-feeding effects to larvae of *Diabrotica undecimpunctata howardi*. Although, in our bioassays some larvae of *D. v. virgifera* moulted to 2nd instar suggesting no inhibitor effects, our assay duration of 5 days might not have been long enough to detect all

possible sublethal effects. Ladd et al. (1984), for example, detected insect growth regulator effects on *Popillia* grubs only within about 20 days after treatment. As for rootworms, however, longer assays are rarely achievable as the known artificial insect diets are suboptimal and contamination rapidly occurs due the non-sterile nature of the available diets (Hibbard, University of Missouri, 2019, pers. comm.; Huynh et al. 2019). Nevertheless, the here-reported LD_{50} levels of azadirachtin of *D. v. virgifera* seem in line with lethal effects to other insect groups, and therefore warranted further investigation.

In a second step, we simulated the efficacy and feasibility of an azadirachtin-based granule application into the soil for corn rootworm control using potted-plant experiments in comparison to standard pesticides. Results showed that standard doses of thiamethoxam-seed coating and tefluthrin-granular soil insecticides applied at maize sowing can well suppress larval populations of *D. v. virgifera* and prevent most root damage (Pilz et al. 2009; Rozen and Ester 2010; Modic et al. 2018; Souza et al. 2020). In contrast, a cypermethrin granular soil insecticide applied at its standard dose at sowing appeared much less effective, and even doubling its dose did not improve efficacy. This confirms variable experiences with cypermethrin-based soil insecticides for rootworm control in field studies (Toth et al. 2020). Unfortunately, also the currently proposed standard dose of 38 g azadirachtin/hectare for granular in-furrow application at sowing appeared not enough to control *D. v. virgifera* or to prevent root damage. However, when increasing doses of azadirachtin, control of larvae and prevention of root damage became evident. At a 5× standard-dose of azadirachtin (200 g/ha), 25 to 67% percent of larvae were killed, about 40% of overall root damage prevented as well as about 67% of heavy root damage. This, as well as the modelled ED_{50} are comparable to or better than the efficacy of most cypermethrin applications. At a 10× standard-dose azadirachtin (380 g/ha), total pest control was achieved as well as the prevention of most root damage. Whether this would be economically feasible is not yet clear. However, such a control efficacy is even better than the efficacies of most applications of tefluthrin granules and comparable to the efficacy of thiamethoxam seed-coatings. This confirms Xie et al. (1991) who applied high doses of azadirachtin as a drench into the sowing furrow in potted-plant trials. This also indicates that both fluid and granular applications into the furrow at maize sowing would lead to control efficacies. As into-furrow applications of agents in maize seem to have little impact on non-targets (limited area treated and below ground) (Babendreier et al. 2015), it is also unlikely that applications of higher concentrations of azadirachtin are environmentally problematic (Boeke et al. 2004). This is also underlined by the biodegradable nature of azadirachtin. Interestingly our experiments showed a slight positive effect of higher doses of neem-granules on plant height and biomass in comparison to similarly effective chemical treatments. Although Xie et al. (1991) did not observe such effects, it may potentially confirm the biofertilizer properties of neem in maize as suggested by Vageesh et al.(2016).

Conclusion

In conclusion, there seems clear potential for the development of a neem-based botanical soil insecticide if the required higher concentrations of azadirachtin in the granule, or potentially fluid formulation can be achieved. The currently suggested standard dosage of 38 g azadirachtin/hectare corresponds to 25 kg granules/hectare. Many commercial applicators for fine granules on sowing machines may deliver not more than 20 kg hectare. Therefore, a higher concentration of azadirachtin in less weight of granules would be needed if the application for rootworm control at larger field scale was to become reality. In a next step, larger open field trials using farmer machinery are suggested towards the development of a practical and effective neem-based soil insecticide for corn rootworm control in maize. Those, experiments should clarify whether for example 50, 200, 380 g or more azadirachtin would be needed/hectare under real farming conditions to suppress *D. v. virgifera* larval populations below threshold and to sufficiently prevent root damage to avoid yield losses. If this is achieved, such product(s) may become a promising, safer alternative in the management of rootworms such as *D. v. virgifera* and potentially other soil insect pests. This could become a replacement of some of the banned soil insecticides or insecticidal seed coatings, and will ultimately help diversify the currently limited integrated pest management toolbox.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43170-021-00044-9.

Additional file 1. Dose-efficacy response of azadirachtin in killing neonates of *Diabrotica v. virgifera* in artificial diet-based bioassays of 96-well plates under standardised semi-sterile laboratory conditions.

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5.5 References

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Authors' contributions

All authors have substantially contributed to conception and design, analysis and interpretation of data, as well as to drafting and revising the article. All approved the final version to be published and agreed to be accountable for all aspects of the work. In addition to above, SzT and ST implemented the experiments and collected the data, MSz and ST conducted the statistical analyses, and ST supervised the study and finalised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Meta-data are available in an additional file to the publication. Raw data are deposited in Zenodo (https://doi.org/10.5281/zenodo.4318642). Any other information is available upon request to the corresponding author.

Declarations

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants or vertebrates performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Consent for publication

All authors agree with this publication.

Competing interests

The authors declared no conflicts of interest.

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Publisher's Note

6. Chapter IV.

On understanding and manipulating the hatching patterns of *Diabrotica virgifera virgifera* eggs to improve design of experiments

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6.1 Abstract and Introduction

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On understanding and manipulating the hatching patterns of *Diabrotica virgifera virgifera* eggs to improve the design of experiments

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Key words: Western corn rootworm, egg development, bioassay, pest control, experimental design, invasive species, Coleoptera, Chrysomelidae, *Diabrotica virgifera virgifera*, maize, *Zea mays*, Poaceae

Abstract

Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae) is a well-studied pest of maize (*Zea mays* L., Poaceae) in North America and Europe. Many studies on its biology, behaviour, or management rely on individuals reared from either field-collected insects or laboratory colonies. Naturally, *D. v. virgifera* eggs require an obligate 8–10-month diapause, which can cause technical challenges such as a deceleration of research activities. To allow better planning of experimentation, we have investigated the survival and temporal hatching patterns of the pest's eggs depending on diapause length as well as post-diapause incubation temperature. Laboratory assays revealed that the highest hatching rates and most synchronized hatching times in a wild diapausing population occurred when eggs were overwintered at the natural diapause length (8–10 months) or shorter (5–7 months) and then incubated at 20–24 °C. Eggs diapaused for only 2 months showed comparably good hatching rates, but hatching patterns appeared more variable. Diapause of <2 or >10 months reduced hatching success, as did low (16 °C) incubation temperatures. For comparison, a well-studied non-diapausing laboratory colony was assessed as well. Data matrices on egg overwintering survival, the start, peak, duration, and end of egg hatching, as well as hatching rates are provided for various diapause lengths and three incubation temperatures for both populations. This information will support scientists in choosing a *D. v. virgifera* colony that fits best their experimental set-up and study conditions, as well as in optimally planning such studies.

Introduction

The Western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is an univoltine leaf beetle (Chiang, 1973; Krysan & Branson, 1983). Its eggs hatch after an obligate diapause at the beginning of the cropping season. The larvae feed nearly exclusively on the roots of maize, *Zea mays* L. (Poaceae) (Branson & Krysan, 1981; Moeser & Hibbard, 2005). This root damage can cause plant lodging, plant growth reduction, and eventually yield losses (Levine & Oloumi-Sadeghi, 1991; Godfrey et al., 1993).

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The pest is hypothesized to have evolved together with maize in Central America or Mexico. It invaded the USA and Canada in the second half of the last century (Gray et al., 2009), and Europe in the 1980s (Miller et al., 2005; Szalai et al., 2011). On both continents, it is causing billions USD or EUR of annual pest management costs (Rice, 2004; Wesseler & Fall, 2010). However, due to those investments, the pest is relatively well managed and large-scale crop losses are relatively rare (Bažok et al., 2021).

The insects naturally overwinter as eggs in the soil under obligate diapause, for 8–10 months depending on the maize growing region (Ball, 1957). This diapause is influenced by environmental factors such as pre-diapause temperatures, overwintering temperatures, post-diapause temperatures, and the time spent in diapause, as well as by genetic and hormonal elements (Denlinger et al., 2012; Gotthard & Wheat, 2019; Kozak et al., 2019). However,

6.2 Materials and Methods

studies of this insect often demand a continuous supply of test individuals, particularly for laboratory or semi-field experiments. A solution for this problem came after Branson (1976a) artificially selected a colony of *D. v. virgifera* which does not require a diapausing phase. Nowadays, several of such laboratory colonies exist. Well-established rearing protocols allow the continuous rearing of those insects and subsequently the continuous availability of individuals for experimentation. A similar approach was followed to obtain non-diapause colonies of other insects such as *Drosophila montana* Stone, Griffen & Patterson (Kankare et al., 2016), *Pieris rapae* (L.) (Park & Kim, 1989), *Ostrinia nubilalis* Hübner (Nordin et al., 1984), and *Delia radicum* (L.) (Kostal & Simek, 1995). However, some argue that such colonies have adapted to laboratory conditions and may no longer well reflect wild populations (Li et al., 2009, 2014).

