Doctoral (PhD) dissertation

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Natural source-agents for control of the invasive alien Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) in maize

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Dedications

This work is dedicated to:

The memory of my beloved mother, Bunga Mari Sembiring, who passed away 5 years ago shortly after I began my work as a lecturer at Universitas Kristen Wira Wacana Sumba in Indonesia. I had promised to make my mother the happiest woman achieving monumental academic success, and I hope I have partly fulfilled that promise. I wish she were alive today to enjoy the fruits of her sacrifices and prayers and to share in the joy of my graduation with a Doctor of Philosophy degree. My dad, Pendapaten Tarigan, you not only worked so hard to provide for the family, but also inculcated in us the principles of hard work and encouraged me to believe in myself. My siblings (Andrew Lopiga Tarigan, Nurbety Br Tarigan (Ph.D candidate) and Aldy Efendi Tarigan, BSc for your support, encouragement, inspiration, affection, love and steadfast partnership for success in my life. You have been my support system.

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My little daughter, Amanda Ánelia Wanjala, my sunshine and support system, was born in Kistarcsa, Hungary. I thank God for having you by my side during my PhD journey.

1. Introduction

1.1 Maize (*Zea mays* L.)

Maize (*Zea mays*) is one of the most important staple crops famously called as queen of cereals in the world (Grote et al., 2021). In Central and South America maize is a major source of carbohydrates. In the United States, maize is used as an alternative food ingredient. The benefits of maize in addition to being consumed by humans, it is also used as animal feed (Erenstein et al., 2022; Njugi et al., 2018) and as an industrial raw material (Ayiti et al., 2022; Chaudhary et al., 2014).

In 2023, Hungary planted maize on roughly 800 thousand hectares, achieving an average yield of about 6.05 metric tons per hectare (KSH, 2023). Throughout Europe, approximately 14.1 million hectares were planted with maize, yielding an estimated 61.4 million metric tons (Huzsvai et al., 2024). This reflects a significant agricultural effort in both Hungary and Europe, highlighting the importance of maize as a staple crop in the region.

The morphology of maize plants consists of roots, stems, flowers and seeds. Maize is a tall plant, single-housed, and per season. Maize plants are C4 plants that want to grow in places open and well lit. C4 crop group is more efficient in utilizing CO_2 necessary in the process of photosynthesis.

1.2 *Diabrotica virgifera virgifera* LeConte

1.2.1 The Origin and biology

Diabrotica virgifera virgifera, commonly known as the western maize rootworm, is believed to have originated in Mexico. Genetic studies indicate that this pest likely spread alongside maize cultivation into North America thousands of years ago, adapting to various new environments as it expanded (Lombaert et al., 2018).

Diabrotica virgifera virgifera, is a univoltine beetle species which has one generation per year. The lifecycle begins with eggs laid in the soil during late summer, which overwinter and hatch into larvae the following spring. The larvae undergo three instars. Larvae then become pupae in the soil, emerging as adults in mid to late summer (Levine & Oloumi-Sadeghi, 1991). Male has longer antenna than female (Spencer et al., 2009). Both male and female of *Diabrotica virgifera virgifera* exhibit a yellow and black striped pattern on their elytra (wing covers). However, males tend to have more pronounced and darker black stripes compared to females, whose stripes are usually more regular and less confluent (EPPO, 2017).

1.2.2 Spread in Europe

The population of *Diabrotica virgifera virgifera* was first detected near Belgrade, Serbia, in 1992. The beetle then moves to Hungary, making induced larvae-damage in 1995. Then around 10 years later it rapidly spread to Central and South-Eastern of Europe (Baufeld and Enzian, 2001). The continuously expanding CSE European outbreak extends from Austria to Ukraine from southern Poland to northern Bulgaria. A number of isolated outbreaks have been detected almost every year since 1998, in various countries including Italy, France, Switzerland, Belgium, the United Kingdom, the Netherlands and Germany (Anonymous 2007; Edwards and Kiss, 2007). It has invaded with total of 32 countries in Europe.

1.2.3 Damage on maize and factors affected its damage

Diabrotica virgifera virgifera started created primary damage on maize roots at the first instar larvae stage (Blandino et al., 2017). First instar larvae feeding the brace roots causing maize plants reduced its ability on absorbing waters and nutrients (Chiang, 1973; Moeser and Hibbard, 2005). Heavy damage by the larvae causing plant lodging and significant yield losses (Toepfer et al., 2010; Szalai et al., 2011). The level of damage caused by larvae on the roots can be measured using the IOWA with 1-6 scale (Davis, 1994). If the larvae created heavy root damage, the measurement can be evaluated using node-injury 0.00 to 3.00 (Oleson et al., 2005). The adult stage feeding on maize silks causing pollination interfering resulting in reduced fertilization and kernel set (Culy et al., 1992; Tuska et al., 2002; Tuska et al., 2003). The level of damage caused by adult depends on the maize variety, cultivating maize with sweet maize variety and do silk cutting to 1 cm resulted in moderate yield reduction (Gyeraj et al., 2023).

1.3 Current situation of controlling *Diabrotica virgifera virgifera*

Diabrotica virgifera virgifera remains a significant concern for maize cultivation in Europe and North America. In hungary, this pest has been detected in 16 out of 19 counties including Borsod- Abaúj-Zemplén, Nógrád, Veszprém, and Zala. It then continued to spread to Slovakian border and westwards to the northern shore of lake balaton (EPPO, 2023). Bacs Kiskun and Csongrád counties were reported as area larvae damaged was observed. Growers use insecticides to control the pest. They were used first the organochlorine insecticides against *Diabrotica virgifera virgifera* in the USA in 1949 then resistance of the pest developed within five years (Ball and Weekman, 1962). Then, it continues with using carbamate, organophosphate, pyrethroid insecticides that also causing pest develop resistance in 2001 (Zhu et al., 2021). In 2003, in USA maize producing crystalline toxins derived from the bacterium *Bacillus thuringiensis* (Bt maize),

was produced and used to control *Diabrotica virgifera virgifera* (Storer et al., 2006). In 2006-2009, the pest population showed resistance to Cry3Bb1 maize and mCry3A maize.

In USA, using crop rotation has less value for *Diabrotica virgifera virgifera* management because the species has lost its fidelity for maize and lays eggs in fields planted with other crops followed by maize (Prasifka et al., 2013). In Europe, crop rotation is an effective tool for managing *Diabrotica virgifera virgifera* (Kiss et al., 2005b). In addition to crop rotation, and soil or foliar insecticide applications are frequently used in the EU to manage *Diabrotica virgifera virgifera* .

Using foliar insecticides to control adult *Diabrotica virgifera virgifera* presents several challenges. These include 1) the absence of appropriate machinery for pesticide application in maize fields in certain regions, 2) the fragmentation of arable land into small plots in some areas, 3) restrictions on aerial pesticide application, 4) wide non-target impacts, and 5) challenges in pesticide registration. Seed coating and the use of soil insecticides are preferred methods for protecting maize because they are less intrusive (Furlan et al., 2002). Chemical control of *Diabrotica virgifera virgifera* larvae has primarily relied on pyrethroid, neonicotinoid, and organophosphate insecticides. The soil insecticide, tefluthrin is particularly frequently used (Rozen and Ester, 2010).

Tefluthrin (applied as granular sometimes fluid) is a synthetic pyrethroid insecticide targeting soil-dwelling pests (Clark et al., 2012). Neonicotinoid insecticide seed treatments have been widely utilized in pest management systems due to their effectiveness in controlling a range of underground pests. Since then, the use of some neonicotinoids was banned under regulation (EU) No 485/2013. Subsequently thiacloprid was recommended for maize seed treatment because of its lower toxicity to honey bees. However, it is not widely used and may also be phased out in the future. Insecticide resistance in *Diabrotica virgifera virgifera* has developed both behaviourally and physiologically. Many insecticides have been withdrawn due to their non-target effects. In Europe, several safer alternative methods have been explored to reduce the population of *Diabrotica virgifera virgifera*. One example is the use of mating disruption with 8-methyl-2-decanolpropanoate (Xie et al., 1992). Other methods include breeding maize hybrids with native resistance and tolerance to *Diabrotica virgifera virgifera*, the attract-and-kill strategy (Schumann et al., 2014), and the application of entomopathogenic nematodes. However, these methods face implementation challenges and have not yet been widely adopted by growers.

1.4 Plant bio stimulants including microbial bio stimulants and their effects on crops

1.4.1 Definition of plant bio stimulants

A plant bio stimulant is defined as a “substance”, “microorganism”, “soil improver”, “plant strengthener”, “phyto stimulators”, or “plant conditioners” excluding nutrients and pesticides when it’s application to plants, or seeds enhances natural processes that give benefit on improving nutrient uptake, nutrient efficiency, tolerance to abiotic stress, or quality and crop yield (Du Jardin, 2005; Rouphael and Colla, 2020; Sharma et al., 2024). Under the new EU Fertilising Products Regulation (EU 2019/1009), bio stimulants are now classified as fertilisers and excluded from the plant protection regulation, can be commercialized more quickly and cost-effectively (Ricci et al., 2019). Plant bio stimulants can be divided into 2 categories as follow: microbial and non-microbial plant bio stimulants (Sharma et al., 2024).

1.4.2 Definition of microbial bio stimulant

Microbial bio stimulants are formulations of ingredients that consists a microorganism or a consortium of microorganisms which can be applied to plants, seeds, or soil (Castiglione et al., 2021; Babalola and Glick, 2012). It can contribute to improve plant health and productivity by promoting beneficial microbial interactions in the rhizosphere (Fadiji et al., 2022), enhancing nutrient availability, and inducing plant defence mechanisms (Farid et al., 2019; Pereira et al., 2021). It also refers to many terms such as “bio stimulators” (Palma et al., 2022), “bio protectors” (Morcillo and Manzanera, 2021), and “bio remediators” (Raklami et al., 2019). It can be made from the ingredient a group of fungi, bacteria, or algae (Johnson et al., 2023).

1.4.3 Effect of microbial bio stimulants on crops

1.4.3.1 Bacterial bio stimulant

Bacterial bio stimulant is one of innovative product with substance of living beneficial bacteria that enhance plant growth and health crops. Many of scientists have been reported the positive effects of bacterial bio stimulants against crops. It gives positive effects on crops by improving nutrient uptake, boosting soil health, and increasing stress tolerance on the crops (Choudhary et al., 2011). For example, *Bacillus amyloquafaciens*, a plant growth-promoting bacteria commonly called PGPB, a free-living in soil, rhizosphere, rhizoplane, and phylosphere bacteria were reported have positive effect on improving nutrient uptake on maize, wheat, rice, vegetables when it is mixed with *Azospirillum lipoferum*. Another example is *Bacillus subtilis* that were reported boosting soil health through mechanism increasing nutrient availability and uptake thus improve the soil structure by producing enzymes for breaking down the organic matter in soil

thus it releasing nutrients to plants for easily absorb (Ortiz and Estibaliz, 2022). *Pseudomonas fluorescens* and *Bacillus amyloliquefaciens* are the examples of bacterial bio stimulants that increasing stress tolerance on the crops by enhancing plant ability to abiotic stresses (drought, salinity or extreme temperature) through releasing hormones and enzymes in the root system of the crop (Inbaraj, 2021; Sangiorgio et al., 2020). *Pseudomonas aeruginosa* also were reported on capability on increasing stress tolerance against maize, wheat and mung beans plants (Sarma et al., 2014; Yasmeen et al., 2021). *Bacillus megaterium* were reported has capability on increasing stress tolerance against maize plants by increasing the ability of root on absorbing water under the salinity conditions (Marulanda et al., 2010).

1.4. 3. 2 Fungal bio stimulants

Fungal bio stimulant is a promising tool in modern agriculture. It divided to two group including mycorrhizal fungi and endophytic fungi. One of familiar fungal bio stimulant is arbuscular mycorrhizal fungi, called AMF, defined as a subset of two or three fungal species that specifically penetrate the cortical cells of plant roots. It reported to have positive effect on increasing plant resilience through improving the nutrient uptake, particularly ability plant on absorbing phosphor from the soil, and enhancing the soil structure and fertility. Furthermore, fungal bio stimulants help plants tolerate to abiotic stresses like drought and salinity by improving water retention and root growth.

Several number of fungal microorganisms with positive effect to plants are *Rhizophagus irregularis* - improving uptake of phosphorus on wheat and maize plants (Renaut et al., 2020) *Trichoderma harzianum* - enhancing root development on rice, soybean, and cucumber plants (Lian et al., 2023; Singh et al., 2023; Yao et al., 2023), *Aspergilus niger* - enhancing the phosphorus availability and uptake on maize, wheat and soybean (Tian et al., 2023, Naeem et al., 2021).

1.4.3. 3 Algae bio stimulant

Algal bio stimulant, a new approach in agriculture, derived from algae containing vitamins, amino acids, and hormones have been shown that enhance plant growth and productivity of crops (González-Pérez et al., 2022). He did reviewed work and highlighted that crop had positively affect including better rooting, higher crop yields, and increased resistance to abiotic stress. One of famous species of alga bio stimulants is *Chlorella vulgaris* were reported to increase biomass and fruit yield against tomato (Chiaiese et al., 2018), wheat, (García-González and Sommerfeld, 2016), and lettuce (Faheed and Fattah, 2008). Another example of algal bio stimulants is *Ascophyllum nodosum* that were reported enhancing growth performance of strawberry plants by reducing the

drought stress, improving growth parameters and fruit quality (Shakya et al., 2023). *Dunaliella salina* revealed the enhancing growth against lettuce and tomato plants by boosting their resistance to environmental stress (Arroussi et al., 2018).

2. Hypotheses and aim of the study

The major goal of this study was to better understand natural source agents for the control of the invasive alien western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in maize to widen the IPM toolbox for growers. One of the aims of this study was to review scientific papers in order to get information on existing or potential biological control properties of microbial plant bio stimulants as well as the knowledge gaps. Secondly, the aim of the study was to establish dose-efficacy-responses and minimum effective dosages of common pesticides against *Diabrotica virgifera virgifera* eggs, larvae, and adults under standardised laboratory conditions to facilitate a better choice of positive controls in standard bioassays on more sustainable control agents. This step was necessary because information on positive controls is often not openly available. Thirdly, and mainly the aim of this study was to better understanding the breadth and diversity of insecticidal and crop-enhancing effects of microbial biofertilizers and yield enhancers through applying experiments under standardised and semi-field conditions. To fulfil the third aim of this study, we tested the microbial bio stimulants agents we previously reviewed for their potential to affect *Diabrotica virgifera virgifera.* By confirming our findings, we aim to identify better control options for *Diabrotica virgifera virgifera* for farmers in the future, following integrated pest management systems. This will help reduce chemical pesticide use and improve the sustainability and resilience of maize cultivation.

1. **Do microbial bio stimulants have biological control properties and what may be their role in modern agriculture? (Chapter I.:** Biological control properties of microbial plant bio stimulant. A review

Commercial bio stimulants have been used in agriculture for decades. In recent years, the number of available products and their use by growers has markedly increased (Sible et al., 2021). This is, on one hand, because plant bio stimulants play a key role in further increasing crop yield and in maintaining long-term soil fertility, which is essential for meeting increasing food demands (Johnson et al., 2023). Many plant bio stimulants have been shown to improve the growth and yield of a crop by 10–40% (Nosheen et al., 2021). On the other hand, plant bio stimulants are usually not regulated under the legislation for plant protection products, which eases their faster and less costly commercialisation (Calvo et al., 2014). However, some microbial plant bio stimulants seem not only to improve soil fertility and/or crop productivity, but may also protect the plant from arthropod pests or plant diseases (Nosheen et al., 2021).

In conclusion, microbial bio stimulants can cause a broad diversity of effects. This leads to inconsistency on whether registered plant bio stimulants are solely stimulants or also have plant

protection properties comparable to plant protection products. In order to better understand the breadth and diversity of effects of microbial bio stimulants on crops, we reviewed such products that are commercially available. We chose countries that (a) actually have elaborated regulatory processes for microbial plant bio stimulants registration separated from plant protection product registration, and (b) have accessible databases of commercial microbial plant bio stimulant, such as Hungary, Switzerland, Spain, France, Canada and Indonesia. **We hypothesized that** many products of microbial plant bio stimulants may have, for example, additional insecticidal or plant defence effects, something that we assessed through reviewing literature databases, such as CAB Direct (CABI, 2022) and Web of Science (Clarivate, 2022). The intention of our review was, however, not to blame bio stimulants for their plant protection properties or to demand different registration processes. Our aim was to raise awareness about the multiple effects of plant bio stimulants on crops, something essential to be understood and considered by growers and other plant health system stakeholders.

2. **What are the microbial bio stimulants that can be useful for soil insect pest control?** (**Chapter II.:** Can microbial plant bio stimulants be useful for insect soil pest control? A review

Microbial plant bio stimulants are products that contain living cells of microorganisms which have the ability to enhance plant characteristics. However, many of them have recently been reported to also have insecticidal properties (Tarigan et al., 2022). Soil insect pests are a major problem in agriculture causing yield losses in many crops.

We hypothesized that some microbial bio stimulants have effect on soil insect pests. We therefore reviewed commercial microbial plant bio stimulants with regard to their effects on soil pests, such as on rootworms, a group with several key soil insect pests in the genus Diabrotica (Coleoptera: Chrysomelidae). This will help to identify multiple effects of products that not only promote plant growth but also offer protection against insect soil pests. By understanding their multiple effects, farmers can potentially reduce the need for chemical pesticides, leading to more sustainable agricultural practices. Additionally, this review aims to highlight gaps in current research and suggest directions for future studies to explore the insecticidal potential of microbial bio stimulants further.

3. **Which positive controls in egg, larva, and adult bioassay of *Diabrotica virgifera virgifera?*** (**Chapter III.:** Methods for high-throughput screening of novel agents against the maize pest, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte is a chrysomelid beetle that is one of the most important pests of maize (*Zea mays* L.) in the USA and Europe (Meinke et al., 2021). Its larvae feed on maize roots which can lead to plant instability, reduced growth and yield losses (Meinke et al., 2021). This pest has 7 developmental stages: egg, three larval instars, pre-pupa, pupa, and adult. *Diabrotica virgifera virgifera* is often difficult to control because its immature stages hide underground, the eggs take a relatively long time to hatch and the larvae feed on and in the maize roots or at least a month (Toepfer et al., 2006). With recent bans on key insecticides and concerns about overuse of remaining options, there is an urgent need for accessible and comparable screening methods. Dipping assays with ready-to-hatch eggs was used for egg bioassay. Artificial diet overlay assays were used for larvae bioassay, Artificial diet- core overlay assays were used for adult bioassay.

We hypothesized that there is one or more insecticides as positive control for egg, larvae and adult bioassay of *Diabrotica virgifera virgifera*. There will be one or more insecticides that act as good positive control that we defined it has high toxicity in small dosages tested to kill egg, larvae or adult with regard to their significance of effects (+ at $p < 0.05$), their least variable dose-response (+ if $X^2 > 300$ and $R^2 > 0.3$), and their highest toxicity (+ if lowest ED_{50}) when we analysed using probit analysis, linear and logarithmic regressions. We evaluated seven common insecticides (imidacloprid, clothianidin, acetamiprid, novaluron, cypermethrin, chlorpyrifos-methyl, spinosad) against eggs, larvae, and adults as potential positive controls for each of the proposed assay methods.

4. **Are microbial bio stimulants able to affect *Diabrotica virgifera virgifera* life stages, increasing the plant performance of maize crop and also preventing the root damage** (**Chapter IV.:** Effect of microbial bio stimulants on maize and its pest, the western corn rootworm, *Diabrotica virgifera virgifera*. **We hypothesized that** 1) some microbial bio stimulants can kill eggs, larvae or adults, 2) some microbial bio stimulants can increase the maize performance, reduce the root damage and the number of living larvae.

Plant bio stimulants are ingredients aimed solely at improving the agronomic performance of plants. Some microbial plant bio stimulants had effect on insect pest. *Diabrotica virgifera virgifera* is a serious pest affecting maize crops in Europe and the USA. We tested ten bio stimulants which represented a group of bacteria (5 species) including *Bacillus amyloliquefaciens*, *Bradyrhizobium japonicum*, *Rhizobium leguminosarum*, *Bacillus subtilis*, *Ensifer melliloti*, a group of fungi (4 species) including *Beauveria bassiana*, *Trichoderma harzianum*, *Trichoderma asperellum, Rhizophagus irregularis*, and a group of algae (1 species) such as *Chlorella vulgaris.* For assessing plant bio stimulants in laboratory, all the products were tested against eggs ready to

hatch, neonate larvae, and adults of *Diabrotica virgifera virgifera* using standard laboratory bioassay methods whereas for assessing the potential effect of microbial bio stimulants to maize crop performances and *Diabrotica virgifera virgifera* larvae under semi filed conditions, all products tested were applied as maize seed treatments. All microbial bio stimulants were diluted with unsterilized tap water. Maize seeds were treated with a diluted microbial bio stimulant using a pipette, applied to the surface area of the seeds, and then immediately covered with soil. Three dosages were applied per treatment, made following the recommended dosages as written in the label of products. Untreated control was served as maize seed treated with unsterilized tap water only. Our findings will help to identify which microbial bio stimulants are most effective in combating *Diabrotica virgifera virgifera* at different life stages, as well as those that can enhance maize crop performance and protect against root damage. This research can lead to the development of more integrated pest management strategies, reducing reliance on chemical pesticides and promoting sustainable agriculture. Additionally, our study will provide valuable insights into the dual- function capabilities of microbial bio stimulant, guiding future research and application in crop protection.

3. Chapter I.

Biological control properties of microbial plant bio stimulants. A review

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3.1 Abstract and Introduction

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REVIEW ARTICLE

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Biological control properties of microbial plant biostimulants. A review

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ABSTRACT

Plant biostimulants, sometimes referred to as biofertilisers or plant enhancers, are ingredients stimulating plant nutrition processes independently of the product's nutrient content with the sole aim to improve the agronomic performance of a plant. Many of these biostimulants contain microorganisms. Although most of these microorganisms are supposed to only promote plant growth, some have plant protection properties. We reviewed commercial microbial plant biostimulants with regard to their potential effects on insects. This revealed 483 different products and 245 microorganisms registered as microbial plant biostimulants in Hungary, Switzerland, Spain, France, Indonesia, and/or Canada (181 ± 157 products, 64 ± 27 species per country). Among the products, 82% contained bacteria (133 ± 106 products), 63% contained fungi (77 ± 59) and 14% contained protista including algae (23 ± 24). About 1/3rd of products contained mixes of either bacteria, fungi, and/or protista; and 48% contained more than one microorganism. About 53% of products (137 ± 121) contained microorganims that had been reported to have insecticidal properties and 36% of species (23 ± 9), although the underlaying mechanisms often remain unknown. About 67% of products (149 ± 133) contained microorganisms reported to defend a plant from insects, and 54% of species (35 ± 10). The most common biostimulant microorganisms with reported insecticidal effects were strains of *Rhizophagus irregularis*, followed by *Bradyrhizobium japonicum*, *Rhizobium leguminosarum*, *Bacillus megaterium*, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *Penicillium bilaiae*, *B. pumilus* and *Ascophylum nodosum*. In conclusion, growers may profit from, but should be made aware of the multiple effects of microbial plant biostimulants.

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* Larger figures are available in the appendices

Introduction

Plant biostimulants may be referred to as biofertilisers, soil conditioners, phytostimulators, biostimulatory agents, plant strengtheners, crop enhancers, or similar. Many different definitions exist (Albrecht, 2019; du Jardin, 2015; Ricci et al., 2019; Yakhin et al., 2017). The European Union defines them as ingredients that stimulate plant nutrition processes independently of a product's nutrient content with the sole aim of improving the characteristics of a plant (EU, 2019). Plant biostimulants are usually applied to planting materials or growing substrates and sometimes as foliar treatments. They are claimed to have the capacity of modifying physiological processes of a plant in a way that provides benefits to nutrient uptake or efficiency, and/or to plant growth or stress tolerance (Yakhin et al., 2017).

Commercial biostimulants have been used in agriculture for decades. In recent years, the number of available products and their use by growers has markedly increased (Sible et al., 2021). This is, on one hand, because plant biostimulants play a key role in further increasing crop yield and in maintaining long-term soil fertility, which is essential for meeting increasing food demands (Nosheen et al., 2021). Many plant biostimulants have been shown to improve the growth and yield of a crop by 10–40% (Nosheen et al., 2021). On the other hand, plant biostimulants are usually not regulated under the legislation for plant protection products, which eases their faster and less costly commercialisation (Daniel et al., 2014).

Many biostimulants are based on microorganisms, and are therefore called microbial plant biostimulants (EU, 2019). Such microorganisms are usually either bacteria, fungi or protista including several types of algae. When inoculated to the seed or the soil, they often systemically colonise the rhizosphere and the interior of a plant, whilst promoting plant growth (Berruti et al., 2016). Others add or activate nutrients in the soil. For example, the fungus *Glomus mosseae* (syn. *Funneliformis mosseae*) (Glomerales: Glomeraceae) is used to enhance growth and yield of crops like groundnuts. This is likely due to increased enzyme activities of the crop (alkaline phosphatase and nitrate reductase) and increased nutrient levels in the soil (nitrogen, phosphorus, potassium) after application of the biostimulant (Pawar et al., 2020). The bacterium, *Bacillus licheniformis* produces auxin, antifungal β-glucanases and siderophores which stimulate seed germination and promote the growth of vegetative plant organs, i.e. roots, stems or leaves, such as in mungbean (Lim, 2009).

However, some microbial plant biostimulants seem not only to improve soil fertility and/or crop productivity, but may also protect the plant from arthropod pests or plant diseases (Nosheen et al., 2021). These can be direct pesticidal effects of an ingredient resulting in destroying or mitigating a pest (Suiter & Scharf, 2008) or indirect effects of plant responses to an ingredient resulting in a better defence to pests (Fürstenberg-Hägg et al., 2013). A classic example is the fungus *Rhizophagus (syn. Glomus) intraradices* (Glomerales: Glomeraceae) which, on one hand, enhances crop tolerance to abiotic stresses and therefore increases yield, such as in tomato (Shirazi et al., 2018; Volpe et al., 2018). On the other hand, it seems to negatively affect the larval development of *Spodoptera exigua* (Lepidoptera: Noctuidae) (Lei et al., 2017; Shrivastava et al., 2015). Similar effects have been observed on *Spodoptera litura* (Lepidoptera: Noctuidae) in black gram (Selvaraj et al., 2020). *Rhizophagus intraradices* is also known to shorten the

3.2 Materials and Methods

development time of nymphs and longevity of adults of *Myzus persicae* (Hemiptera: Aphididae) (Mardani-Talaee et al., 2017), or to reduce oviposition of *Lissorhoptrus oryzophilus* (Coleoptera: Curculionidae) on rice (Cosme et al., 2011). The suggested reason is that *Rh.. intraradices* increases the quantity of plant defence metabolites such as phenolics, lignin, superoxide dismutase, peroxidase, catalase, phenylalanine ammonia lyase, or polyphenol oxidase. Next to insecticidal, also fungicidal effects have been reported, such as against *Rhizoctonia solani* (Cantharellales: Ceratobasidiaceae) in beans (Hafez et al., 2013); or even nematocidal effects such as against *Meloidogyne javanica* (Tylenchida: Heteroderidae) in pistachio (Mehdinejad et al., 2021).

Other microorganisms in plant biostimulants may be beneficial to insects. An example is the alga *Chlorella vulgaris* (Chlorellales: Chlorellaceae) which, on one hand, enhances the germination of tomatoes (Bumandalai & Tserennadmid, 2019) or enhances the growth and stress resistance in tomatoes and guar plants, likely through increasing β-1,3 glucanase activity, and remodelling phenylalanine ammonia lyase, lipoxygenase and activities of antioxidant enzymes (Kusvuran & Kusvuran, 2019; Rachidi et al., 2019). On the other hand, *C. vulgaris* has been reported to increase growth and productivity of bee families (Eremia et al., 2013), or to increase numbers of adults produced by *Forcipomyia taiwana* (Diptera: Ceratopogonidae), a haematophagous insect pest of humans (YiPey, 2018).

In conclusion, microbial biostimulants can cause a broad diversity of effects. This leads to inconsistency on whether registered plant biostimulants are solely stimulants or also have plant protection properties comparable to plant protection products.

In order to better understand the breath and diversity of effects of microbial biostimulants on crops, we reviewed such products that are commercially available. We chose countries that (a) actually have elaborated regulatory processes for microbial plant biostimulant registration separated from plant protection product registration, and (b) have accessible databases of commercial microbial plant biostimulants, such as Hungary, Switzerland, Spain, France, Canada and Indonesia. We hypothesised that many products of microbial plant biostimulants may have, for example, additional insecticidal or plant defence effects, something that we assessed through reviewing literature databases, such as CAB Direct (CABI, 2022) and Web of Science (Clarivate, 2022). The intention of this review was, however, not to blame biostimulants for their plant protection properties or to demand different registration processes. Our aim was to raise awareness about the multiple effects of plant biostimulants on crops, something essential to be understood and considered by growers and other plant health system stakeholders.

Material and methods

In contrast to plant protection products, not many countries have a detailed registration processes and/or an accessible and searchable database for plant biostimulants. We tried to review biostimulants from a representative number of countries. We succeeded to extract details from all microbial plant biostimulants registered in Hungary (NEBIH, 2020), Switzerland (Federal Office of Agriculture BLW, 2020), Spain (Ministry of Agriculture, Fisheries and Food, MAPA, 2020), France (L'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail Anses, 2020), Indonesia

(Direktorat Pupuk dan Pestisida, 2020) and Canada (Canadian Food Inspection Agency, 2020). In Hungary microbial plant biostimulants are referred to as 'Yield enhancers: microbiological preparation' (Termésnövelők, mikrobiológiai készítmények) (Haller et al., 2021). In Switzerland these are referred to as 'Fertilizers: based on microorganisms for soil, seed or plant treatments' ('Duenger: Kulturen von Mikroorganismen zur Behandlung von Boeden, Saatgut oder Pflanzen'). In France these can be found under 'Fertilizers and growth promoters: fungal or bacterial preparation' ('MFSC Matières Fertilisantes et Supports de Culture: préparation fongique / préparation bactérienne') and in Spain under 'Fertilizer products: Mycorrhizae, Non-mycorrhizal microorganisms, Fertilizers with non-mycorrhizal microorganisms, Mixture of microorganisms, Fertilizer with microorganisms' (Consulta de productos fertilizantes:4401 Micorrhizas / 4403 Microorganismos no micorrícicos / 4404 Abono con microorganismos no micorrícicos / 4405 Mezcla de microorganismos / 4406 Abono con microorganismos). In Indonesia, they are registered under 'organic biofertilizer: biofertilizer and soil enhancers: organic' ('Rekap pupuk organik: Pupuk hayati dan pembenah tanah, Hayati') without a separate category for microbial plant biostimulants. In Canada, they are registered under 'fertilizers: registered supplements', without a separate category for microbial plant biostimulants. For each product, microorganism species, orders, families, product trade name, and usage were recorded where available.

Once all microbial plant biostimulants had been extracted, each individual microorganism was reviewed for its potential effects on insects. We used the literature data bases of CAB Direct 1917to 2020 (CABI, 2022) and Web of Science 1973 to 2020 (Clarivate, 2022). We searched abstracts of scientific publications containing information on effects of a microorganism using the search terms 'name of microorganism species' AND 'insect'. Data were then averaged across countries, and descriptive statistics were applied.