Therefore, a number of researchers prefer to work with individuals from field-collected *D. v. virgifera* or recently established colonies of wild and diapausing populations. Consequently, egg diapause and development as well as hatching dynamics have been relatively well studied (Branson, 1976b, 1978; Krysan, 1982; Schaafsma et al., 1991). Numerous studies describe specific aspects, but there is no clear overview of egg overwintering and hatching dynamics. For example, diapause length seems to influence some parameters of egg survival and hatching, but others less so. On the one hand, *D. v. virgifera* eggs collected from various locations in South Dakota, USA, have no hatching problems when undergoing no, or short periods of up to 12 weeks, cooled storage at 5 °C (58–68% hatching success) (Branson, 1976b). Also, Krysan (1982) observed comparable hatching rates when storing eggs at 7 °C for 0, 2, 4, or 6 weeks. On the other hand, Krysan (1982) reported a delay in hatching as a result of a lack of diapause; this is, a hatching start of up to 31 days compared with 19–20 days after a 2.7-month diapause. Similarly, Branson (1976b) reported a 14–15 day period after 2.7 months of diapause, up to 60 days when no diapause had been initiated. The opposite, a too long diapause, also seems problematic. Branson (1976b, 1978) reported that 1 year of cooled storage of eggs causes a loss in viability of up to 80%. In conclusion, egg diapause length as well as temperature seems to influence *D. v. virgifera* egg hatching. This suggests egg development processes take place during cool diapause. Despite all these studies, it remains difficult for researchers to precisely predict the egg hatching success and patterns for *D. v. virgifera* populations for various egg storage conditions.

After diapause, temperature plays a role in post-diapause egg development and therefore in defining egg hatching patterns. Eggs need a certain amount of degree days above a base temperature to develop, that is, 9–11 °C for *D. v. virgifera* (Schaafsma et al., 1991). Again, there are numerous, but scattered, studies. For example, only 23% of eggs hatched when incubated at a low temperature (12 °C) and 12% at a high temperature (32 °C) (after diapause at 7.5 °C for 2 months) compared to 52% at 24 °C (Schaafsma et al., 1991). Wilstermann & Vidal (2013) reported only 14% hatching at 14 °C post-diapause incubation temperature. Wilde (1971) reported that eggs incubated at 16 or 20 °C hatched about 1.5–2× slower than eggs incubated at 25 °C. Again, it would be desirable to have a clear overview of all those effects.

As so many factors may influence egg diapause, survival, and hatching patterns, researchers need to understand those processes for better planning of experiments. This is crucial for diapausing *D. v. virgifera* populations and colonies, as they have only one generation per year. Therefore, researchers usually have a stacked stock of diapausing eggs in overwintering conditions and then incubate eggs under higher temperatures when needed. It is, however, often uncertain how long such eggs should remain in diapause, and which post-diapause incubation temperatures are to be chosen for optimal hatching. Obviously, a synchronized hatching would be desirable, with a well-timed and narrow start, a fast-reached peak, and a fast termination of hatching of an egg batch.

To facilitate the planning of experiments with this pest, we have investigated diapause and hatching patterns of *D. v. virgifera* eggs depending on diapause length and post-diapause incubation temperature under controlled laboratory conditions. We hypothesized that diapause length may be shortened to a certain extent without compromises in hatching rates and hatching synchrony, which would allow more frequent experimentation. We also hypothesized that there might be an optimal post-diapause incubation temperature. Eggs from a wild diapausing *D. v. virgifera* population [here the Central South-eastern European (CSE) population] were assessed, as well as from a non-diapausing laboratory colony from USDA ARS for comparative reasons, although this colony has been well-studied (Li et al., 2009, 2014; Bermond et al., 2021). Findings on optimal conditions for egg overwintering survival, synchronized egg hatching patterns, and overall hatching rates will help to better plan experiments in future research.

Materials and methods

Origin and handling of *Diabrotica virgifera virgifera*

Beetles from the wild CSE *D. v. virgifera* population (Miller et al., 2005) were collected from highly infested maize fields in southern Hungary in 2017 (near Mártély, Csongrád-Csanád County) and 2018 (near Kondoros,

6.3 Results

Békés County). Beetles were reared under standardized laboratory conditions at 23–25 °C and 40–60% r.h. according to Jackson (1986) and Branson et al. (1988). The obtained eggs were held for pre-diapause development at around 24 °C during 2–3 weeks. Eggs were then sieved (300 µm mesh) and washed in clean water containing <0.5% NaOCl. Eggs were stored in clean sterile river sand in Petri dishes at 6–8 °C for diapause in dark conditions (see diapause details below). The stored eggs were periodically checked for possible contamination with a Science ETD-201 stereomicroscope (10× magnification; Bresser, Rhede, Germany). In case of fungal or bacterial contamination, sample dishes were excluded. If too dry, a few ml of sterile tap water was sprayed onto the sand to keep the soil moist (15–30% wt/wt). Some eggs were used directly without diapause for the corresponding experiment (see below).

The non-diapause (ND) laboratory colony of *D. v. virgifera* was obtained from USDA-ARS Laboratories (Brookings, SD, USA) where it had been reared for nearly 300 generations (C Nielson, pers. comm.). Those *D. v. virgifera* were reared in our Hungarian laboratories under similar conditions as the wild colony described above, except that no diapause was applied and eggs were directly used for experiments.

Experimental set-up

Egg batches from the wild colony of *D. v. virgifera* were overwintered at 6–8 °C for eight diapause durations: no diapause, 0.5–1.5, 2–4, 5–7, 8–10 (i.e., natural diapause length), 11–13, 14–16, and >17 months of diapause. Eggs from the ND colony were not exposed to cool temperature periods and used directly. To start hatching experiments, egg batches were sieved and washed in clean water containing <0.5% NaOCl. On average, 38 ± 18 (mean ± SD) eggs from each batch were pipetted in water onto a 5-cm-diameter filter paper in each of 12 Petri dishes. Four Petri dishes of eggs from each batch were incubated on the moist filter paper (no free water visible) in the dark at each of three temperatures (16 ± 0.5, 20 ± 0.5, and 24 ± 0.5 °C) and 50–60% r.h. (respective incubators: Friocell 22 and 222; MMM Group, München, Germany; MIR-153; Sanyo, Osaka, Japan).

In total 3–13 experimental series were implemented per diapause length category, *D. v. virgifera* colony type, and post-diapause incubation temperature. Series were started on a monthly basis using new rearing batches of eggs ($n \geq 12$).

Data collection and analysis

Egg overwintering survival was assessed through counting healthy and dead eggs under a Science ETD-201 stereomicroscope (10× magnification; Bresser) at the beginning of each experiment. An egg was considered healthy if of creamy colour, non-empty, and with an intact skin, that is, with a honeycomb surface structure (Modic et al., 2005). During egg incubation, hatching of larvae was assessed every 2nd or 3rd day. Hatched larvae were removed from the dishes with clean forceps. After the last eggs had hatched, we still incubated the eggs for two more weeks to assure termination of hatching.

Egg overwintering survival and hatching rates, as well as the beginning, peak, termination, and duration of egg hatching were calculated per Petri dish, and then averaged among the four corresponding Petri dishes of the same treatment. Dishes that did not lead to a minimum of six hatched larvae were not used for calculating those averages, because of a potential overweighting of proportional data originating from dishes with few eggs over dishes with many eggs.

Raw data were standardized to 100 eggs per dish to visualize temporal hatching dynamics. Mean values were used to plot start, peak, duration, and termination of egg hatching per diapause category. Synchrony of hatching was, next to a visual assessment, also characterised through calculating the standard deviation (SD) and coefficient of variation of each of those steps of hatching.

Basic diagnostic plots were used to examine assumptions of the normal distributions of the residuals and homoscedasticity (Faraway, 2016). Generalized additive models (GAMs) were applied to analyse the effect of diapause length (eight categories) and post-diapause incubation temperature (16, 20, and 24 °C) on the beginning, peak, termination, and duration of egg hatching as well as on hatching rates. General linear hypothesis testing (GLHT) followed by post-hoc Tukey honestly significant difference tests were applied to detect variability in egg hatching patterns between the eight diapause length categories, three incubation temperatures, and two colony types.

R v.3.5.2 (R Core Team, 2018) was used for the data visualization and statistical analysis with the following packages: ggplot2 (Wickham, 2016), multcomp (Hothorn & Bretz, 2008), and mgvc (Wood, 2017).