Results

Microbial plant biostimulants

In total, 483 commercial products of microbial plant biostimulants are registered in Hungary (116 products), Switzerland (176), Spain (58), France (71), Indonesia (182), and/or Canada (483) in 2020 (see table in supplementary materials) with an average of 181 ± 157 SD products per country (median 146; Figure 1). These contain a total of 245 registered microorganism species, with 64 ± 27 per country (median 66). In Hungary, 103 such microorganisms are registered in plant biostimulants, followed by Canada (88 species), Indonesia (69), Switzerland (66), France (37) and Spain (36). Bacteria appeared to be the most common microorganisms used in these plant biostimulants (41 ± 19 species /country, 62% of biostimulants; median 41) followed by fungi (21 ± 7, 32%; median 24) and protista including algae (5 ± 2, 6%; median 4). This is also true for the number of products; 133 ± 105 products contained bacteria (82%; median 108), 77 ± 59 fungi (63%; median 56), and 24 ± 2 protista (14%; median 15).

Some biostimulants contain several microorganism groups mixing fungi, bacteria, and/or protista (Figure 2). About 29% of products (214 ± 116 SD) contain bacteria-fungi mixes, 0.5% contain bacteria-protista mixes (4 ± 3), 0.3% fungi-protista mixes (3 ± 4) and 1.3% mixes of all three groups (9 ± 9).

3.3 Results and Discussion

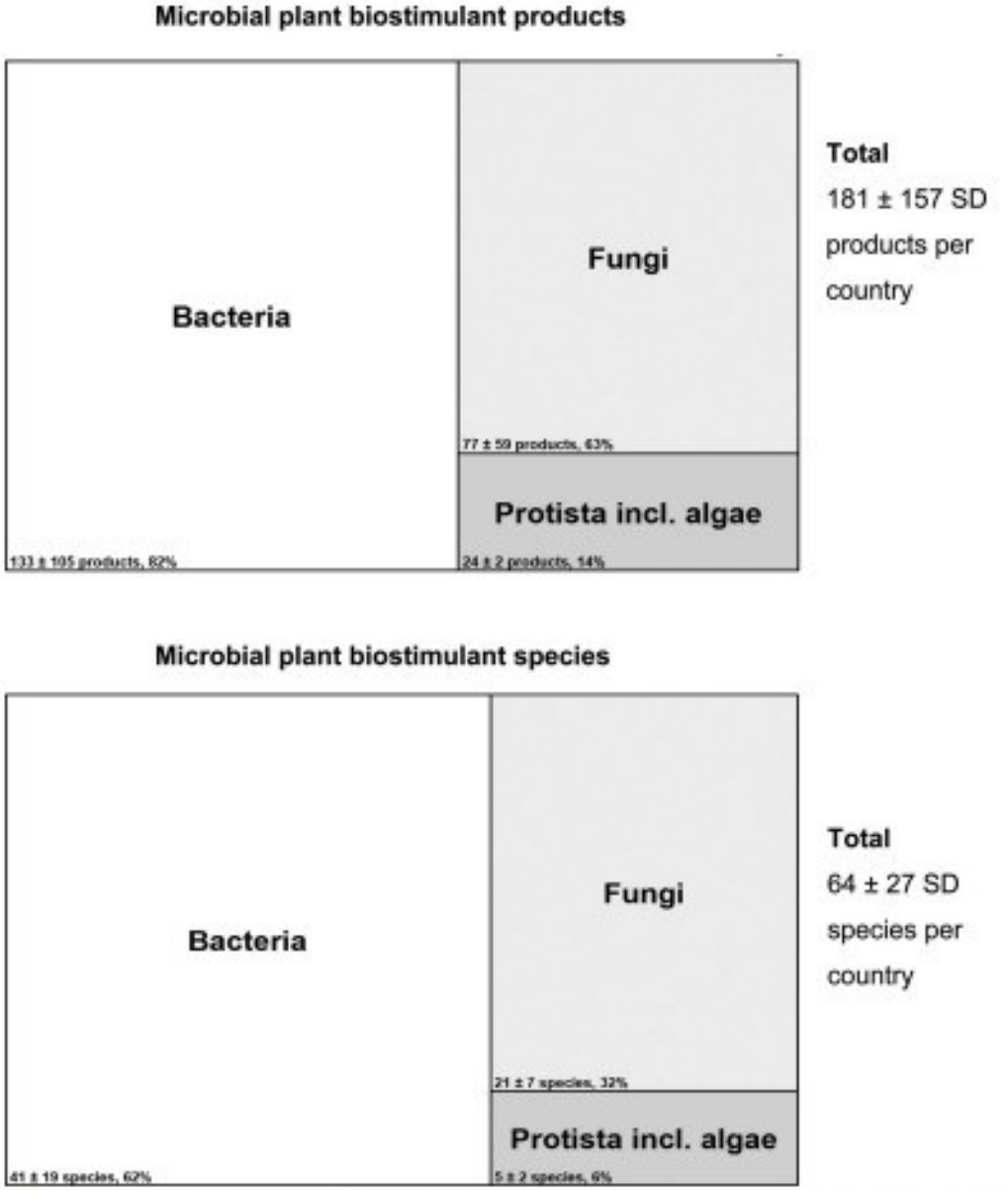

Figure 1. Commercial microbial plant biostimulants averaged per country. In total, 483 biostimulant products with 245 microorganism species from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada) by 2020. Some products contain several microorganisms from bacteria and fungi and/or protista.

About half of the biostimulants contain several microorganism species (48%, Figure 2). Around 2–16% of products either contain two, three, four, five, six, or even seven microbial ingredients. Few products (around 0.1–0.7%) contain more than seven and even up to sixteen microorganism species in a single product. On average, 52% of products (96 ± 120: median 48) contain only one microorganism species.

Bacterial biostimulant products contain species from at least 18 orders (Table 1). Most bacterial products are based on organisms from the order of Rhizobiales followed by

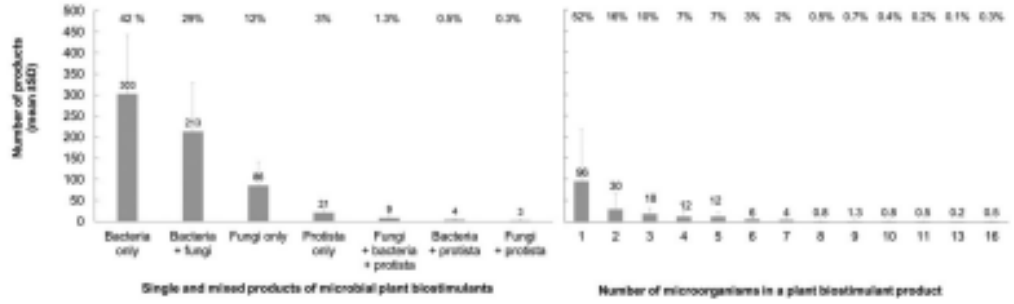

Figure 2. Average numbers and percentages of products of registered microbial plant biostimulant per country containing single or multiple microorganism groups and single or multiple species. 483 products with 245 microorganism species from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada) by 2020. Error bars = standard deviation.

Bacillales, Pseudomonadales, Rhodospirillales. The most dominant bacterial species in biostimulant products is *Bradyrhizobium japonicum* (Rhizobiales: Bradyrhizobiaceae), followed by *Rhizobium leguminosarum* sometimes referenced as *Bradyrhizobium japonicum* (Hyphomicrobiales: Rhizobiaceae), *Bacillus megaterium, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus pumilus* (all Bacillales: Bacillaceae), *Pseudomonas fluorescens* (Pseudomonadales: Pseudomonadaceae), *Azospirillum brasilense* (Rhodospirillales: Azospirillaceae), and *Azotobacter chroococcum* (Pseudomonadales: Pseudomonadaceae).

Fungal products contain species from 14 orders (Table 1). Most fungal products are based on organisms from the orders of Glomerales followed by Hypocreales, Eurotiales, and Saccharomycetales. The most dominant fungal species in biostimulant products is *Rhizophagus irregularis* (syn. *Glomus irregulare, Rhizoglomus irregulare)* (Glomerales: Glomeraceae). This is followed by *Penicillium bilaiae* (Eurotiales: Trichocomaceae), *Trichoderma harzianum* (recently some strains re-classified as *Trichoderma asperellum*), *Glomus mosseae* (Glomerales: Glomeraceae), *Trichoderma virens* (syn. *Gliocladium flavofuscum, Gliocladium virens, Trichoderma flavofuscum, Hypocrea virens*) (Hypocreales: Hypocreaceae). This is then followed by *Claroideoglomus etunicatum* (Glomerales: Claroideoglomeraceae), *Phanerochaete chrysosporium* (Polyporales: Phanerochaetaceae), *Saccharomyces cerevisiae* (Saccharomycetales: Saccharomycetaceae), and *Trichoderma reesei* (Hypocreales: Hypocreaceae).

Products of protista including algae contain species from at least seven orders. Most products came from the order of Fucales followed by Chlorellales, Sphaeropleales, and Chlamydomonadales. The most dominant protista species in biostimulant products is *Ascophylum nodosum* (Fucales: Fucaceae) followed by *Chlorella vulgaris* (Chlorellales: Chlorellaceae), *Chlamydomonas reinhardtii,* (Chlamydomonadales: Chlamydomonadaceae) and *Sargassum hemiphyllum* (Fucales: Sargassaceae).

Microbial plant biostimulants with effects on insects

Many microorganisms in commercial plant biostimulants appeared to have, next to their plant stimulating functions, also insecticidal and/or insect plant defense properties (Figure 3). In few cases, such microorganisms can be also beneficial to insects, such as

Table 1. Ten most common orders, genera, and species of microorganisms registered in products of microbial plant biostimulants.

	# Products per country							
	Mean	SD		Mean	SD		Mean	SD
Bacteria								
Orders [18]			Genus [55]			Species [153]		
Rhizobiales	66	89	*Bacillus*	100	52	*Bradyrhizobium japonicum*	21	38
Bacillales	61	36	*Rhizobium*	29	46	*Rhizobium leguminosarum* [1]	19	44
Pseudomonadales	36	43	*Pseudomonas*	27	31	*Bacillus megaterium*	18	14
Rhodospirillales	23	29	*Bradyrhizobium*	26	43	*Bacillus subtilis*	16	13
Lactobacillales	14	19	*Azospirillum*	24	29	*Bacillus amyloliquefaciens*	15	18
Actinomycetales	11	15	*Azotobacter*	23	31	*Bacillus licheniformis*	9	8
Enterobacterales	2	4	*Lactobacillus*	17	19	*Bacillus pumilus*	9	11
Cellvibrionales	2	4	*Rhodopseudomonas*	8	15	*Pseudomonas fluorescens*	8	8
Burkholderiales	2	2	*Streptomyces*	7	12	*Azospirillum brasilense*	6	7
Bifidobacteriales	0.8	1.0	*Paenibacillus*	3	4	*Azotobacter chroococcum*	5	10
Fungi								
Orders [14]			Genus [40]			Species [79]		
Glomerales	32	36	*Glomus*	27	39	*Rhizophagus irregularis* [2]	26	36
Hypocreales	23	12	*Trichoderma*	26	16	*Penicillium bilaiae*	9	18
Eurotiales	15	18	*Saccharomyces*	12	18	*Trichoderma harzianum* [5]	8	9
Saccharomycetales	13	18	*Penicillium*	11	18	*Glomus mosseae* [3]	4	5
Polyporales	2	4	*Rhizophagus*	10	7	*Trichoderma virens* [4]	4	9
Helotiales	2	1.4	*Aspergillus*	5	9	*Trichoderma asperellum* [5]	3	4
Boletales	1.3	2	*Funneliformis*	3	4	*Claroideoglomus etunicatum*	3	4
Diversisporales	1.0	2	*Claroideoglomus*	3	5	*Phanerochaete chrysosporium*	2	4
Agaricales	0.8	1.3	*Rhizopogon*	3	6	*Saccharomyces cerevisiae*	2	2
Sordariales	0.3	0.8	*Phanerochaete*	2	4	*Trichoderma reesei*	2	4
Protista including algae								
Orders [7]			Genus [11]			Species [13]		
Fucales	9	20	*Ascophylum*	8	19	*Ascophylum nodosum*	8	19
Chlorellales	2	4	*Chlorella*	2	4	*Chlorella vulgaris*	2	4
Sphaeropleales	0.7	1.6	*Scenedesmus*	0.7	2	*Algae (unspecified)*	0.5	1.2
Chlamydomonadales	0.5	1.2	*Chlamydomonas*	0.5	1.2	*Chlamydomonas reinhardtii*	0.5	1.2
Phaeophyceae	0.2	0.4	*Phaeophyceae*	0.5	1.2	*Sargassum hemiphyllum*	0.5	1.2

(*Continued*)

Table 1. Continued.

	# Products per country							
	Mean	SD		Mean	SD		Mean	SD
Klebsomidiales	0.2	0.4	*Sargassum*	0.5	1.2	*Chlamydopodium fusiforme*	0.3	0.8
Chordariales	0.2	0.4	*Chlamydopodium*	0.3	0.8	*Durvillea potatorum*	0.3	0.8
			Durvillea	0.3	0.8	*Scenedesmus armatus*	0.3	0.8
			Eckolina	0.2	0.4	*Scenedesmus obtisausculus*	0.3	0.8
			Klebsormidium	0.2	0.4	*Eckolina maxima*	0.2	0.4
All microbials								
Orders [39]			Genus [105]			Species [245]		
Bacillales	11	3	*Bacillus*	100	52	*Rhizophagus irregularis*	26	36
Rhizobiales	10	8	*Rhizobium*	29	46	*Bradyrhizobium japonicum*	21	38
Glomerales	8	2	*Pseudomonas*	27	31	*Rhizobium leguminosarum*	19	44
Pseudomonadales	6	3	*Glomus*	27	39	*Bacillus megaterium*	18	14
Lactobacillales	6	5	*Bradyrhizobium*	26	43	*Bacillus subtilis*	16	13
Hypocreales	6	2	*Trichoderma*	26	16	*Bacillus amyloliquefaciens*	15	18
Boletales	5	3	*Azospirillum*	24	29	*Bacillus licheniformis*	9	8
Actinomycetales	4	5	*Azotobacter*	23	31	*Penicillium bilaiae*	9	18
Cellvibrionales	4	0	*Lactobacillus*	17	19	*Bacillus pumilus*	9	11
Enterobacterales	3	3	*Saccharomyces*	12	18	*Ascophylum nodosum*	8	19

Note: In total, 483 products and 245 microorganism species by 2020 reviewed from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada). Average product numbers shown per country. In brackets [ ] total number.

1 syn. *Bradyrhizobium japonicum*

2 syn.*Glomus intraradices*, *Rhizophagus intraradices*, *Glomus irregulare*, *Rhizoglomus irregulare*, *Glomus irregulare*

3 syn. *Funneliformis mosseae*

4 syn. *Gliocladium flavofuscum*, *Gliocladium virens*, *Trichoderma flavofuscum*, *Hypocrea virens*

5 some strains of *Trichoderma harzianum* re-classified as *Trichoderma asperellum*.

serving as a food source. For the full list of microorganisms and their effects on insects refer to supplementary material table.

Briefly, 36% of microorganism species (23 ± 9 SD), registered as microbial plant biostimulants, were reported in the literature to have insecticidal properties (Figure 3). Consequently, about half of the commercial products of microbial biostimulants may have insecticidal properties (53%, 137 ± 121). Most of those products and species of plant biostimulants with insecticidal properties originate from the bacterial kingdom (15 ± 5 thus 36% of species; 101 ± 91 thus 56% of products). This is followed by fungi (8 ± 4, 37% of species; 51 ± 41, 58% of products), and finally protista including algae (1 ± 1, 38% of species, 2 ± 4, 19% of products).