Results

Both length of overwintering diapause and post-diapause incubation temperature influenced the hatching patterns of *D. v. virgifera* eggs (Figure 1, Table 1). For the wild diapause strain, the most similar patterns and highest synchronization of hatching of eggs occurred when overwintered at 8–10 months (i.e., the natural diapause length) or 5–7 months. The non-diapause colony

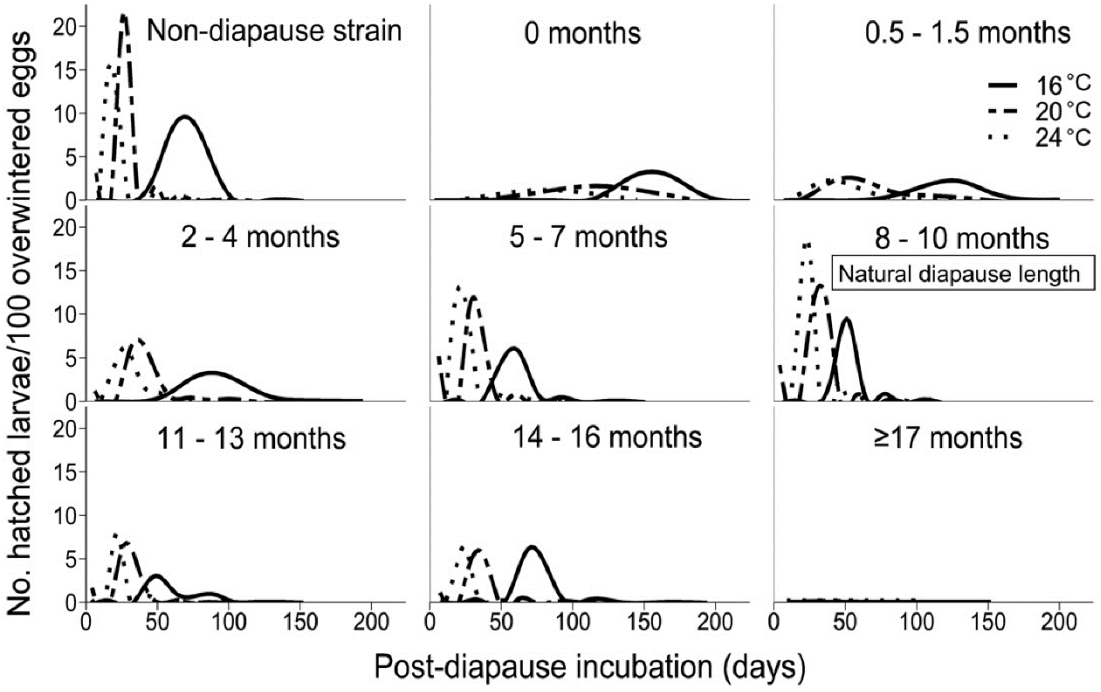

Figure 1 Temporal hatching patterns of *Diabrotica virgifera virgifera* eggs in response to various lengths (months; indicated above the panels) of overwintering diapause and three post-diapause incubation temperatures (16–24 °C). A diapausing wild Central South-eastern European population of *D. v. virgifera* was assessed; a non-diapause laboratory colony of USDA ARS was included in the upper left panel for comparison (n = 8–48 Petri dishes with 20–100 eggs, i.e., 3–13 replications of four Petri dishes). Loess smoothed trendlines are fitted.

displayed high synchronization in the absence of diapause (Figure 1).

In more detail, the length of the overwintering diapause (eight categories), as well as incubation temperature (16, 20, or 24 °C), influenced the beginning of hatching of *D. v. virgifera* eggs (GAM: diapause length: $F_{7.9,417.1} = 47.6$; temperature: $F_{3,417.1} = 2281$, both $P<0.001$), the peak (diapause length: $F_{7.7,580.3} = 128.8$; temperature: $F_{3,580.3} = 2282$, both $P<0.001$), the duration (diapause length: $F_{6.7,418.3} = 64$; temperature: $F_{3,418.3} = 535$, both $P<0.001$), and the termination of hatching (diapause length: $F_{8,417} = 125$; temperature: $F_{3,417} = 2568$, both $P<0.001$), as well as hatching success (diapause length: $F_{8\cdot7,660.2} = 30$; temperature: $F_{3,660.2} = 617$, both $P<0.001$) (Table 1).

Eggs from wild *D. v. virgifera* that underwent 8–10 months of diapause started to hatch around the same time as eggs that diapaused for shorter (5–7 months) or longer (11–17+ months) periods: within 19–20 days at 24 °C, within 27–28 days at 20 °C, and within 46–57 days at 16 °C. However, the shorter the diapause, the less synchronized the eggs started to hatch. After a very short diapause (<2 months), eggs started to hatch later, and they started latest when not diapaused at all (Figure 2B, Table 1).

Eggs of the non-diapause laboratory colony started to hatch at comparable times as those of the diapausing colony exposed to a diapause of natural length, except when they were kept at a low incubation temperature (16 °C), which led to a later and less synchronized start of hatching (mean ± SD = 62 ± 12 days) (Table 1).

At 24 °C, egg hatch from wild *D. v. virgifera* peaked already 3–5 days after the start of hatching, regardless of diapause length; that is, egg hatch peaked within 21–25 days after the start of incubation at 24 °C. At 20 °C, egg hatch peaked 3–10 days after the start of hatching (i.e., within 30–38 days after the start of incubation). At 16 °C, egg hatch peaked 1–11 days after the start of hatching (i.e., 50–68 days after start of incubation). In general, the shorter the diapause, the less synchronized was the peak of egg hatching. After a very short diapause (<2 months), egg hatch peaked later, and hatching peaked latest when not diapaused at all (Figure 2C, Table 1).

Egg hatch of the non-diapause laboratory colony peaked at comparable times as those of the diapausing colony: 18 ± 1 days after incubation at 24 °C (3 days after the start of hatching) and 27 ± 3 days at 20 °C (4 days after hatching started). At low temperatures (16 °C), synchronization was low, with a small peak after 70 ± 11 days only (Table 1).

Table 1 Hatching characteristics (mean ± SD) of *Diabrotica virgifera virgifera* eggs in response to various lengths of overwintering diapause and three post-diapause incubation temperatures. A diapausing wild Central South-eastern European population of *D. v. virgifera* was assessed; a non-diapause laboratory colony of USDA ARS was included for comparison (n = 8–48 Petri dishes with 20–100 eggs, i.e., 3–13 replications of four Petri dishes)