Among the most frequently found bacteria in biostimulants having insecticidal properties are strains of *Bradyrhizobium japonicum*, followed by *Rhizobium leguminosarum*, *Bacillus megaterium* and *Bacillus subtilis* (Figure 4). For example, there are on average 21 ± 38 products containing *B. japonicum* per country. Next to its plant stimulating properties, this bacteria was reported to also attack insects such as *Callosobrochus maculatus* (Coleoptera: Chrysomelidae), a pest of cowpea grains (Naseri & Hamzavi, 2021) or *Phthorimaea operculella* (Lepidoptera: Gelechiidae), a pest of potatoes (Murray et al., 2010). *Rhizobium leguminosarum* was, for example, reported to negatively affect *Sitona lineatus* (Coleoptera: Curculionidae), a pest of peas (Vankosky et al., 2011). *Bacillus megaterium* was reported to negatively affect *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) on oil palms and dates (Francesca et al., 2015), *Xyleborus dispar* (Coleoptera: Scolytidae) in hazelnuts (Sezen et al., 2008), and *Aphis pomi* (Hemiptera: Aphididae) in apples (Aksoy & Ozman-Sullİvan, 2008). Finally, *B. subtilis* was reported to reduce *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) in potatoes (Sorokan et al., 2016).

Among the most frequently found fungi in biostimulants with insecticidal properties are strains of *Rhizophagus irregularis* (Glomerales: Glomeraceae) followed by *Trichoderma harzianum, T. virens, and T. asperellum*, and then *Saccharomyces cerevisiae* (Saccharomycetales: Saccharomycetaceae), *Aspergillus niger*, *Aspergillus oryzae* (both Eurotiales: Trichocomaceae), *Trichoderma viride* and *Beauveria bassiana* (Helotiales: Orbiliaceae) (Figure 4). For example, there are on average 16 ± 31 products containing *R. irregularis* per country. Next to its plant-stimulating properties, this species was reported to negatively affect insect pests such as *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on maize (Yan et al., 2021). The second most used fungus, *T. harzianum* negatively affects *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae), a pest on common beans (Gad et al., 2020) and *Nezara viridula* (Hemiptera: Pentatomidae), a pest on soybean (Alınç et al., 2021). *Trichoderma virens* for example negatively affects *Gryllotalpa gryllotalpa* (Orthoptera: Gryllotalpidae), a pest on cowpea, soybean, and other crops (Veena-Bhamrah, 2007). *Trichoderma asperellum* attacks *Helopeltis theivora* (Hemiptera: Miridae), a pest on tea (Kumhar et al., 2020). *Saccharomyces cerevisiae* attacks *Anopheles arabiensis* (Diptera: Culicidae), a pest on humans (Makhanya et al., 2020). There are many more examples, as represented in the table in the supplementary materials.

The most frequently found protista or algae in biostimulants with insecticidal properties are *Chlorella vulgaris* or *Chlamydomonas reinhardtii* (Chlamydomonadales: Chlamydomonadaceae) (Figure 4). *Chlorella vulgaris*, for example, negatively affects larvae of

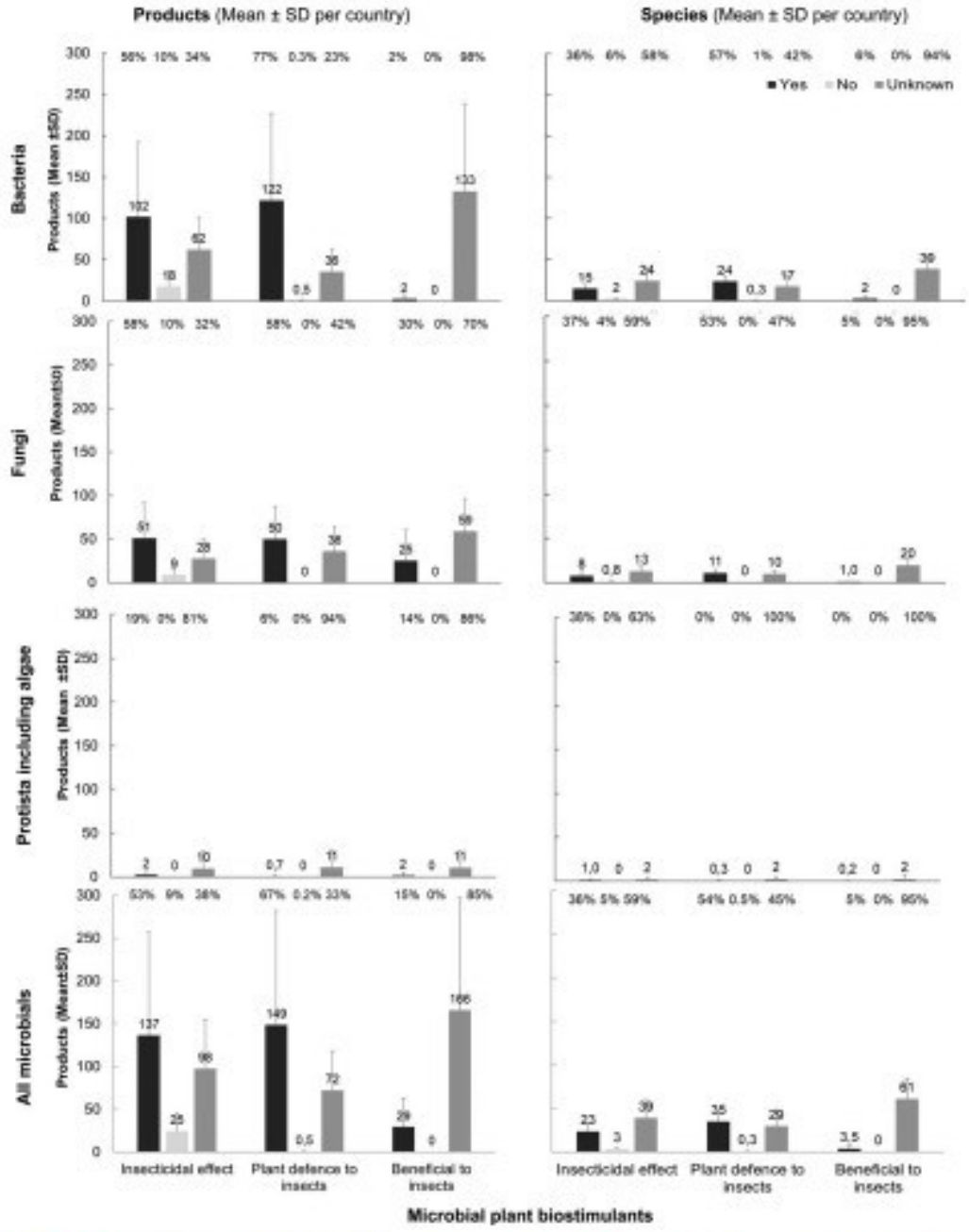

Figure 3. Average numbers and percentages of commercial microbial plant stimulants with potential effects on insects. Insecticidal, plant defence and beneficial effects of microorganism species with regard to insects reviewed in the literature as per Web of science (Clarivate 2022) and CAB Direct (CABI, 2022), but strain level information rarely available. In total, 483 products with 245 microorganism species reviewed from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada) by 2020. Averages per country shown with standard deviation as error bars.

Chironomus riparius (Diptera: Chironomidae) (Purushothaman & Mol, 2020); and *C. reinhardtii* affects *Aedes aegypti* (Diptera: Culicidae) both being pests of humans (Fei et al., 2021).

About 54% of microorganism species (35 ± 10 species) registered as microbial plant biostimulants, were reported in the literature to improve plant defence properties (Figure 3).

Among the most frequently used bacteria with reported plant-defence properties to insects are strains of *Bradyrhizobium japonicum, Rhizobium leguminosarum, Bacillus megaterium, Bacillus subtilis, Bacillus amyloliquefaciens* (Figure 4). For example, *Bradyrhizobium japonicum* was reported to improve the defence of tobacco and potatoes to *Phthorimaeae oppercullella* (Lepidoptera: Gelechiidae) (Murray et al., 2010), likely through adding certain proteins to the cell vacuoles of the crop. The same bacteria seems to also improve the defence of soybean to certain insect pests, such as *Epilachna varivestis* (Coleoptera: Coccinelidae) (Pulido et al., 2019), to collembola like *Folsomia candida* or *Tulbergia granulate* (both Collembola: Isotomidae) (Lussenhop, 1993) or to *Helicoverpa zea* (Lepidoptera: Noctuidae) (Dean et al., 2014). The latter likely due to the induction of defense signalling pathways reducing feeding preferences (Dean et al., 2014).

Among the most frequently used fungi with plant-defence properties to insects are strains of *Rhizophagus irregularis, Trichoderma harzianum, Trichoderma asperellum, Glomus mosseae, Phanerochaete chrysosporium* (Figure 4). For example, the inoculation of *R. irregularis* to the roots of black gram can induce the release of defence metabolites from the plant such as phenolics, lignin or superoxide radical quenching enzymes (superoxide dismutase, peroxidase, catalase, phenylalanine ammonium lyase, polyphenol oxidase). Those reduce the feeding activity of *Spodoptera litura* (Lepidoptera: Noctuidae) (Selvaraj et al., 2020). Another example is *Trichoderma harzianum* inoculated to the roots of tomato crops reduces the growth of *Nezara viridula* (Hemiptera: Pentatomidae) through an increase in jasmonic acid (Alınç et al., 2021). There are hardly any protista that seem to induce plant defence to insects. Algae in general are sometimes claimed to have such properties (Bouissil et al., 2020; Singh et al., 2009). For example, *Fucus spiralis* (Fucales: Fucaceae), which is a commercial biostimulant from Morocco, was reported to induce defence of date palms to *Oligonychus afrasiaticus* (Trombidiformes: Tetranychidae) through eliciting the activity of sulphated polysaccharides (Bouissil et al., 2020).

About 8% of microorganism species (32 ± 36 species), registered as plant biostimulants, were reported in the literature to be beneficial to insects (Figure 3). For example, among the bacteria, *Micrococcus roseus* was reported beneficial to *Odontotermes obesus* (Blattodea: Termitidae) through producing endogenous and exogenous cellulase in the gut of the termite increasing its feeding activity (Sarkar et al., 1988). Another example is *Lactobacillus plantarum* (Lactobacillales: Lactobacillaceae) being beneficial to *Dacus ciliatus* (Diptera: Tephritidae) on pumpkin, primarily through reducing development time of larvae (Rempoulakis et al., 2018). It appears also beneficial to *Apis mellifera* (Hymenoptera: Apidae) through competing out the bee pathogen *Paenibacillus larvae* (Bacillales: Paenibacillaceae) (Daisley et al., 2020). Also, *Lactobacillus casei* (Lactobacillales: Lactobacillaceae) has probiotic effects in the digestive tract of honey bees, such as of *Apis mellifera carpatica* (Hymenoptera: Apidae) (Pătruică & Mot, 2012). Finally, *Lactobacillus acidophilus* helps *Galleria mellonella* (Lepidoptera: Pyralidae), a pest of bee colonies, to compete out the *Galleria* pathogen *Candida albicans* (Saccharomycetales: Saccharomycetaceae) (Vilela et al., 2015).

The only used fungi in biostimulants that was reported to be beneficial to certain insects is *Rhizophagus irregularis*. This is astonishing, as the same fungus, seems, as

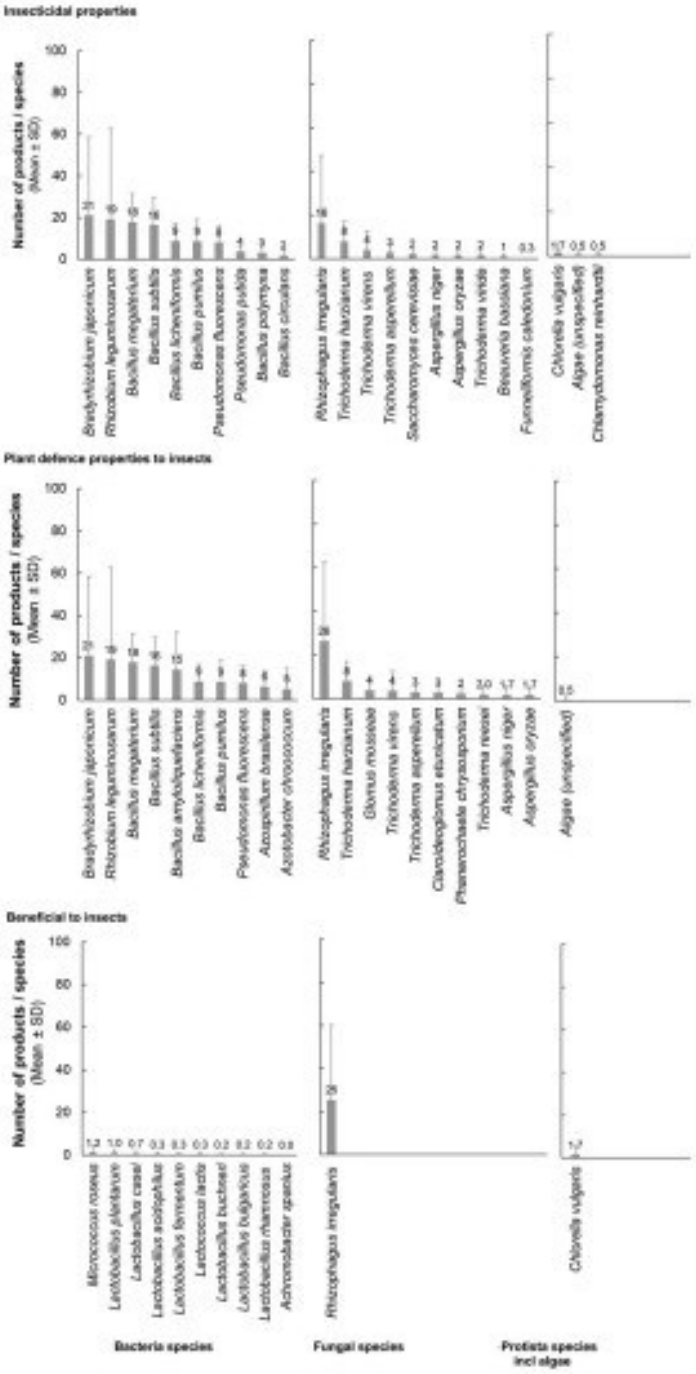

Figure 4. Most common microorganisms in commercial plant biostimulants with potential side effects on insects. Insecticidal, plant defence and beneficial effects of microorganism species with regard to insects reviewed in the literature as per Web of science (Clarivate 2022) and CAB Direct (CABI 2022), but strain level information rarely available. In total, 483 products with 245 microorganism species reviewed from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada) by 2020. Averages per country shown with standard deviation as error bars.

reported above to have insecticidal or plant defence effects against some insects. However, this arbuscular mycorrhizal fungal species when inoculated to the roots of tomato crops seems to improve the foraging of the predatory mirid bug, *Macrolophus pygmaeus* (Hemiptera: Miridae) towards the crop and prey, although reasons behind this mechanism remain unknown (Prieto et al., 2017). *Rhizophagus irregularis* inoculated to the roots of alfalfa can increase the crop`s hormones levels (e.g. β-1,3 glucanase, thaumatin-like protein, ethylene response factor 1, gibberellin 20-oxidase, GA 2-oxidase) which then increase the feeding and fitness of the pea aphid, *Acyrthosiphon pisum* (Garzo et al., 2018).

There are only two protista or algae species in biostimulants that have been reported beneficial to insect, this is, *Chlorella vulgaris* and *Chlamydomonas reinhardtii* (Chlamydomonadales: Chlamydomonadaceae). This is astonishing as the same species have been also reported to negatively affect some insects (see above). However, when an algal suspension of *Chlorella vulgaris* is added to the feed for bee colonies this can increase the growth and productivity of bees hives (Eremia et al., 2013). This alga species was also added as a feeding additive for experimental colonies of the fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae) whilst increasing the body mass and travel distances of the flies (Shuang et al., 2019).