Incubation temperature (°C)	Colony	Diapause duration (months)	n	Egg hatching								
				Start (days after diapause)	CV	Peak (days after diapause)	CV	End (days after diapause)	CV	Duration (days)	CV	Rate (%)
16	Non-diapausing	0	8	62 ± 12B	0.19	70 ± 11B	0.16	90 ± 11B	0.12	30 ± 11A	0.37	85 ± 16B
	Diapausing	0	12	122 ± 37eC	0.30	155 ± 21eC	0.14	186 ± 12eC	0.06	65 ± 35aB	0.54	34 ± 11bcA
		0.5–1.5	20	93 ± 12d	0.13	113 ± 17d	0.15	151 ± 21d	0.14	59 ± 23a	0.39	31 ± 15bc
		2–4	36	70 ± 10c	0.14	84 ± 19c	0.23	125 ± 19c	0.15	56 ± 18a	0.32	40 ± 18c
		5–7	36	52 ± 7ab	0.13	57 ± 10ab	0.18	76 ± 13ab	0.17	26 ± 11b	0.42	37 ± 26c
		8–10[1]	40	46 ± 3aA	0.07	50 ± 5aA	0.10	66 ± 10aA	0.15	21 ± 10bA	0.48	38 ± 24cA
		11–13	48	56 ± 14a	0.25	57 ± 14ab	0.25	72 ± 20ab	0.28	17 ± 10b	0.59	19 ± 15b
		14–16	8	57 ± 11abc	0.19	68 ± 7b	0.10	92 ± 22b	0.24	36 ± 24b	0.67	38 ± 14bc
		≥17	24	53 ± 0abc	0.00	70 ± 6abc	0.09	67 ± 0ab	0.00	15 ± 0ab	0.00	2 ± 4a
20	Non-diapausing	0	8	24 ± 2A	0.08	27 ± 3A	0.11	54 ± 16A	0.30	31 ± 17A	0.55	86 ± 16B
	Diapausing	0	12	60 ± 19cB	0.32	93 ± 26cB	0.28	131 ± 16dB	0.12	72 ± 31cB	0.43	34 ± 22bcA
		0.5–1.5	20	36 ± 9b	0.25	61 ± 21b	0.34	92 ± 23c	0.25	57 ± 18c	0.32	40 ± 12bc
		2 4	36	28 ± 5a	0.18	38 ± 12a	0.32	64 ± 22b	0.34	37 ± 20b	0.54	43 ± 15c
		5–7	36	27 ± 3a	0.11	32 ± 5a	0.16	46 ± 9a	0.20	19 ± 9a	0.47	47 ± 25c
		8–10[1]	40	28 ± 2aA	0.07	31 ± 2aA	0.06	42 ± 10aA	0.24	15 ± 11aA	0.73	49 ± 28cA
		11–13	48	27 ± 3a	0.11	30 ± 5a	0.17	42 ± 16a	0.38	16 ± 15a	0.94	26 ± 19b
		14–16	8	28 ± 1a	0.04	33 ± 1a	0.03	58 ± 20ab	0.34	31 ± 20ab	0.65	32 ± 11bc
		≥17	24	No data		37 ± 5a	0.14	No data		No data		2 ± 4a
24	Non-diapausing	0	8	16 ± 1A	0.06	18 ± 1A	0.06	42 ± 21B	0.50	27 ± 22B	0.81	86 ± 13B
	Diapausing	0	12	50 ± 24cB	0.48	56 ± 19dB	0.34	116 ± 10dC	0.09	67 ± 25cC	0.37	28 ± 11bcA
		0.5–1.5	20	26 ± 7b	0.27	41 ± 13c	0.32	77 ± 20c	0.26	51 ± 19c	0.37	32 ± 13bd
		2–4	36	20 ± 1a	0.05	25 ± 6ab	0.24	54 ± 15b	0.28	35 ± 16a	0.46	42 ± 18cde
		5–7	36	20 ± 2a	0.10	23 ± 7a	0.30	34 ± 14a	0.41	15 ± 13b	0.87	49 ± 22e
		8–10[1]	40	19 ± 2aA	0.11	22 ± 6aA	0.27	29 ± 7aA	0.24	11 ± 7bA	0.64	48 ± 30deA
		11–13	48	19 ± 1a	0.05	21 ± 3a	0.14	29 ± 7a	0.24	11 ± 7b	0.64	23 ± 18b
		14–16	8	20 ± 3ab	0.15	23 ± 3ab	0.13	40 ± 14ab	0.35	21 ± 15ab	0.71	34 ± 15be
		≥17	24	26 ± 0ab	0.00	34 ± 13bc	0.38	33 ± 0ab	0.00	8 ± 0ab	0.00	4 ± 6a

Means within a column and within an incubation temperature group followed by different lowercase letters (comparison of wild diapausing population among diapause lengths) or by different uppercase letters (comparison of laboratory non-diapause and wild diapause colony) are significantly different (Tukey HSD test: $P<0.05$). CV = coefficient of variation.
[1]Natural diapausing length.

6.4 Discussion

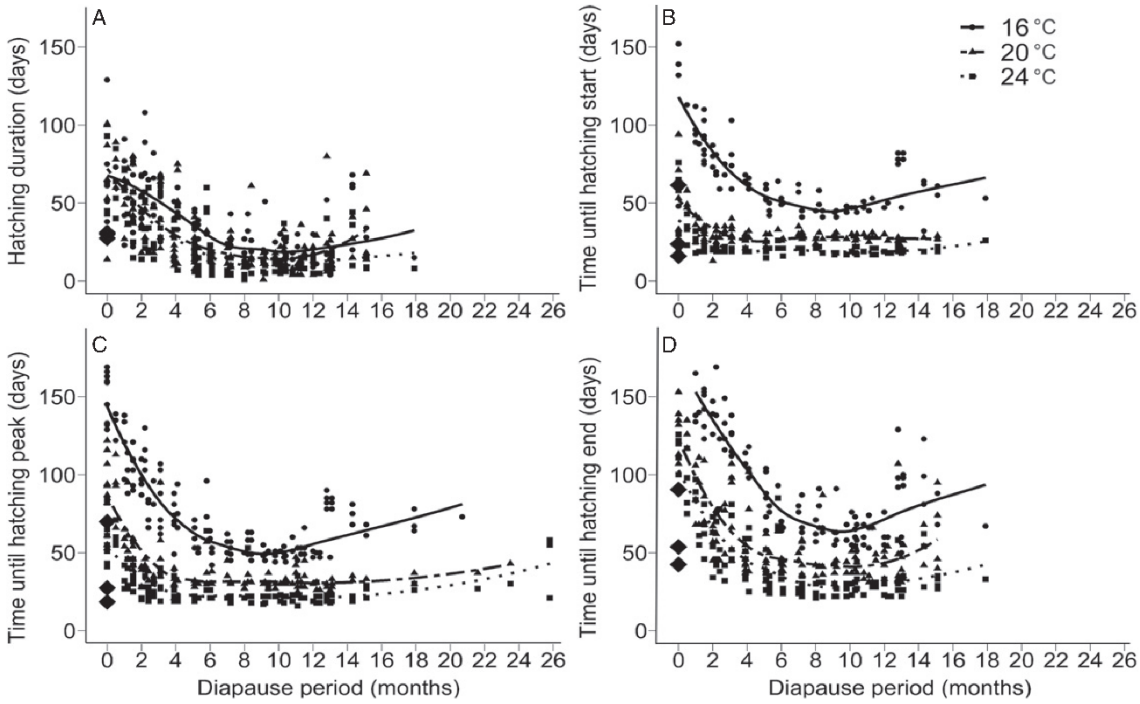

Figure 2 *Diabrotica virgifera virgifera* egg hatching (A) duration, (B) start, (C) peak, and (D) end in response to various lengths of overwintering diapause and three post-diapause incubation temperatures (16–24 °C). A diapausing wild Central South-eastern European population of *D. v. virgifera* was assessed; a non-diapause laboratory colony of USDA ARS (diamond shapes) was included for comparison (n = 8–48 Petri dishes with 20–100 eggs, i.e., 3–13 replications of four Petri dishes). Loess smoothed trendlines are fitted.

Eggs from wild *D. v. virgifera* that underwent 8–10 months of diapause or that were in diapause shorter (5–7 months) or longer (11–17+ months) hatched most synchronized, that is, within the shortest time period: at 24 °C within 11–21 days, at 20 °C within 15–31 days, and at 16 °C within 17–36 days. In contrast, eggs hatched within longer time periods if diapause had been shortened to 2–4 or <2 months, or in the absence of diapause (Figure 2A, Table 1). Eggs from the non-diapause laboratory colony hatched within a slightly longer period than eggs from the diapausing colony at a higher temperature: 27 ± 2 days at 24 °C. This was comparable at lower temperatures: 31 ± 17 days at 20 °C, and 30 ± 11 days at 16 °C (Table 1). Eggs from the non-diapause laboratory colony usually stopped hatching later than those from the diapausing colony (Figure 2D, Table 1).

Overall, 68 ± 7% of eggs of wild *D. v. virgifera* successfully overwintered at 6–8 °C. Overwintering survival did not depend on the length of the overwintering diapause (GLM: $F_{1,622} = 0.36$, $P = 0.55$), except when diapaused for >16 months ($F_{1,70} = 168$, $P<0.001$) (Figure 3A). Only about half of those successfully overwintered eggs hatched at 24 or 20 °C, regardless of being diapaused for 8–10 months or shorter (2–7 months) (Figure 3B, Table 1). At 16 °C, slightly fewer larvae hatched (GLHT, 16 vs. 24 °C: $Z = -2.4$, $P = 0.04$; 16 vs. 20 °C:, $Z = -3.1$, $P = 0.005$). Also, in the total absence of diapause, a few eggs still hatched. Hatching success gradually decreased with prolonged diapause periods >11 months; for example, <10% of eggs hatched if they had been overwintered for >16 months (Figure 3B). Eggs of the non-diapause laboratory colony hatched more successfully than those of the diapausing colony, that is, 85–86% regardless of incubation temperature (Figure 3B, Table 1).

Discussion

Numerous researchers work with *D. v. virgifera* larvae. Particularly under laboratory and semi-field conditions, this demands precise timing of the hatching of eggs, as well as a good hatching rate. Therefore, it is crucial to have beforehand knowledge about egg hatching patterns and how those may change due to external factors. As only limited information is available (Table 2), we have provided a detailed analysis of the effects of egg diapause length, as well as post-diapause incubation temperature, on the success and temporal patterns of egg hatching. We think this technical information will be valuable for future research.

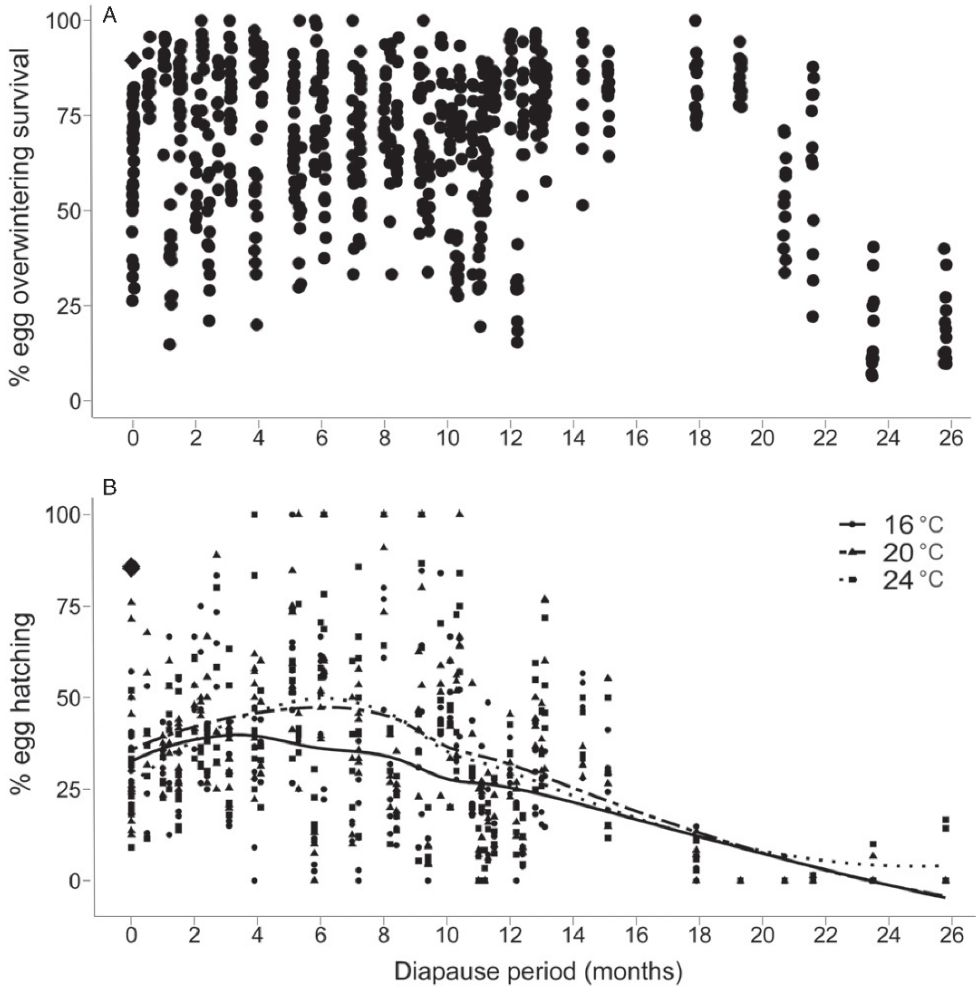

Figure 3 (A) Overwintering survival (%) and (B) hatching rate (%) of successfully overwintered viable eggs of *Diabrotica virgifera virgifera* eggs in response to various lengths of overwintering diapause and three post-diapause incubation temperatures (16–24 °C). A diapausing wild Central South-eastern European population of *D. v. virgifera* was assessed; a non-diapause laboratory colony of USDA ARS (diamond shapes) was included for comparison (n = 8–48 Petri dishes with 20–100 eggs, i.e., 3–13 replications of four Petri dishes). Loess smoothed trendlines are fitted.

Our results revealed that eggs from a diapausing population generally hatch at the highest rate and most synchronized when overwintered at their natural diapause length (8–10 months), but also after shorter periods (5–7 months), at 20–24 °C. A shorter period widens the time frame of use of the eggs for experimentation. Comparable hatching success was recorded for eggs diapaused for even shorter periods (2–4 months), although hatching patterns appeared less synchronized, making such eggs less suitable for experimentation. Diapause for <2 or >10 months negatively affected several egg hatching parameters, as did lower incubation temperatures (16 °C). Those conditions may be less practical for certain experimental set-ups.

In more detail, our data show that eggs diapausing at 6–8 °C for >16 months slowly start to die, which is confirmed by Branson (1976b). Oppositely, if eggs are diapaused for ≤12 months, overwintering survival remains around 67–73%, suggesting these eggs are suitable for experimentation. This seems similar to field conditions, as Levine et al. (1992) showed that eggs diapaused under field conditions for 12 months are hardly losing viability (60–96%).

After successfully overwintering, the question was how diapause length and post-diapause temperature may affect hatching rates. In our study, the post-diapause eggs of the wild *D. v. virgifera* population hatched at rates of 38–49% on average across experiments after overwintering at 6–8 °C for 2–10 months. This is slightly lower than hatching rates reported from other studies, such as 63% by Branson (1976b), 53 ± 10% by Branson (1978), 68 ± 8% by

Table 2 Published information on egg overwintering and hatching of *Diabrotica virgifera virgifera*

Colony	Type	Origin	Diapause duration (months)	Diapause temperature (°C)	Incubation temperature (°C)	Egg hatching					Source
						Start (days after diapause)	Peak (days after diapause)	End (days after diapause)	Duration (days)	Rate (%)	
Diapausing	Wild	USA (MN)	3.7	5	20	24	No data	66	42	No data	Wilde (1971)
Diapausing	Wild	USA (MN)	3.7	5	25	15	No data	64	49	No data	Wilde (1971)
Non-diapausing	Laboratory	USA	0	20–22	20–22	24	No data	No data	25	73	Branson (1976a)
Diapausing	No data	USA	0	5 ± 0.5	20–22	51	No data	No data	59	68	Branson (1976b)
Diapausing	No data	USA	2.7	5 ± 0.5	20–22	19	No data	No data	15	64	Branson (1976b)
Diapausing	Laboratory	USA	3.7	7.5	25	No data	No data	No data	No data	54	Branson (1978)
Diapausing	Laboratory	USA	12	7.5	25	No data	No data	No data	No data	33	Branson (1978)
Diapausing	Wild	USA (SD)	5	8.5	25	No data	No data	No data	No data	68	Fisher (1989)
Diapausing	Wild	Canada	6-11	7.5	20	No data	No data	No data	30	50	Schaafsma et al. (1991)
Diapausing	Wild	Canada	6-11	7.5	24	No data	No data	No data	18	52	Schaafsma et al. (1991)
Non-diapausing	Laboratory	USA (SD)	0.2	4	25	No data	No data	No data	No data	ca. 72	Geisert et al. (2019)
Non-diapausing	Laboratory	USA (SD)	0.9	4	25	No data	No data	No data	No data	ca. 62	Geisert et al. (2019)

Fisher (1989), or 52% by Schaafsma et al. (1991). The slightly lower hatching rate in our study may be explained by experimental differences among studies, as it is not always easy to define a viable egg (Modic et al., 2005). Alternatively, population genetics and phenotypic variation may differ between the European and USA populations (Li et al., 2009, 2014). Our results, as well as those of Branson (1976b), showed that a shortened diapause of down to 2 months does not negatively influence hatching rates compared with the natural diapause length of 8–10 months, which could widen the experimental usage of such eggs. In contrast, a diapause of <2 or >17 months reduces hatching success. This is in contrast to Branson (1976b), who reported that field-collected, diapausing eggs from the USA may already start losing their viability when in diapause for >3.6 months at 5 °C. However, a 5 °C overwintering temperature may have been too low. A later study of Branson (1978) performed at 7.5 °C resulted in findings similar to ours. In conclusion, both *D. v. virgifera* populations from USA and Europe seem to tolerate diapause lengths shorter and longer than natural to some extent.

Similar tendencies and patterns are found when assessing temporal hatching details, such as start, peak, duration, or end of hatching. The here-studied eggs of the wild European population started to hatch in the range of 19–28 days after diapause at 20–24 °C, which seems comparable with *D. v. virgifera* collected from fields in the USA. For example, field-collected diapausing eggs from Minnesota started to hatch after 15 days at 25 °C when they had been in diapause for 3.6 months at 5 °C (Wilde, 1971). Levine et al. (1992) placed diapaused eggs from Illinois into a simulation chamber for 6 months where the temperature was adjusted according to previous year field temperatures, averaging 5.7 °C. After egg incubation at 21 °C, the first eggs hatched after 23 days. Branson (1976b, 1987) held eggs for 2.7 months at 5 °C and recorded a start of hatching after 19–20 days of incubation at 20–22 °C, or after 14 days at 25 °C when in diapause for 6 months at 7–8 °C.

As stated above, our data showed that eggs which were not diapaused or diapaused only for a very short period started to hatch later and over longer periods, thus, were less synchronized. Branson (1976b) too had observed that short diapause lengths of 0 3 weeks delayed egg hatching. For example, after only 1 week cooling at 5 °C, eggs started to hatch 31 days later, compared to 19–20 days later when in diapause for 12 weeks. Branson (1976b) also reported such a delay in egg hatching when eggs were in diapause for too long (13 months), something we did not see in our study, not even after 17 months of diapause.