Discussion

This review revealed an enormous variety of microbial-based biostimulants available for growers to improve their crop production. These commercial products appeared to be based on at least 245 different microorganism species, as reviewed here from six representative countries. This largely outpaces the number of registered microbial biopesticides (CABI, 2022). This might be due to the usually easier registration processes of biostimulants than of plant protection products (Daniel et al., 2014; Huber, 2017). Also, their relatively easy application as seed coating, granules or simply as sprays may have contributed to their success. Finally, and potentially most prominently, they seem to indeed improve yields by 10–40% and this in many different cropping systems and under different conditions (Nosheen et al., 2021).

Interestingly, bacteria species seem to contribute more to biostimulant products for agriculture than fungi or other microorganism groups. For example, 82% of products contain bacteria, and only 63% contain fungi and even less contain protista including algae (14%). Interestingly, and potentially problematically, many biostimulants contain bacteria-fungal mixes (29%), and some contain bacteria-protista mixes or fungi-protista mixes or even mixes of all three groups. Although, such mixes may increase chances of products to reach crop enhancements under diverse agro-ecological conditions, it makes it difficult to understand and attribute observed effects to certain microorganisms. Our results showed that only half (52%) of all the 483 reviewed products contained only a single microorganism species. In contrast, many products contain multiple organisms, and few products contain even up to 16 different microorganisms. This is critical, as multiple effects can, as stated before, not be discriminated any more, regardless of being positive for crop production or potentially negative. Scientific literature somewhat adds to this problem as it mainly reports about studies of single microorganisms in biostimulants and rarely about any synergistic or antagonistic effects of combinations. Exceptions are

for example reported by Berendsen et al. (2018) who studied how three bacteria, *Stenotrophomonas* sp. WCS2014-113, *Xanthomonas* sp. WCS2014-23, *Microbacterium* sp. WCS2014-259, inoculated together to the root of *Arabidopsis thaliana*, can change the microbiome on the roots therein inducing systemic resistance against downy mildew, *Hyaloperonospora arabidopsidis* (Peronosporales: Peronosporaceae) and enhancing growth of the plant (Berendsen et al., 2018). Also the re-colonization of cleaned *Arabidopsis thaliana* roots with the most complex multi-kingdom microbiome (bacteria, fungi, and oomycetes) resulted in most plant growth compared with less complex microbiomes (Durán et al., 2018). Another example showed that increasing the richness of microorganisms on pea roots (here with seven bacteria and one applied fungus, *Trichoderma guizhouense*) can increase antagonistic effects between the highly diverse organisms of the root microbiome therein reducing some pathogenic root diseases, as well as can improve the accumulation of plant biomass compared with similarly diverse microbiomes without those added microorganisms (Wang et al., 2022).

We reviewed and summarised some of the multiple effects of biostimulants, such as to insects. Interestingly, many microbial plant biostimulants seem not only to have crop-enhancing properties as defined by Albrecht (2019); du Jardin (2015); Ricci et al. (2019), but also have some indirect effects such as by improving the plant defence to insects (Arpaia et al., 2017). This amounts to around half of all biostimulant products and to 1/3rd of species reported to have some insecticidal properties and to around 2/3rd of products and half of species reported to increase plant defences to insects. Some do both, being insecticidal to some insects, and improve the defence of some crops to other insects, such as do *Bradyrhizobium japonicum, Rhizobium leguminosarum, Rhizophagus irregularis, Trichoderma harzianum,* or *Trichoderma virens*.

The most common microorganisms found in commercial biostimulants are, at the same time, also the most commonly reported ones for adverse effects on insects (compare Table 1 with Figure 4). This is particularly true for bacteria, and slightly less prominent for fungi. There is hardly anything known about negative effects of protista or algae in biostimulants on insects.

To add to the confusion, few microorganisms in biostimulants seem to negatively affect some insects whilst being beneficial to others, or beneficial when applied to a different crop system. For example, some strains of the plant stimulant *Rhizophagus irregularis* were reported to negatively affect insects such as *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on maize (Yan et al., 2021). However, when this plant stimulant was inoculated to the roots of tomatoes, this seems to have improved the foraging behaviour of the predatory mirid bug, *Macrolophus pygmaeus* (Hemiptera: Miridae) towards the crop and prey (Prieto et al., 2017). When inoculated to alfalfa, this increases certain hormones levels (e.g. β-1,3 glucanase, thaumatin-like protein, ethylene response factor 1, gibberellin 20-oxidase, GA 2-oxidase) that can increase the feeding and fitness of pea aphids, *Acyrthosiphon pisum* (Garzo et al., 2018). Another example is the alga *Chlorella vulgaris* which, on one hand, negatively affects larvae of *Chironomus riparius* (Diptera: Chironomidae) (K & Purushothaman & Mol, 2020), on the other hand, positively affects the productivity of bees hives (Eremia et al., 2013) as well as of *Drosophila melanogaster* (Diptera: Drosophilidae) when added to their food (Shuang et al., 2019).

Unfortunately, many of the here-reviewed studies are based on laboratory experimentation with results difficult to extrapolate to field conditions. Published information

about the level of such insecticidal or plant defence effects under field conditions is scare, and for many microorganisms non-existent. Another problem is that studies as well as commercialised products often do not state the strain of the considered microorganism. However, strains of the same microorganism species can differ considerably. A prominent example are the different insecticidal protein compositions of strains of some *Bacillus* species (Ch. Sallaud, 2022, pers. comm). For example, *Bacillus thuringiensis kurstaki* or *aizwai* mainly contain toxins specific to lepidopteran caterpillars, whilst *B. thuringiensis israelenisis* are more specific to dipterans, and *B. thuringiensis tenebrionis* to few coleopterans (Jabeur, 2022). Moreover, *B. thuringiensis* changes the secretion of insecticidal proteins during its different growth phases. The vegetative growth phase is known for producing secreted insecticidal protein (Sip reassigned as Mpp by Crickmore et al. (2020)) and vegetative insecticidal proteins (Vip), whereas parasporal crystalline δ-endotoxins, such as cytolytic toxin (Cyt) and crystal toxin (Cry), are produced during sporulation and the vegetative stationary phase (Palma et al., 2014). In detail, Vip1 and Vip2 proteins are binary toxins with some coleopteran activity whereas Vip3 have some lepidopteran activity (Bhalla et al., 2005;Estruch et al., 1996;Yu et al., 1997). Another study showed the different effects of strains of the same bacterium *Rhizobium alamii* on the diversity of root-associated microbiota in rapeseed plants, one strain reducing the alpha-diversity of the microbiota, whilst another strain mainly modifying the beta-diversity (Tulumello et al., 2021).

Those examples emphasise the complexity of those interactions and effects. We therefore urge scientists, companies, and regulators to consider the importance of the strain level of bacterial and fungal microorganisms in research and commercialisation.

Finally, many scientists tend to preferably present positive results, i.e. confirming a hypothesis whereas the negative results, such as the lack of effects, are less often published (Scudellari, 2015). There are comparatively few publications that report the lack of effects or the rejection of a hypothesis. As a consequence, some of the here-reviewed organisms and products remain uncertain with regard to potential effects additional to their plant enhancing properties, and even those are not always clear.

Although it is not surprising and also generally known that microbial plant biostimulants have multiple effects, it somewhat perturbs a well-targeted and specific use in crop production. We believe that effects of biostimulants on insects should be better studied under field conditions and products also labelled accordingly. Some of the microorganisms registered as biostimulants are at the same time, or in other countries, also registered as plant protection products. Some prominent examples are *Bacillus subtilis, Trichoderma harzianum,* or *Trichoderma asperellum* registered as fungicidal biopesticides, and *Beauveria bassiana* as insecticidal biopesticides (CABI, 2022; NEBIH, 2020). Many of those are even pre-dominantly plant protection agents and less a biostimulant. However, the intention of our review was not to blame biostimulants for their plant protection properties or to demand similarly complicated and costly registration processes as for plant protection products (Daniel et al., 2014; Huber, 2017). Our aim was to better understand and to raise awareness about the multiple effects plant biostimulants may have to crops, something essential to be understood and considered by growers, companies, or researchers, and something we can profit from.

In conclusion, many commercial microorganism-based biostimulants have, next to their crop and yield enhancing effects, also plant protection properties. They therefore

contribute to the biological management of insect pest populations. Such effects should be better studied, and growers should be made aware of it.

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Data availability statement

The data of this review are presented in supplementary online materials.

Informed consent for publication

Informed consent was obtained from all participants included in the study.

Authors' contributions

ST, SIT, SzT, MS and JK jointly developed the study. ST, JK, GT and MS supervised the study. SIT extracted the registered biostimulants from Indonesia and Canada; SzT from Spain and Hungary, ST from France and Switzerland. SIT reviewed all products and species for insecticidal or plant defence effects. SIT, ST, SzT conducted the analysis. SIT wrote the manuscript with support from all authors.

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4. Chapter II.

Can microbial plant bio stimulants be useful for insect soil pest control? A review

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4.1 Abstract and Introduction

Microbial and Nematode Control of Invertebrate Pests
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Can microbial plant biostimulants be useful for soil insect pest control? A review

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Abstract: Plant biostimulants are ingredients with the sole aim to improve the agronomic performance of a plant. We listed 483 commercial plant biostimulants based on 245 microorganism species from six countries that have a detailed registration processes and/or an accessible and searchable database for plant biostimulants. Subsequently, we reviewed the found microbial plant biostimulants with regard to their effects on soil insects pests such as for example on rootworms (*Diabrotica* spp., Coleoptera: Chrysomelidae) via databases of scientific literature. About 66 % of products (154 ± 133) contain microorganisms reported to directly or indirectly affect insect pests, which is 53 % of species (34 ± 8) used in biostimulants. Among them, about 30 % of products (103 ± 86) contain microorganisms reported to affect soil insect pests, which is 44 % of the used species (19 ± 6). At least 20 % of products (41 ± 46) contain microorganisms reported to affect rootworms, which means 9 % of the species (6 ± 2). In conclusion, growers should be made aware of the multiple effects of microbial plant biostimulants.

Key words: biofertilizer, yield enhancers, *Diabrotica v. virgifera*, biopesticide

Introduction

Microbial plant biostimulants are products that contain living cells of microorganisms which have the ability to enhance plant characteristics. However, many of them have recently been reported to also have insecticidal properties (Tarigan et al., 2022). Soil insect pests are a major problem in agriculture causing yield losses in many crops. We therefore reviewed commercial microbial plant biostimulants with regard to their effects on soil pests, such as on rootworms, a group with several key soil insect pests in the genus *Diabrotica* (Coleoptera: Chrysomelidae).

Materials and methods

We listed 483 microbial plant biostimulants registered in Hungary, Switzerland, Spain, France, Indonesia and Canada (Tarigan et al., 2022). Each of the found 245 microorganisms was reviewed for its potential direct or indirect effects on soil insect pests including rootworms, which are major pests in the genus of *Diabrotica*. We used the literature databases of CAB Direct (CABI, 2022) and Web of Science (Clarivate, 2022).

* Larger figures are available in the appendices

4.3 Results and discussion

Results and discussion

Many microorganisms found in commercial plant biostimulants may, according to the scientific literature, also have effects on soil insect pests. Briefly, about 44 % of microorganisms (19 ± 6 SD species) registered as microbial plant biostimulants, were reported to affect soil insect pests; this is about 30 % of the commercial products (103 ± 86 products) (Figure 1).

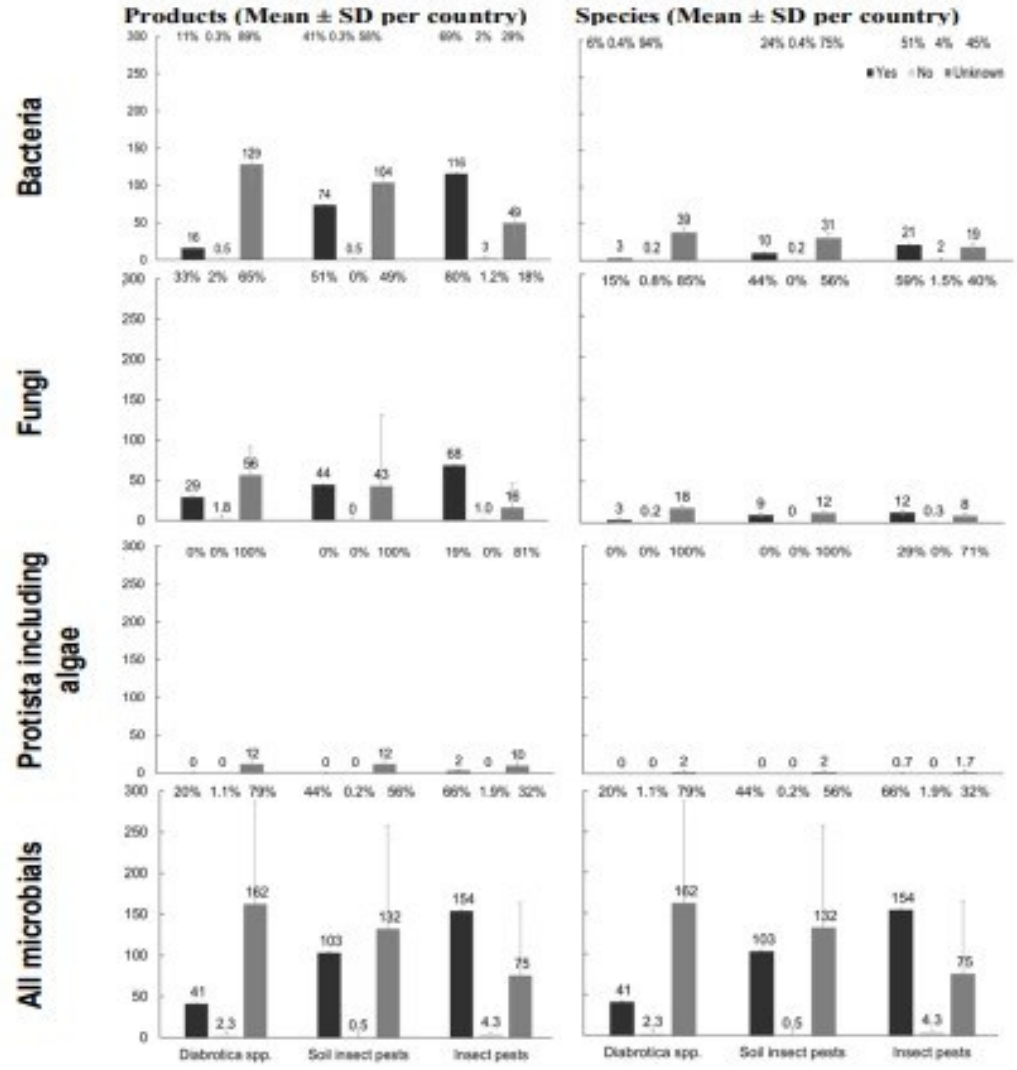

Figure 1. Average numbers and percentages of commercial microbial plant stimulants with potential effects on *Diabrotica* species (rootworms), soil insect pests, and insect pests in general, reviewed as per Web of Science (Clarivate, 2020) and CAB Direct (CABI, 2020). In total, 483 products reviewed with 245 microorganisms from Hungary, Switzerland, Spain, France, Indonesia, Canada, as per 2021.

Among the most frequently used bacteria with reported effects to soil insects, are strains of *Bacillus thuringiensis* and *Pseudomonas fluorescens*. *Bacillus thuringiensis*, which is a well-known insect pathogen (Bowen et al., 2021), should probably not appear as an ingredient in biostimulants. Also some *P. fluorescens* strains have insecticidal effects, such as against *Leptinotarsa decemlineata* (Erarslan and Kotan, 2021).

Among the most frequently used fungi with reported effects to soil insects, are strains species of *Rhizophagus irregularis* (syn. *Glomus intraradices, Rhizophagus intraradices, G. irregulare, Rhizoglomus irregulare, G. irregular*), *Glomus mosseae* (syn. *Funneliformis mosseae)* and *Beauveria bassiana*. For example, *R. irregularis* inoculation is known to reduce infestation of wheat by *Mayetiola destructor* (Prischmann-Voldseth et al., 2020). *Beauveria bassiana* is a well-known insecticidal fungus that should not appear in biostimulants.