We reported hatching peaks around 3 weeks after incubation at 24 °C and 4–5 weeks after incubation at 20 °C. In both cases, this means usually 3–5 days after the start of egg hatch. This corresponds to findings of Branson (1976b, 1987), who observed a 5-day peak (when in diapause for 3.6 months at 5 °C) or a 2-day peak (when in diapause for 6 months at 7 8 °C) for eggs after hatch start. Not only the start of egg hatching, and confirming findings by Branson (1976b, 1987), also the hatching peak was less synchronized and occurs late for eggs diapaused for too short or too long. In any case, the hatching peak can be well predicted for experimentation using the data obtained in this study.

A similar picture can be seen when considering the duration of hatching. In our study, eggs most consistently hatched over a period of 11–26 days when in diapause at natural length (8–10 months), but also at shorter (5–7 months) or longer periods (11–13 months). This is comparable to the reported 15 days of hatching of Canadian *D. v. virgifera* eggs at 20 °C when in diapause for 3.6–3.7 months (Schaafsma et al., 1991). However, when eggs were overwintered too short (e.g., <2 months in our study, or <2.7 months in Branson, 1976b), hatching will happen over longer periods, something to consider when using such eggs for experiments.

As for post-diapause incubation temperatures, we suggest to use temperatures between 20 and 24 °C. Lower temperatures lead to less synchronized, longer, and later hatching periods, as do too short diapause periods. This is in line with findings from Wilde (1971), who reported that eggs incubated at 16 °C hatched 27 days later than eggs incubated at 25 °C (42 vs. 14 days). Schaafsma et al. (1991) reported a hatch start after 47, 28, 17, 14, and 16 days when incubated at 16, 20, 24, 28, and 32 °C, respectively. All this is not surprising, as eggs need certain temperature sums above a base temperature (i.e., degree days) of development to growth to larvae. However, our data now allow precise prediction of hatching at various temperatures.

Overall, for a wild *D. v. virgifera* population, egg diapause for 0–16 months does not seem to negatively influence egg overwintering survival. Diapause for 2 10 months seems not to affect hatching rates at 20–24 °C. Diapause for 5–13 months seems not to affect hatching start, peak, and duration when incubated at 20 24 °C. Lower incubation temperatures (16 °C) should be avoided, as well as a short diapause of <2 and a long diapause of >10 months. Our study does not provide insight into performance parameters of the hatched larvae as we needed to remove the neonates from the hatching dishes and therefore often destroyed them during handling.

6.5 References

As many researchers work with laboratory colonies of *D. v. virgifera* selected for non-diapause, we have included such a colony as a comparison in our study. Egg hatching has been more successful for the non-diapause colony (85 86%) than for the European diapausing colony, as well as those of other diapausing colonies from the USA (Branson, 1976a; Krysan & Branson, 1977). This is in line with the literature (Li et al., 2009, 2014), indicating that *D. v. virgifera* from non-diapause colonies may have been selected for higher fecundity and/or fertility over the many generations in the laboratory. Our results also showed that hatching patterns of eggs of the non-diapause colony incubated at 20–24 °C are usually similar to hatching patterns of the wild diapause population diapaused at the natural length of 8–10 months. Only at low incubation temperature (16 °C), synchronization and hatching rates decreased in the non-diapause colony. This is not surprising, as the non-diapause colonies are usually reared at 21–24 °C and may therefore be adapted to those conditions (Geisert et al., 2019).

Despite the obvious technical advantages of using *D. v. virgifera* from non-diapausing colonies, it appears that insects from wild diapausing populations may be easier to use than originally thought. Researchers do not necessarily need to wait the 8–10 month natural diapause before starting experimentation, which renders working with diapause populations more flexible. Eggs that overwintered for 2–10 months and incubated post-diapause at 20–24 °C may be used without compromising egg survival, hatching rates, or hatching patterns. If eggs under such conditions are not available, researchers may refer to our dataset to estimate egg hatching patterns for their experiments.

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Author contributions

Szabolcs Toth: Conceptualization (equal); Data curation (lead); Formal analysis (equal); Funding acquisition (equal); Investigation (lead); Methodology (lead); Project administration (lead); Visualization (equal); Writing – original draft (lead); Writing – review & editing (lead). Mark Szalai: Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Visualization (supporting); Writing – review & editing (supporting). Stefan Toepfer: Conceptualization (equal); Data curation (equal); Formal analysis (supporting); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (supporting); Writing – original draft (supporting); Writing – review & editing (equal).

Data availability statement

The data that support the findings of this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.5180429. Also, the analysis with the data alongside is openly available in Github at https://github.com/DiabroticaHULab/hatchingDvv.

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7. General discussion and conclusions

Chapter I. – How does the efficacy of entomopathogenic nematodes and soil insecticides change against *Diabrotica v. virgifera* pest populations during the cropping seasons? In other words, what is the effect of time on treatment efficacies?

Generally, cypermethrin, tefluthrin and the *H. bacteriophora* were able to control the pest population measuring thought the total adult beetle captures with gauze cages. The overall efficacies were around 33 – 42% with relatively high standard deviations. If we separate the total beetle emergence to males and females, they are also effectively control them within the same efficacy range as for total beetles. Variability in chlorpyriphos's efficacy indicated that it can only controls females sufficiently.

In our study, we used two common pyrethroid active substances (cypermethrin and tefluthrin). We found that tefluthrin's efficacy slightly increases over time to control females, they can better control late than early female larvae. However, this increase is so small, that it's cannot be detected at 25, 50, 75% and peak cumulative adult emergence compared to control. We found no such effect for the cypermethrin. Oppositely, there was some slight indications that chlorpyriphos can better control early than late females, again a closer look on the cumulative adult emergence compared to control indicated no such effect. Interestingly, no temporal effect found for *H. bacteriophora*, it seems there is no improvement on their efficacies, the hypothesized propagation of them in the *Diabrotica. v. virgifera* larvae is not reflected in the beetle emergence later in the cropping seasons.

In conclusion, all tested agents were able to control the pest population under variable field conditions and in most case across fields and years. the used treatments had any temporal effects in their efficacies during the cropping season. Other environmental and biotical factors may play larger roles to determine treatment's low or higher level of efficacies.

Chapter II. – Which abiotic and biotic factors may influence and how the efficacies of entomopathogenic nematodes and soil insecticides at reducing *Diabrotica v. virgifera* pest populations and preventing root damage under field conditions?

In general, we detected that the used control methods, namely the seed coated clothianidin, the granule cypermethrin and tefluthrin and the water-sprayed *H. bacteriophora* can reduce pest populations and prevent general and heavy root damage, with relatively high variability. Results showed that 20% - 30% of the cases treatments were unable to control pest populations and in 5% - 20% unable to prevent heavy root damage.

In our analysis, we investigated 6 biotic and 20 abiotic factors influence on the previously mentioned three soil insecticide efficacies on the pest population management and prevention of the root damage assessed with two different damage scales, one which better detect general root damage and the other the heavy root damage. We concluded that there are very few factors which are influence treatment efficacies. However, the following factors may interfere with the chemical treatments efficacies under field conditions:

Clothianidin was slightly less effective at controlling *Diabrotica v. virgifera* with increasing $CaCO_3$ and humus content of the soil, and at preventing root damage at high bulk density. Late maize sowing and late treatment in April or May as well as high soil moisture in July improved the prevention of the heavy root damage. Cypermethrin was more effective to prevent heavy root damage, when an increased amount of clay of the soil was there. Tefluthrin was less effective controlling *Diabrotica v. virgifera* with increasing soil moisture in July, but slightly more effective with increased amount of cumulative rainfall in July. Moreover, it's efficacy to prevent heavy root damage was less effective with increased sand content of the soil.

Interestingly, we were not able to detect any relationships between the *H. bacteriophora*'s efficacies on controlling pest population and protect maize root damage and between the investigated 12 biotic and 20 biotic factors. Nevertheless, there were some promising indicators in the point of view of the field usage of the nematodes, for example all mortality and virulence indicators showed lack of influence on their efficacies, meaning the quality of the nematodes was satisfying during the field application process. A wide range of water amount (133 L to 558 L) can be used to apply nematodes without compromising their efficacies, thus lower water amount can be used, thus farmers needs to carry less water quantities to the field.

In conclusion, there are very few indications that the investigated abiotic and biotic factors causing the variability of the treatment efficacies. This indicates that environmental factors individually are not effects treatments efficacies in a major way, thus they universal usage is recommended. Our results may suggest that there are complex multi-interactions exists between the factors which are acting on each other or together and thus their added effect act on negatively on the treatment's efficacies. These kind of fine interactions we are not able to detect. As for the variability of the nematodes efficacy, it is possible that microbial communities of the soil and the belowground fauna has larger role to determine their efficacies as nematodes themselves living creatures. However, these statements are remaining hypothetical.

Chapter III. – Can the botanical azadirachtin sufficiently kill *Diabrotica v. virgifera* larvae and prevent root damage to become an alternative, new candidate that can replace conventional soil insecticides?

Bioassays conducted in the laboratory revealed that after 3 days of the experiments the LD_{50} was 22.3 µg azadirachtin/ml which corresponds to 0.45 µg/neonate larvae. After 5 days this LD_{50} was 19.3 µg azadirachtin/ml which means 0.39 µg/first to second instar larvae. Due to their high and rapid kill, no sub-lethal effect was observed.

The greenhouse experiments showed that the proposed standard dose of the granular formulated 38 g azadirachtin/hectare applied at sowing into the furrow are not able to control *Diabrotica v. virgifera* or prevent root damage. However, 10x of the standard dose can suppress pest populations as well as protect the maize roots. This was better than the efficacy of the cypermethrin-based granules and comparable to tefluthrin-granules or the thiomethoxam seed coatings. In this case, the ED_{50} was 92 g azadiracthtin/ha to control *Diabrotica v. virgifera*, for preventing general root damage it was 220 g/ha and for preventing the heavy root damage it was 52 g/ha.

In conclusion, we demonstrated that azadiracthin based granular products could replace phased-out soil insecticides, thus farmers may can continue they traditional ways of controlling this serious pest under field conditions. However, more increased active ingredient containing granules and large field trials needed before this solution could be a part of farmer's plant protection toolkit.

Chapter IV. – What is the shortest diapause length and most practical incubation temperatures which still do not compromises proper hatching rates and a good hatching synchrony of *Diabrotica v. virgifera* eggs?

Our experiments showed that, eggs from wild diapausing population hatched most synchronized when they underwent 8 – 10 months diapause (the natural diapause length) or shorter (5 – 7 months) or longer (up to 13 months).

As for optimal incubation temperatures: both 20 and 24 °C could be used. Lower incubation temperature i.e. 16 °C causes less synchronized egg hatching, meaning that the hatching periods are longer and later, similarly to very short (i.e. < 2 months) diapausing time.

In conclusion, our analysis revealed that researchers do not need to wait necessarily 8 – 10 months (the natural diapause length). Eggs which have been underwent 2 – 10 months and after that incubated at 20 – 24 °C can be used without compromising egg survival, hatching rates and

hatching patterns. Diapause for <2 or >10 months negatively affected several egg hatching parameters, as did lower incubation temperatures (16 °C), thus are not recommend.

In summary, we investigated the effect of time, abiotic and biotic factors on the variable efficacies of different soil insecticides, seed coating and a biological control agent. We found that these effects are only slightly influence the treatment efficacies so reasons behind their variability to manage this insect pest are still remaining unknown, further research needs to be conduct to reveal the solution. Azadiracthin based granule insecticides could be used against *Diabrotica v. virgifera* to manage its population and root damage. Wild, diapausing *Diabrotica v. virgifera* eggs with reduced time in diapause and incubated in 24 °C could be used for trials and bioassays without compromising the timing and successfulness of an assay. Moreover, they are could be used for assays in the same way as the laboratory, non-diapausing *Diabrotica v. virgifera.* strain.

8. New scientific results

- I have justified that soil insecticides as tefluthrin, cypermethrin and the clorpyriphos, seed coating as the clothianidin and biological control agent as the *H. bacteriophora* entomopathogenic nematodes are able to reduce *Diabrotica v. virgifera* populations and prevent maize root damage under field conditions in various years and fields with a high variability in their efficacies.

- I discovered that the temporal effects of the above listed treatments are not the cause of their variable efficacies against controlling *Diabrotica v. virgifera* populations.

- I have detected that the abiotic and biotic factors are only slightly influence of the efficacies of the above mentioned treatments efficacies against *Diabrotica v. virgifera.* populations and preventing general and heavy root damage.

- I have discovered that the azadirachtin based soil insecticide granules are able to induce high mortality among *Diabrotica v. virgifera* larvae under laboratory conditions. Also, it is able to reduce *Diabrotica v. virgifera* larvae populations and prevent root damage under greenhouse conditions with an elevated dosage compared to the recommended one.

- I have determined that the diapausing phase of the eggs of the wild populations of *Diabrotica v. virgifera* can be reduced until almost to 2 months compared to the natural diapause length of 8 to 10 months without compromising it’s successful and synchronized hatching at 20 – 24 C°.

9. Summary

The subtribe Diabroticina (Coleoptera: Chrysomelidae) contains a range of pest species which are causing economic losses to field and horticultural crops, mainly on the American continent. However, one species successfully invaded Europe, namely the western corn rootworm *Diabrotica v. virgifera* LeConte, which causes damage in maize (*Zea mays* L.). Since its appearance and spread from the 1990's, the way of cultivating maize in Europe has been changed. Farmers had to give up continuous plantation of maize, and they had to use diverse plant protection methods and practices to avoid damages by this invasive, alien pest. These methods are assembled from crop rotation, synthetic chemical insecticides against larvae in the soil and against the adults above ground, and different biological control methods (for example entomopathogenic nematodes). However, these methods have been frequently reported to lead to variable efficacies against this pest. Although many laboratory studies exist, reasons behind variable efficacies under field conditions are limited and warranted investigation.

In this thesis, we investigated why soil insecticides, seed coatings and entomopathogenic nematodes may have variable efficacies at reducing *Diabrotica v. virgifera* pest populations and at preventing root damage. Firstly, we focused on the possibility that different granular insecticide treatments as well as entomopathogenic nematodes have temporal effects when controlling pest populations. In other words, the time of peak activity of a treatment in the soil as well as the temporal pattern of hatching of the larval population may cause those variable efficacies under field conditions. **(Chapter I.)**. Results revealed that there seems no major evidence that the occasionally occurring low efficacies of treatments are due to temporal effects. We only found few indications that chlorpyrifos may slightly lose and tefluthrin slightly increase efficacy over time, but this temporal change was minor and difficult to detect. The cypermetrin and the *H. bacteriophora* successfully reduced larvae over time in the different cropping seasons. Next, we explored whether different abiotic factors (up to 22 tested) and biotic factors (up to 10) are the reasons behind the observed variability of efficacies of the treatments in controlling pest populations and preventing root damage **(Chapter II.)**. Results showed that only a few factors influence soil insecticides and seed coatings. For example, clothianidin was slightly less effective at controlling *Diabrotica v. virgifera* with increasing $CaCO_3$ and humus content of the soil, and at preventing root damage at high bulk density. Late maize sowing as well as high soil moisture in July slightly improved the prevention of heavy root damage. Cypermethrin was slightly more effective in preventing heavy root damage at an increased amount of clay in the soil. Tefluthrin was slightly less effective at controlling *Diabrotica v. virgifera* with increasing soil moisture in

June, but slightly more effective with increased amount of cumulative rainfall in July. Its efficacy to prevent heavy root damage was less effective with increased sand content of the soil. No such factors were found to influence *H. bacteriophora*. In conclusion, most studied factors seem not to have major effects on any of the treatments, and reason behind variability in efficacies remain still somewhat little understood. Therefore, in **Chapter III.** we tried to come up with a new, environmentally friendly way and practical solution for farmers to control *Diabrotica v. virgifera*. In this case we used granules of the botanical azadirachtin against the pest's larvae. We tested this product under laboratory and greenhouse conditions. We concluded that this product can effectively kill *Diabrotica v. virgifera* larvae at a level comparable with other chemical insecticides. Applying dose-efficacy trials, we established the 3 and 5 day LD_{50} and LD_{90} values. In the greenhouse experiments, we used potted-plant trials to repeat and reconfirm our findings from the laboratory. We sowed and treated the plants in the same moment, i.e. prior larvae hatch, imitate field conditions. We found that this control method effectively controls the larvae. Moreover, it can protect the maize roots from root damage. However, the applied standard dosage was not able to control larvae populations and protecting maize roots. In contrast, a 10x concentration lead to total pest control as well as the prevention of root damage. Bioassays using larvae of this maize pest need precise hatching information to plan for the right amount of larvae at the right time **(Chapter IV.)**. To allow this, we have investigated the survival and temporal hatching patterns of the pest's eggs depending on diapause length as well as post-diapause incubation temperature. We used eggs from a wild diapausing European population and for comparison a laboratory non-diapausing population. After conducting laboratory assays we provided data matrices on egg overwintering survival, the start, peak, duration and end off egg hatching, as well as hatching rates. We found that the highest hatching rates and most synchronized hatching times in a wild diapausing population occurred when eggs were overwintered at the natural diapause length (8–10 months) or shorter (5–7 months) and then incubated at 20–24 °C. Eggs diapaused for only 2 months showed comparably good hatching rates, but hatching patterns appeared more variable. Diapause of <2 or >10 months reduced hatching success, as did low (16 °C) incubation temperatures. Experimentation can be started earlier with eggs from the wild population of *Diabrotica v. virgifera*, because the diapause length can be reduced.