With regard to rootworms, several microorganisms in commercial plant biostimulants may support protection against this pest group (Figure 1). At least 9 % of microorganisms (6 ± 2 species), registered as biostimulants, were reported in the literature to affect rootworms, most of them through indirect effects. This relates to about 20 % of commercial products potentially affecting rootworms (41 ± 46 products). Most of those originate from the bacterial kingdom (3 ± 1, thus 6 % of species; 16 ± 13, thus 11 % of products) followed by fungi (3 ± 2, 15 % of species; 29 ± 35, 33 % of products). Among the bacteria with potential effects on rootworms are strains of *Bacillus pumilus*, *Azospirilium brasiliense*, *B. thuringiensis*, or *Pseudomonas chlororaphis*. For example, the *B. pumilus* strain INR-7 is known to repel rootworms *(*Disi et al., 2018). Another example is *Azospirilium brasiliense* where rootworm preferentially orient toward roots of non-inoculated plants versus inoculated roots which emit the repellent (E)-β-caryophyllene (Santos and Pen, 2014). Among the fungi are strains of *Rhizophagus irregularis*, *Saccharomyces cerevisiae*, *Beauveria bassiana*, *Metarhizium brunneum* (syn. *Metarhizium anisopliae*), and *Myceliophthora thermophila*. For example, *R. irregularis* was reported to render rootworms prone to predation by natural enemies, probably through an indirect effects by a modified endorhiza microbial community (Dematheis et al., 2013).

It needs to be noted that the diverse effects of microorganisms in biostimulants often depend on the specific strain of a microorganism, which is often neither stated on the product label nor in the reviewed scientific studies in the literature.

Nevertheless, it became clear that many microbial plant biostimulants have multiple effects including the control of insects, something growers should be made aware of.

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5. Chapter III.

Comparative analysis of the suitability of insecticides as positive controls for screening new compounds against the different life stages of the invasive maize pest, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae).

Insects (Submitted)

5.1 Abstract and Introduction

insects

MDPI

Article

Comparative analysis of suitability of insecticides as positive controls for scrceening novel agents against the different life stages of the maize pest, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)

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Simple Summary: Researchers are looking for new ways to control the western corn rootworm, a beetle attacking maize in North America and Europe. We tested seven insecticides to see how well they work against the eggs, larvae, and adults, and how practical they are for comparative studies. Using bioassays, we found that imidacloprid and clothianidin can most consistently reduce egg hatching and kill larvae during hatching, while imidacloprid is best against the larvae, and finally acetamiprid against the adult beetles. These findings are important for developing novel ways to protect crops and for better understanding how resistant corn rootworms might have become against frequently used insecticides. Since some insecticides are banned and others are overused, finding new ways to control insect pests like corn rootworms, is important.

Abstract: The chrysomelid beetle *Diabrotica virgifera virgifera* (western corn rootworm), poses a threat to maize crops in North America and Europe, requiring the development of novel, effective, and less disruptive crop protection agents. With recent bans on key insecticides and concerns about overuse of remaining options, there is an urgent need for accessible and comparable screening methods. We propose comparative high-throughput screening methods against the eggs, larvae and adults of this pest, emphasizing the importance of suitable positive controls tailored to the specific bioassay types. We evaluated seven common insecticides (imidacloprid, clothianidin, acetamiprid, novaluron, cypermethrin, chlorpyrifos-methyl, spinosad) against eggs, larvae, and adults as potential positive controls for each of the proposed assay methods. Dipping assays with ready-to-hatch eggs revealed several ingredients to cause mortality; but imidacloprid and clothianidin might be most suitable as a positive control due to a robust dose-response in reducing egg hatching and causing mortality of hatching neonates. Larval bioassays using artificial diet overlays revealed mortality caused by all insecticides, with imidacloprid exhibiting best dose-mortality response as well as sublethal effects. Adult bioassays using artificial diet-core overlays revealed mortality caused by all insecticides, with acetamiprid exhibiting best dose-mortality response. The provided ED_{50}, ED_{80} values and dose-response equations offer valuable insight for researchers in selecting appropriate positive controls for screening new crop protection agents or assessing resistance levels against different life stages of this pest.

Keywords: western corn rootworm, bioassay methodology, egg bioassay, larval bioassay, adult bioassay

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* Larger figures are available in the appendices

1. Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte is a chrysomelid beetle that is one of the most important pests of maize (*Zea mays* L.) in the USA and Europe [1,2]. Its larvae feed on maize roots which can lead to plant instability, reduced growth and yield losses [1]. This pest has 7 developmental stages: egg, three larval instars, pre-pupa, pupa, and adult. Adult females lay 300-400 eggs in the top 5-20 cm of soil between the roots of maize [4], and these eggs then go through a period of diapause. The neonate larvae (L1) burrow through the soil to search for and feed on the maize roots before metamorphosing into the second larval stage (L2) and finally into the third larval stage (L3). During these stages, the larvae feed on root tissue. The larvae then pupate in the soil, and adults emerge about 1 week later. Adults can cause damage to the maize silks, kernels, and leaf tissue [3].

Diabrotica v. virgifera is often difficult to control because its immature stages hide underground, the eggs take a relatively long time to hatch and the larvae feed on and in the maize roots or at least a month [5]. In addition to crop rotation, chemical pest control has been a common option for controlling this insect for decades. Farmers apply either granular or occasionally liquid soil insecticides in the seed furrow or insecticide-coated seeds to control the insect larvae. Alternatively, farmers occasionally spray insecticides over large areas of the leaves against the adult insects in order to reduce egg laying and thus the damage caused by the larvae in the following season.

Organochlorine insecticides were among the first to be used by farmers in the USA against adult *D. v. virgifera* in the 1950s, but the pest soon developed resistance [6]. Carbamate and organophosphate insecticides were then introduced, and then pyrethroid insecticides [7]. Organophosphate insecticides like chlorpyrifos-methyl are used in some countries to control corn rootworm larvae in maize byinhibiting cholinesterase activity in the insects' nervous tissue ultimately killing them [8]. The pyrethroid insecticides like permethrin, tefluthrin and bifenthrin have been used to control the larvae or adults of *D. v. virgifera* bybinding and interrupting voltage-gated sodium channels, disrupting the insect's central nervous system and causing death [9]. Later, neonicotinoid insecticides were added to the chemical toolbox against corn rootworms [10]. They interact with nicotinic acetylcholine receptors in the neurons of insects [11]. These include imidacloprid, acetamiprid, thiamethoxam and clothianidin, which have been widely used [12]. Most of them had been recently banned in a number of countries due to their high toxicity to bees [13]. Among the newest groups of insecticides that can potentially be used against *D.v.virgifera* are spinosyns and insect growth regulators. Insect growth regulators such as novaluron are substances that disrupt the life cycle of insects and are therefore not harmful to adult pollinators or non-growing stages of other non-targets insects [14]. For example, novaluron has been reported to reduce the viability of *D.v.virgifera* eggs and also has a transovarial effect in adults [14]. Spinosyns, derived from*Saccharopolyspora* bacteria, include compunds like spinosad, a mix of spinosyns A and D [15], and has been shown to reduce adult *D. v. virgifera* populations below the economic threshold [16].

However, the number of chemical insecticides available has steadily declined due to high human toxicity concerns, problems with non-target groups, or the development of resistance. For example, organochlorines, carbamates, most organophosphates and highly toxic pyrethroids such as tefluthrin have been largely banned from use against corn rootworms in most regions [17]. Others, such as neonicotinoids, appear to be toxic to bees and accumulate in the environment [18]. This led, as stated above, to their ban in several countries. As a result, farmers in many maize-growing areas are struggling to control soil insects such as the corn rootworm due to limited options. In addition, reports indicate that insecticides like organophosphates, carbamates, pyrethroids and neonicotinoids have encountered resistance in populations of *D. v. virgifera* [72,73,74,25,75,76].

Therefore, researchers are trying to develop safer, and more sustainable integrated management solution for *D.v.virgifera*. One step is to assess lethal or effective doses/concentrations (LD, ED, LC, EC) of potential novel plant protection agents, comparing them to exisiting active ingredients and formulations [19]. In addition, such estimates are crucial for evaluating insecticide resistant in pest populations. Both industry and public research conduct high-throughput screening of novel substances, including potential biopesticides, against all life stages, but especially the larvae of this pest. These largely follow common standard protocols, but these are often not published. For *D.v.virgifera*, for example, [20,21], have described bioassay methods for screening active ingredients against the larvae using diet-overlay experiments under standardized laboratory conditions. [22] provided bioassay methods for evaluating toxicity to adults based on diet core overlay experiments. [23] provided some bioassay methods for evaluating the effects of agents on eggs by immersion of the eggs and subsequent incubation on filter paper.

Negative and positive controls are needed for most of the different types of bioassays when screening novel agents or active ingredients [24]. Negative controls are usually the formulation only, buffers, water, or no treatment at all. Positive controls are usually effective standard insecticides that are commercialized and commonly used by growers. For the different types of screening tests, however, different positive controls and doses are might be required depending on the area of application, treatment method, number of targeted individuals and their developmental stages, as well as on the assessed parameter. Unfortunately, these details are not always openly available. Astonishingly, also the lethal dose (LD) of insecticides that kill, for example, 50% or 80% of *D.v.virgifera* eggs, larvae or adults are not always published for a range of bioassay situations. This limits comparability when screening novel agents against insect pest.

Therefore, this study aimed to propose methods for high-throughput screening of agents against this serious maize pest. We asssed the dose-response of several common insecticides against the different stages of *D.v.virgifera* under different bioassay methods in standardised conditions to establish suitable positive controls. We also aimed to establish ED_{50} or ED_{80} values for the different situations, the latter being an important value for comparative screenings. Ultimately, we hope that our results

5.2 Materials and Methods

will help researchers in companies and the public to select the most appropriate positive control for a certain standard laboratory bioassay whilst screening for novel agents against *D.v. virgifera* or evaluating levels of resistance development.

2. Materials and Methods

2.1 Tested commercial insecticides

In this study, seven commercially available insecticides were tested in standard screening assays to determine their dose-responses on different life stages of *D. v. virgifera* (Table 1). These were the neonicotinoids imidacloprid, clothianidin and acetamiprid, the pyrethroid cypermethrin, the organophosphate chlorpyrifos-methyl, the insect growth regulator novaluron, and the spinosyn spinosad. Imidacloprid acts systemically. Clothianidin and acetamiprid have systemic and translaminar properties. In contrast, cypermethrin, chlorpyrifos-methyl, novaluron and spinosad mainly act on contact.

We examined the effects of seven insecticides at 5 or more concentrations on eggs, larvae, and adults. All agents were commercial products and diluted in sterile tap water to the required doses. Doses in µg ml^{-1} and µg per experimental arena are presented in Table 1 and in Fig. 1 to 4. For example, 10000 µg imidacloprid or novaluron prepared per ml correspond to 0.2 µg applied per arena of egg bioassays and to 0.2 µg active ingredient (a.i.) per insect egg, and 100 a.i. per mg insect. For example, 20 µg imidacloprid per ml corresponded to 0.4 µg per arena of larvae bioassays and to 0.4 µg a.i. per individual larva and to 1 µg a.i. per mg larva. For example, 7500 µg imidacloprid per ml corresponded to 300 µg per arena of adult bioassay and to 100 µg per individual adult (with three adults per well) and to 10 µg a.i. per mg adult.

Table 1. Specifications of common insecticides tested for their suitability as positive controls in screening bioassays against different life stages of *Diabrotica v. virgifera* under standardized laboratory conditions. There are three experimental repetitions per treatment and dose for egg bioassays, and two to five repetitions for larvae and adult bioassays.

Insecticide group	**Active ingredients Chemical formula**	**Trade name**	**Active ingredient concentration in product**	**Formulation**	**Tested dosage range**		
					eggs µg ml^{-1} µg $arena^{-1}$ µg mg $insect^{-1}$	larvae µg ml^{-1} µg $arena^{-1}$ µg mg $insect^{-1}$	adults µg ml^{-1} µg $arena^{-1}$ µg mg $insect^{-1}$
Organophosphates	Chlorpyrifos-methyl (O,O-dimethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate)	Reldan 22EC	225 mg ml^{-1}	liquid	0.1-200 0.002-4 0.001-2	0.06-6000 0.0012-120 0.003-300	7.5-2000 0.3-80 0.07-3
Neonicotinoids	Imidacloprid (N-[1-[(6-chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide)	Confidor 200SL	200 mg ml^{-1}	liquid	0.1-10000 0.002-200 0.001-100	0.02-20 0.0004-0.4 0.001-1	7.5-7500 0.3-300 0.01-10
	Acetamiprid (N-[(6-chloro-3-pyridyl)methyl]-N'-cyano-N-methyl-acetamidine)	Mospilan 20SG	200 mg g^{-1}	granule	0.075-50 0.0015-1 0.00075-0.5	0.002-2 0.00004-0.04 0.0001-0.1	0.075- 50 0.003- 20.0001- 0.06

	Clothianidin (1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine)	Poncho 600FS	600 mg ml^{-1}	liquid	1-10000 0.02-200 0.01-100	0.06-6000 0.0012-120 0.003-300	7.5-2000 0.3-80 0.07-3
Insect growth regulators	Novaluron ((RS)-1-[3-chloro-4-[[(2,5-dimethylphenyl)amino]carbonyl]aminophenyl]-3-(2,6-difluorobenzoyl)urea)	Rimon 10SC	100 mg ml^{-1}	liquid	0.1-10000 0.002-200 0.001-100	1-5000 0.02-100 0.05-250	7.5-75000 0.3-3000 0.01-100
Spinosyns	Spinosad (2R,3aR,5aR,5bS,9S,13S,14R,16aS,16bR)-2-[(6-deoxy-2,3,4-tri-O-methyl-α-L-mannopyranosyl)oxy]-13-[(2R,5S,6R)-5-(dimethylamino)-6-methyl-2-methylsulfanyl-1,3-dioxan-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,15,16a,16b-hexadecahydro-14-methyl-1H-as-indaceno[3,2-d]oxacyclododecin-7-ol)	Laser Duplo	480 mg ml^{-1}	liquid	0.1-1000 0.002-20 0.001-10	0.02-20 0.0004-0.4 0.001-1	7.5-75000 0.3-3000 0.07-100
Pyrethroids	Cypermethrin (R)-α-cyano-3-phenoxybenzyl (1RS,3RS; 1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate)	Supra 50EC	50 mg ml^{-1}	liquid	0.1-10000 0.002-200 0.001-100	0.08-8000 0.0016-160 0.0008-80	7.5-750 0.3-30 0.01-1
		Sherpa 100EC	100 mg ml^{-1}	liquid	0.1-10000 0.002-200 0.001-100	0.08-8000 0.0016-160 0.0008-80	7.5-750 0.3-30 0.01-1

2.2. *Rearing and handling of D.v.virgifera*

A non-diapause laboratory colony of *D. v. virgifera* was obtained from USDA-ARS Laboratories (Brookings, SD, USA) where it had been reared for around 300 generations (C Nielson, 2020, pers. comm.). The individuals of this colony are therefore supposed to be susceptible to most pest management agents [25]. Insects were reared under standardized laboratory conditions according to [22,26].

Briefly, eggs laid into soil-filled dishes in adult gauze cages were collected every week and then sieved and washed with clean water containing <0.5% NaOCl through a 300 µm mesh sieve. Eggs were stored for 7-10 days in the incubator (23-25°C). One day before a bioassay, the ready-to-hatch eggs were again washed and sieved. The eggs were then ready to use for egg bioassay purposes.

For larvae bioassay purposes, similar procedures were used, then followed by re-mixing the eggs into sterile moist sand placed onto slightly moist tissue paper into a dish. This allowed clean hatching conditions for new neonates and their use for larvae bioassays as described below.