In conclusion, soil insecticides, seed coating and entomopathogenic nematodes can be used to protect the maize in the cropping season with variable efficacies. Reasons behind the inconsistencies are difficult to explain. A good, alternative solution could be an azadirachtin-based soil insecticide. Experiments can be started earlier with the wild population of *Diabrotica v. virgifera*.

10. Összefoglalás

A Diabroticina altörzs (Coleoptera: Chrysomelidae) tagjai között számos rovarkártevő található, melyek képesek hatalmas gazdasági károkat okozni szántóföldi és kertészeti kultúrákban egyaránt, főleg az amerikai kontinensen. Egy fajuk képes volt az európai kontinensen is megtelepedni. Ez a rovar az amerikai kukoricabogár (*Diabrotica v. virgifera* LeConte), amely így Európa egyik legnagyobb kukorica (*Zea mays* L.) károsítójává vált. Az 1990-es években jelent meg, majd kezdett el folyamatosan terjedni, ezzel módosítva az európai kukoricatermesztést. A gazdálkodók nem termeszthették folyamatosan, egymás utáni években a kukoricát immáron és új növényvédelmi eszközöket és technológiákat voltak kénytelenek alkalmazni, bevetni ezen idegenhonos kártevő ellen. Ezek a védekezi módszerek a következőkből állnak: vetésváltás; szintetikus kémiai rovarölő szerek használata a lárvák ellen a talajban és a földfelszínen az imágók ellen; egyéb biológiai védekezési módszerek (pl. entomopatogén fonálférgek, entomotogén gombák, növényi eredetű inszekticidek). Mindezek ellenére ezen védekezési módszerek gyakran vezetnek a kezelések hatákonyságának ingadozásához, így az ezek mögött álló hatások felderítése különösen fontos.

Ezen értekezés céljai között szerepelt megérteni, hogy a különböző kémiai talajfertőtlenítő szerek, csávázószerek és az entomopatogén fonálférgek hatékonysága miért változékony a kukoricabogár populáció ellen valamint a gyökérkártétel megelőzésében. Először, megvizsgáltuk annak a lehetőségét, hogy a különböző rovarölőszerek és az entomopatogén fonálférgek eltérő időbeli hatékonysággal rendelkeznek-e a kukoricabogár lárvái (az imágó fogásokon keresztül mérve) ellen. Lehetséges, hogy a lárvapopulációk kelési dinamikájának (kései vagy korai lárvamegjelenés) eltéréseit nem mindig tudják időben követni, ezáltal hatékonyságukban különbség mutatkozik szántóföldön **(I. fejezet)**. Eredményként azt találtuk, hogy a klórpirifosz hatékonysága kissé emelkedik, míg a teflutriné kissé csökken az éveken belül. A cipermetrin és a *H. bacteriophora* fonálférgek hatékonysága kiegyenlítettnek modható az egész szezonban, hatásuk képes nyomonkövetni a lárvák kelési dinamikáját. Elmondható tehát, hogy az időnként tapasztalt alacsonyabb hatékonyság a kukoricabogár ellen nem köthető az alkalmazott védekezési módszerek időbeli hatékonyságához. Ezután, megvizsgáltuk azt, hogy lehetséges-e, hogy a különböző abiotikus (21 és 22) és biotikus (5 és 10) faktorok hatása az alkalmazott kezeléskre okozza azok hatékonyságbeli változékonyságát a lárvapopuláció ellen és a gyökérkártétel megelőzésében **(II. fejezet)**. Azt találtuk, hogy viszonylag kevés külső tényező van hatással az alkalmazott kezelések hatékonyságára. A klotianidin kevésbé volt hatékony a *Diabrotica v. virgifera* ellen, ha a talaj $CaCO_3$ és humusz tartalma magas volt, valamint a gyökérrágás megelőzése csökkent magas talaj tömegsűrűség mellett. Azonban a kései kukoricavetés és magas júliusi talajnedvességtartalom

növelte a klotianidin hatékonyságát az erős gyökérkártétel megelőzésében. A cipermetrin hatékonysága magasabb volt az erős gyökérkártétel megelőzésben, ha a talaj agyagtartalma magas volt. A teflutrin hatékonysága csökkent a kukoricabogár ellen, ha a júniusi talajnedvesség magas volt, viszont hatékonysága javult, ha a kumulatív esőmennyiség magas volt júliusban. Ezen rovarölőszer hatékonysága az erős gyökérkártétel megelőzésére nőtt, ha a talaj homoktartalma magas volt. Az entomopatogén fonálférgek *H. bacteriophora* hatékonysága és a vizsgált faktorok egyike között sem találtunk kapcsolatot. Emiatt, a **III. fejezet**ben megpróbáltunk egy új, a környezetet sem terhelő megoldási lehetőséget megvizsgálni a kukoricabogár ellen, melynél az is célunk volt, hogy a védekezés azonnal bevethető legyen a mezőgazdaságban és a gazdák gyakorlati szempontból is könnyen használhassák. Ebben az esetben egy azadiraktint tartalmazó granulátumot vizsgáltunk a károsító lárvái ellen laboratóriumi és üvegházi körülmények között. Biotesztek segítségével megállapítottuk, hogy ezen szer lárvicid hatása hasonló a konvencionális inszekticidekhez. Dózis-hatás vizsgálatokban megállapítottuk az azadiraktin LD_{50} és LD_{90}-es értékeit. Az üvegházban a vetés és a kezelések időpontja egybeesett, és két héttel a kukoricabogár petéivel való mesterséges fertőzés előtt történt, ezzel szimulálva a szántóföldi körülményeket. Azt tapasztaltuk, hogy az azadiraktin granulátum magas lárvicid hatással bír, valamint képes megvédeni a kukorica gyökeret az általános és erős gyökérkártételtől. Azonban, ez csak akkor volt elmondható, hogy ha a dózis 10x-esre emeltük az ajánlott, standard dózishoz képest. A különböző biotesztek, kísérletek precíz tervezéséhez és végrehajtásához egy meghatározott időpontban megfelelő mennyiségű lárva kell, hogy rendelkezésére álljon (**IV. fejezet**). Ehhez szolgáltattunk információt azáltal, hogy megvizsgáltuk, hogy a peték diapauzális fázisának hossza és a poszt-inkubációs hőmérséklete hogyan befolyásolja a peték túlélését és a peték kelésének időbeli mintázatát. Ehhez egy európában is megtalálható, szabadföldön fellelhető, diapauza igényes kukoricabogár populációinak petéit használtuk, összehasonlítva a labortenyésztett, diapauza fázist nem igénylő populáció egyedeinek petéivel. A laboratóriumi tesztek eredményét vizsgálva részletes információ kapható arról, hogy a diapauzális fázis hossza (0-tól több mint 17 hónap, kategórikus) és a poszt-inkubációs hőmérsékletek (16, 20, 24 °C) hogyan hatnak a peték áttelelésének sikerességére, a lárvakelés kezdő időpontjára, csúcsára, hosszára, és végpontjára, valamint a kelési arányra. Azt találtuk, hogy a legtöbb lárva és a leginkább szinkronizált kelés a szabadföldi populáció esetén 8-10 hónap (természetes diapauza hossza) után következett be, de hasonló eredényeket kaptunk rövidített, 5-7 hónap diapauza után is. Mindkét esetben a 20-24 °C poszt-diapauzális inkubációs hőmérsélet bizonyult optimálisnak. Azok a peték, amelyek csak 2 hónapnyi diapauzális időszakon mentek keresztül, szintén jó kelési arányt produkáltak, de kelési mintázatuk változatosnak bizonyult. Azon peték esetén, amelyek 2 hónapnál kevesebbet, vagy 10 hónapnál többet töltöttek a diapauza állapotában, valamint azon peték esetén, amelyek 16 °C-on

inkubálódtak, a kelési arány nagymértékben csökkent, így ezek nem voltak alkalmasak további vizsgálatra. A *Diabrotica v. virgifera* vadon élő populációjából származó petékkel már korábban is megkezdhető a kísérletezés, mert a diapauza hossza csökkenthető.

Összefoglalva, a talajfertőtlenítőszerek, a csávázószer és az entomopatogén fonálférgek változó hatékonysággal, de hosszabb távon is képesek védelmet nyújtani a kukoricabogár ellen a szántóföldön. Hatékonyságuk változékonysága mögött meghúzódó okokat nehéz megmagyarázni. Jó alternatív megoldás lehet egy azadiraktint alapú talajfertőtlenítőszer. A kísérletek már korábban megkezdhetők a *Diabrotica v. virgifera* vadon élő populációjával.

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12. External links

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