For adult bioassay purposes, ready to hatch eggs were transferred to maize plant trays aged 7 days old. After another 7 days, larvae had reached L2 stage. Then those maize-root larvae blocks were cut into 4 pieces and transferred each to a new secondary maize tray. Those older larvae were incubated for another 2 weeks until pupation Then the maize trays with the

pupae were transferred to beetle emergence cages. Beetles emerged from these cages were maintained in adult rearing cages with agar as water source and pumpkin and artificial diet as food source [22] until use for adult bioassays.

2.3. Bioassay with different life stages of Diabrotica v. virgifera

2.3.1. Egg bioassays

To access effects and dose-responses of commonly used insecticides on eggs, we applied standard screening methods under controlled semi-sterile conditions following methods of [21]. Each insecticide was prepared in seven concentrations, this is 10000, 5000, 1000, 100, 10, 1, 0.1 µg active ingredient ml^{-1} (Table 1 and Fig.1 to 4). Active ingredients as specified on the product labels underwent serial dilutions using sterile tap water. Dilutions of 1 ml per tube were stored overnight in eppendorf tubes at 3 to 5°C until ready for use. Sterilized tap water served as the untreated control. Ready-to-hatch eggs were washed and placed onto a 300 µm sieve. Thereafter, clusters of clean eggs were transferred using 2 cm-long stainless-steel spoons. The stainless-steel spoon was dipped in 70% ethanol and sterilized tap water for 3 seconds before being used to transfer eggs to another treatment tube.

Eggs were transferred to the 200 ml of treatments in the eppendorf tubes and then soaked for 1 hour. Then 20µl with 10 to 20 eggs were pipetted onto a filter paper in a petri dish (150 mm×25 mm). Then 100 µl of sterilized tap water was added for moisture. The pipette tip was replaced between treatments. The eggs been transferred were counted per filter paper and dish (15± 8). The eggs were then incubated in the dishes at 23-25°C for 7 days, when the experiment was terminated. Egg hatching, mortality of newly hatching larvae, and days until start of egg hatching were observed under stereo microscope and recorded. Data were collected at 1,3, 5 and 7 days after treatments.

2.3.2. Larvae bioassays

To assess the effect and dose-responses of commonly used insecticides on neonates of *D. v. virgifera*, we applied artificial diet-overlay bioassays under controlled semi-sterile conditions. Those are standard screening methods for novel agents as used by many researchers [21,27,28]. Each insecticide was prepared in at least six concentrations (Table 1 and Fig.1 to 4). Active ingredients as specified on the product labels underwent serial dilutions using sterile tap water, such as for example 20, 10, 5, 2, 0.2, and 0.02 µg imidacloprid ml^{-1} up to 50000 to 0.002 µg novaluron ml^{-1}.

Sterilized tap water served as the untreated control. 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 had a volume of 330 µl, with a diameter of 5 mm, a height of 10 mm, and a surface area of 0.34 cm^2. Each treatment was applied to 8 wells of each.

In detail, 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 [21,29,30,31].

This diet recipe consisted of grinded maize roots and food color, 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 grinded maize roots was grinded and added to 88 ml fluid 60 to 70°C agar. After blending and cooling to 55 to 60°C, 0.75 g grinded lyophilized maize roots were added as well as 0.1 g green food color for better larvae observation. 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 of the diet were pipetted into each 330 µl well, filling each to approximately 2/3rd of its capacity. Plates containing the diet were left to dry in a laminar flow cabinet for 45 minutes and then stored overnight at temperatures ranging from 3 to 5°C.

The following day, treatments were applied. This is, 17 µl of a treatment was applied to the 0.34 cm^2 diet surface reaching good coverage and therefore forcing the after-placed larvae to feed through (10 to 100 µl pipette Biohit TM Proline). To prevent edge effects, the sequence of treatments was alternated for every other plate. Following application, the plates were allowed to dry for a duration of 1 to 1.5 hours and were subsequently cooled for 1 hour in a refrigerator set at temperatures between 23 to 25°C.

Each well received one neonate larva, carefully placed on the diet surface using a fine artist brush. A vigorous and visibly healthy larva was selected, lifted from the end of the abdomen with the brush, maneuvered towards a well surface, and allowed to crawl off the brush onto the diet. To avoid systematic errors, larvae were not arranged in treatment column order but rather in a rectangular pattern. After every 12 individual larvae, the brush was cleaned using 70% ethanol followed by sterile tap water. The filled plate was sealed with an optically clear adhesive qPCR seal sheet (#AB-1170, Termo Scientific, USA, or #BS3017000, Bioleader, USA), enabling data assessments without the need to open the plate. Four to five holes were carefully made with fine 00-insect pins into the seal per well to facilitate aeration. The plates, housing the larvae, were then incubated in a dark, ventilated incubator at a temperature of 23-25 °C and a relative humidity of 50 to 90% for a period of 5 days .

We assessed mortality and stunting larvae within 3 and 5 days. Those parameters were visually assessed through the clear seals of the bioassay plates using a stereomicroscope (10× magnification, SMZ-B4, Optec, Chongqing, China). Data from a plate were only accepted when the natural mortality threshold of 37.5% in the untreated control had not been reached, i.e., no more than 3 dead of 8 larvae per column of wells per treatment. This is in contrast to common practices with other insects in bioassays where the quality acceptance is <10% natural background mortality [32]. However, this is rarely achievable with rootworm larvae as the artificial diets known to date remain suboptimal [69].

2.3.3. Adult bioassays

To access the effect and dose-responses of common insecticides on *D.v.virgifera* adult, artificial diet-overlay bioassays with different doses were performed under controlled, semi-sterile conditions . These are standard screening methods for new active ingredients used by many researchers [23,33]. Each insecticide was prepared in at least six concentrations (Table 1

5.3 Results and Discussion

and Fig. 1 to 4). Active ingredients as specified on the product labels underwent serial dilutions using sterile tap water. Sterilized tap water was used as untreated control.

In detail, each bioassay consisted of 6 polystyrene plates of 6 wells each (Eppendorf® 0030720016). Each treatment was applied to 3 wells of each plate per bioassay. The adult diet for a bioassay had been prepared 1-7days before treatment and adult infestation. The diet was prepared under semi-sterile conditions following methods of [70,30]. For 200 ml of diet, 16.5 g sucrose, 9 g cellulose, 8 g casein, 6 g soy flour, 2.5 g yeast, 0.6 g Wesson salt mic and 0.15 g cholesterol diet was grinded and added to 165 ml fluid 60 to 70 °C agar. After blending and cooling to 55 to 60°C, 6 g grinded wheat germ was added as well as 0.0064 g chlortetracycline and 0.0064 g streptomycin sulphate were added. Thereafter, 5.5 ml glycerol were added to reach temperature between 50-55°C. Then, the diet was poured out to 5-6 sterile 11 mm Petri dishes. The plates with diet were allowed to dry for up to 15 minutes under laminar flow cabinet then stored at 3 to 5°C overnight.

The following day, a core of the diet was initially transferred to each well using flamed iron core-cutter (1 cm diameter) under a laminar flow. A core diet was placed each of the 6 wells of the plates. Approximately 40 µl of the treatments were then applied across the surface of diet core (0.34 cm^3). The concentrations used can be seen in Fig. 4. Adult were subsequently transferred from the rearing cage into the wells of the 6-well plates containing the diet and treatments using a tube aspirator. For ease of transfer, the adults were cooled in a fridge for 4 to 7 minutes. Each well plate received 3 to 4 adults. Plates were sealed and incubated at 23-25°C, 50–90% r.h, L: D 12:12. Adult mortality were recorded on days 1,3, 5, 7 of experiment.

2.4. Data analyses

To allow comparisons between experiments, data were standardized to the data of the corresponding negative control, usually sterilized tap water, as follows: standardized data = 100 × (data in negative control - data in treatment)/maximum (data in control or in treatment). The distributions of the data were investigated using histograms and QQ normal and detrended normal probability. Skewness and kurtosis of residuals was observed for normality of influences of treatments on eggs, neonates, or adults. Equality of variances was assessed using Levene's test. Multiple comparisons were performed using the Tukey HSD post hoc test for data with equal variances and the Games-Howell post hoc test for data with unequal variances. For each tested insecticide probit, linear and logarithmic regression models were fit to the dose-response data and the best fit evaluated based on the p, X^2, and R^2 values (Fig.1-4). In case of significant probit, linear or logaritmic relathionships, doses leading to 50% or 80% of relative effects ($ED_{50,80}$) were calculated (Table 2). IPM SPSS Statistical 22 software was used [34].

3. Results

3.1 Laboratory efficacy of commercial insecticides against *D.v.virgifera* eggs

Six of the seven tested insecticides caused some egg mortality, reflected in decreased hatching rates o, except spinosad (Fig. 1, Table 2, 3). However, usually high doses of active

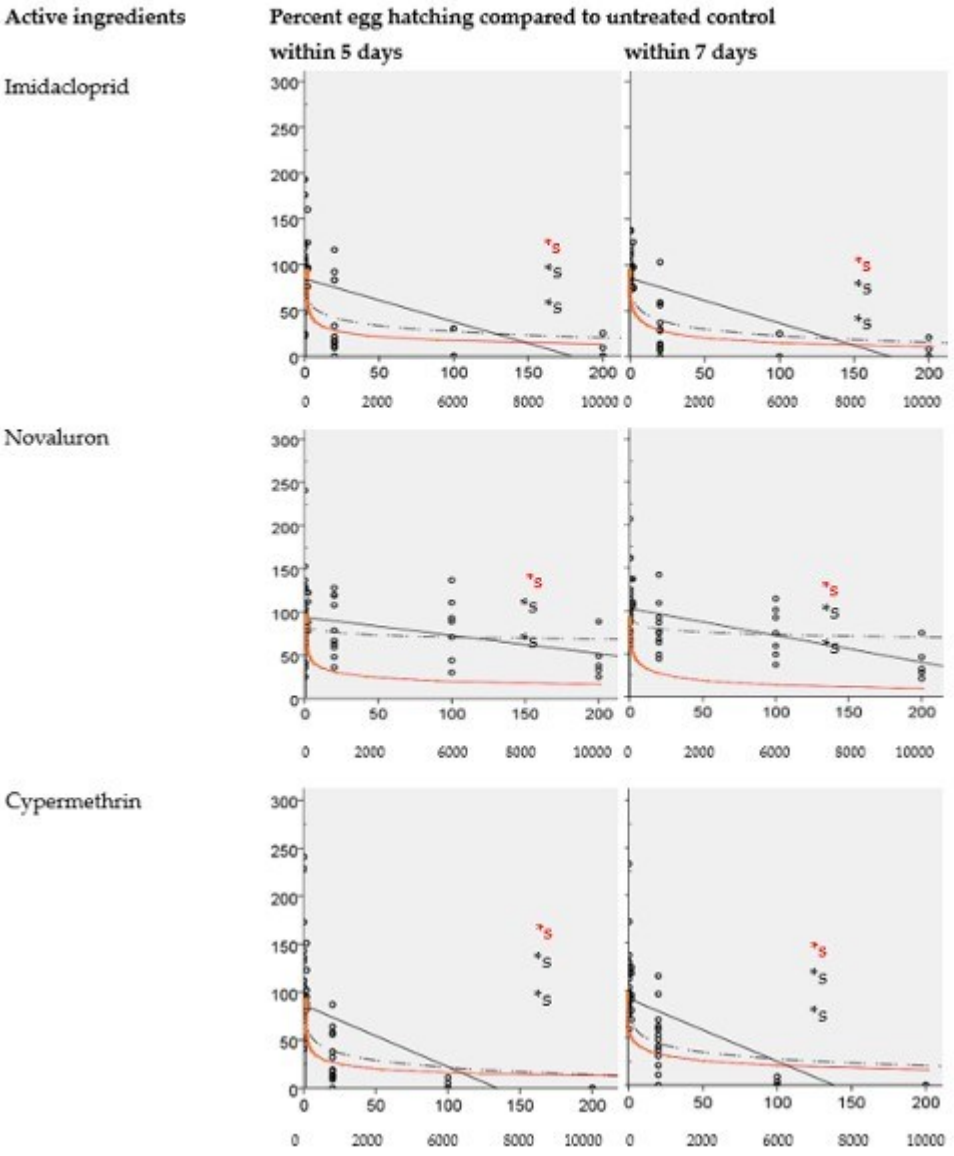
ingredients were needed to affect eggs, thus none of the insecticides tested showed high toxicity to eggs.

Imidacloprid and clothianidin were the insecticides that showed a good dose-response in reducing egg hatching in causing mortality of neonates hatching from treated eggs, as well as in delaying egg hatching, and are therefore proposed as the most suitable positive controls.

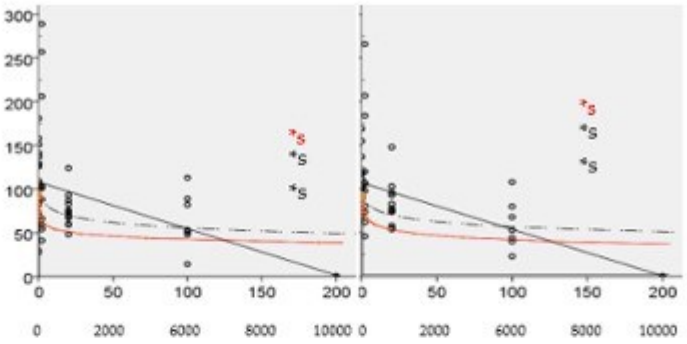
In detail, the best, i.e. least variable dose-efficacy response curves with regard to egg hatching were found for imidacloprid, novaluron, cypermethrin, clothianidin, chlorpyrifos-methyl, and acetamiprid (refer to highest and significant X^2 and R^2 in Table 2, 3).

All tested insecticides caused some mortality to the neonates hatching from treated eggs. Among them, 4 insecticides (imidacloprid, cypermethrin, clothianidin, and spinosad) had comparably high toxicity. The best, i.e. least variable dose-efficacy responses curves with regard to affecting neonates hatching from treated eggs was found for imidacloprid, clothianidin, cypermethrin, and spinosad.

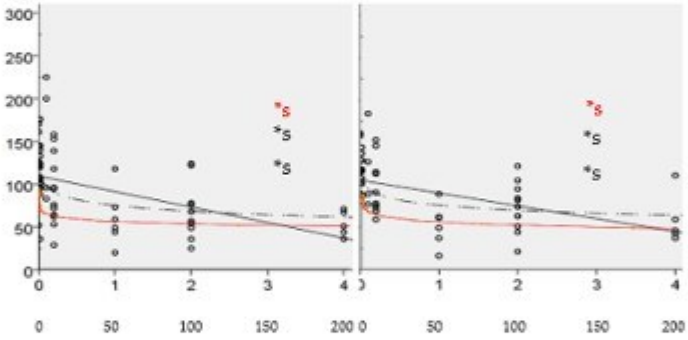
There were only three insecticides that were able to slightly delay egg hatching, this is imidacloprid, clothianidin, and spinosad; but all at unrealistically high doses only (Table 2,3).


Active ingredients
Percent egg hatching compared to untreated control
within 5 days
within 7 days
Imidacloprid
Novaluron
Cypermethrin
300
250
200
150
100
50
0
0
50
100
150
200
0
2000
6000
8000
10000
*S

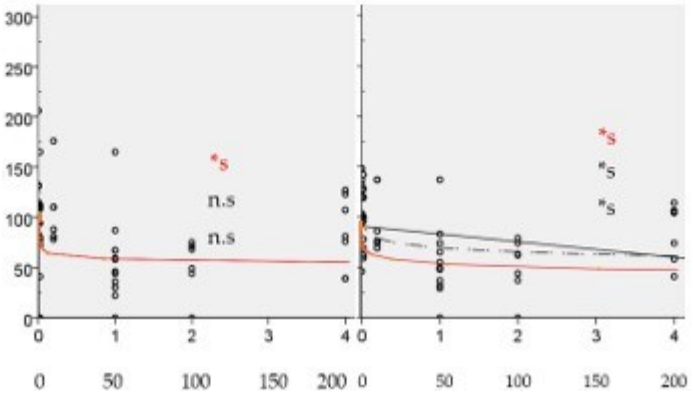
Clothianidin



Chlorpyrifos-methyl



Acetamiprid



Spinosad

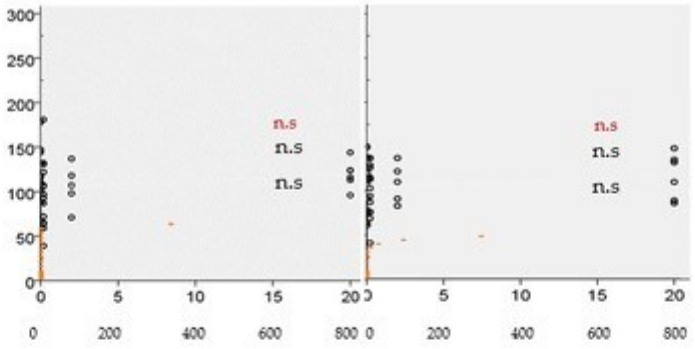
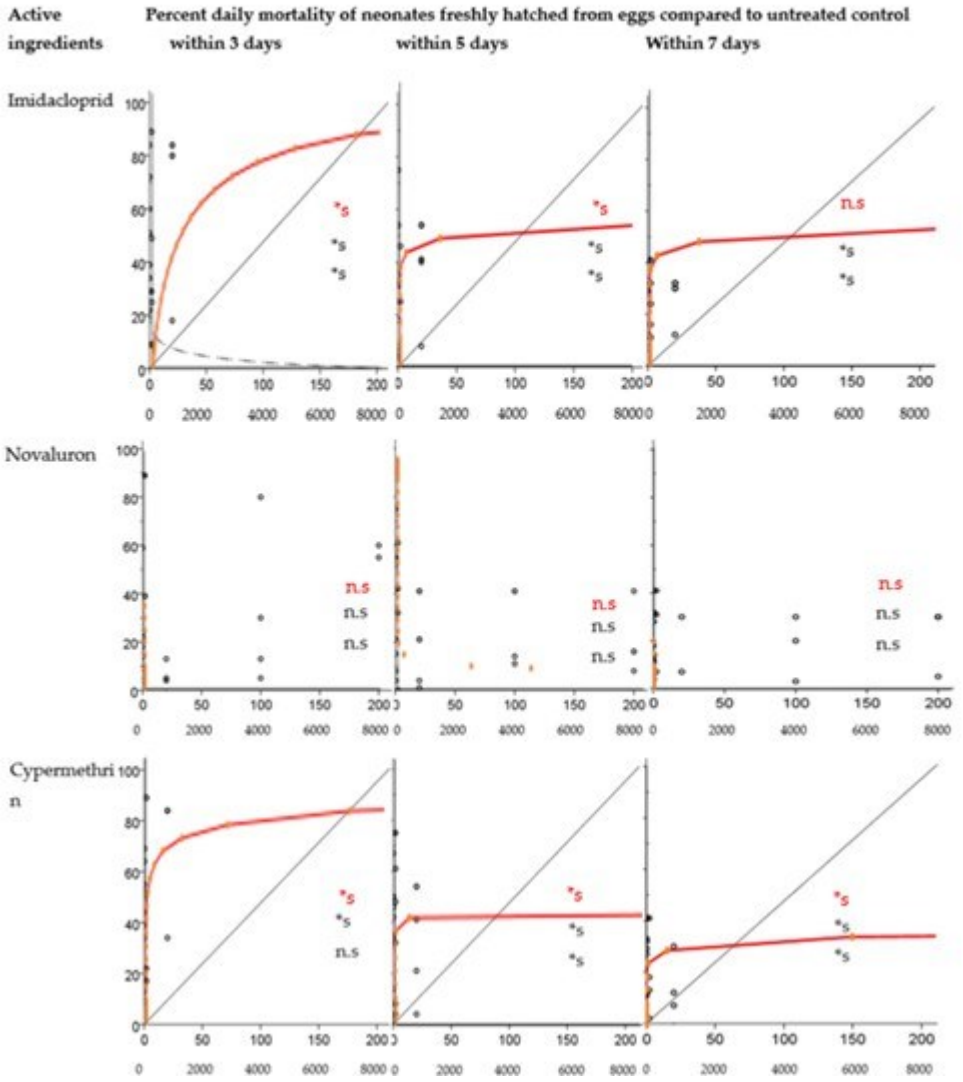
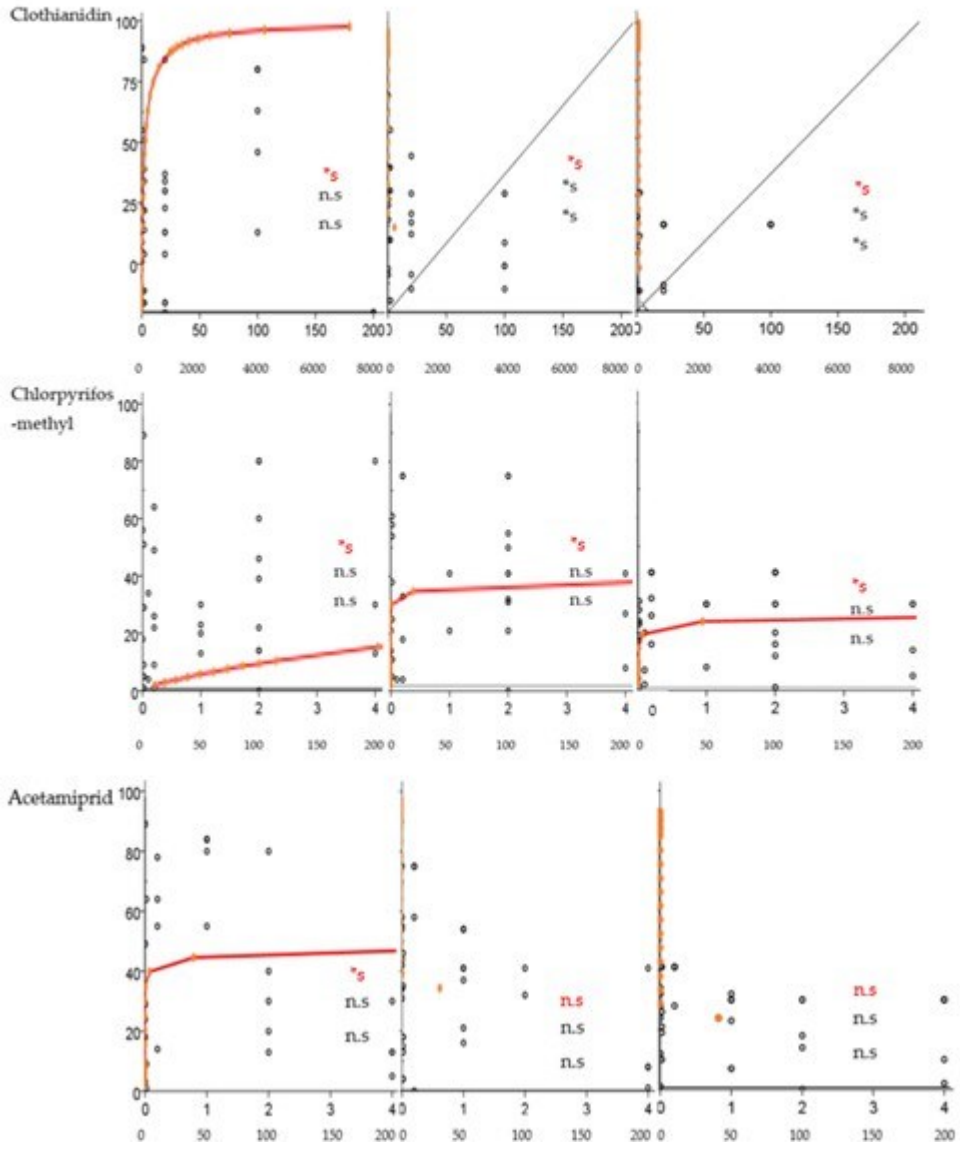


Figure 1. Hatching rate (%) of *Diabrotica v. virgifera* eggs treated with different concentrations of active ingredients of insecticides through dipping treatments. The bioassays were conducted by transferring the treated eggs to filter paper in petri dishes under laboratory conditions (23-25^{0}C, 50-90% r.h.). Reduced hatching reflects egg mortality. The y-axis represents the percent egg hatching compared to the untreated control. Primary x-axis represents the dose tested in µg per arena, and secondary x-axis represents the dose tested in µg per ml. There are three experimental repetitions per treatment and dose. Probit regression (red colored-line), linear regression (black colored-line), and logarithmic regression (black colored-dashed line) fit to dose-response and presented if significant at $p < 0.05$ (*s.); n.s. indicates a non-significant regression and no line is therefore presented.


Active ingredients
Percent daily mortality of neonates freshly hatched from eggs compared to untreated control
within 3 days
within 5 days
Within 7 days
Imidacloprid
Novaluron
Cypermethrin
*s
n.s


Clothianidin
Chlorpyrifos
-methyl
Acetamiprid
*s
n.s

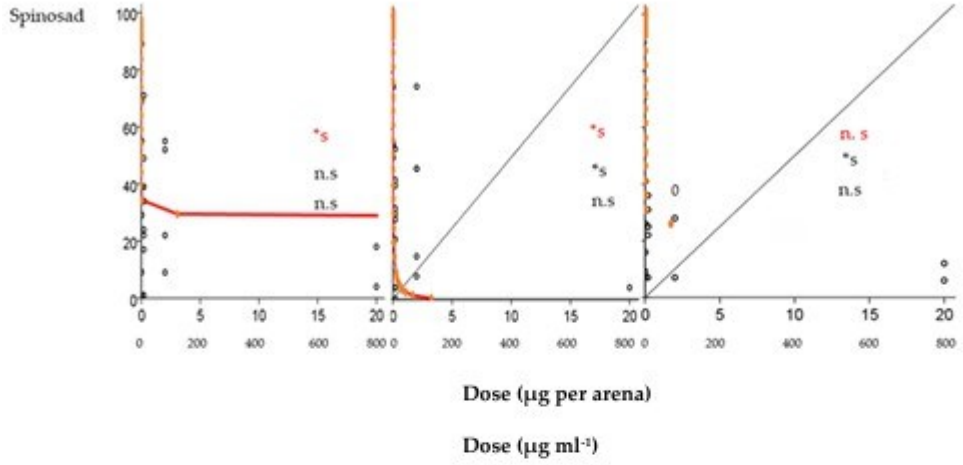
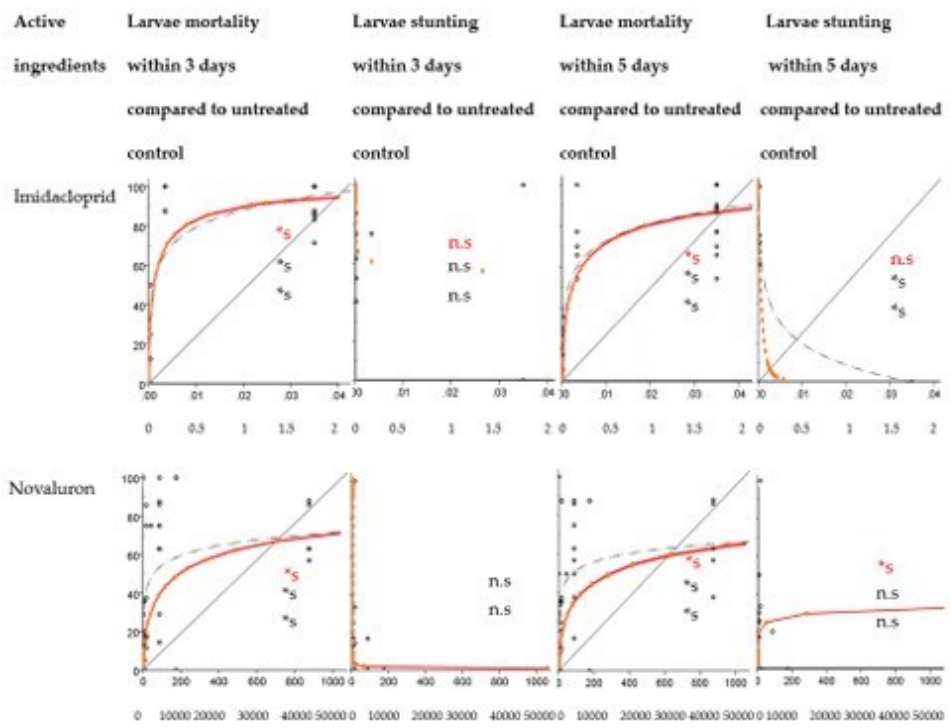


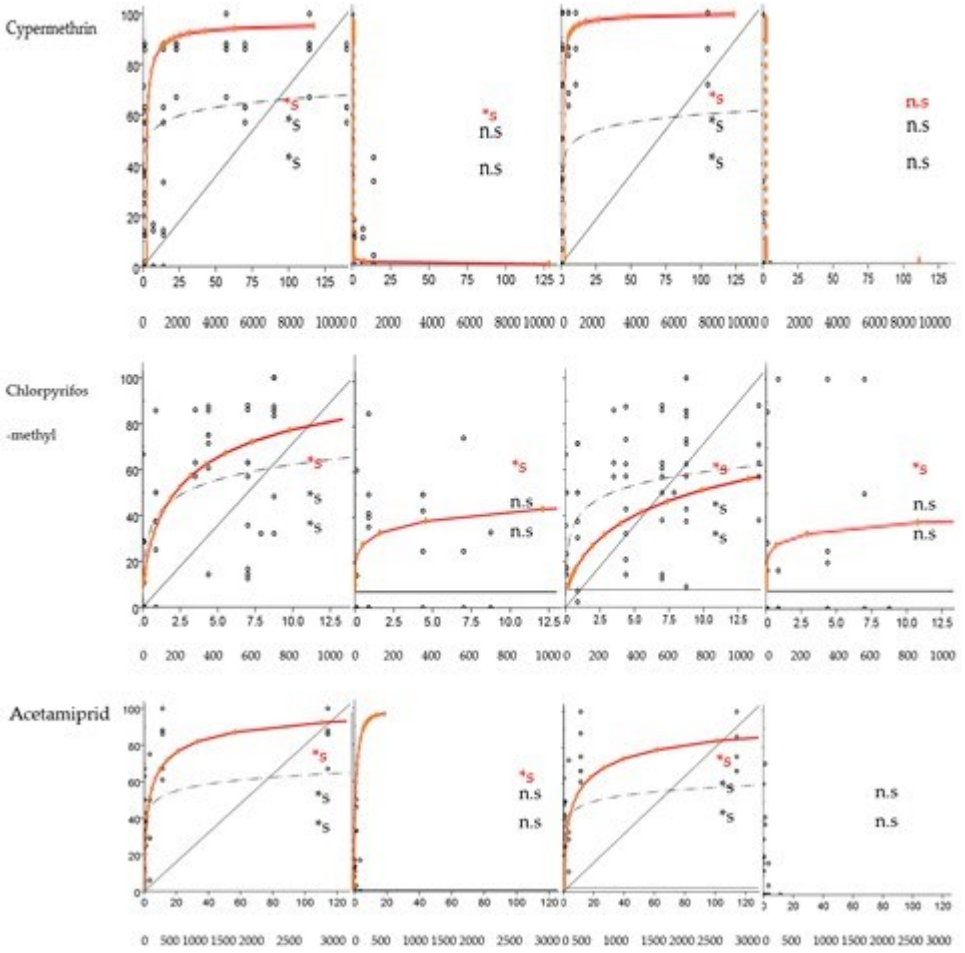
Figure 2. Mortality of *Diabrotica v. virgifera* larvae during hatching from eggs treated with different concentrations of active ingredients of insecticides through dipping treatments. The bioassays were conducted by transferring the treated eggs to filter paper in petri dishes under laboratory conditions (23-25ºC, 50 to 90% r.h.). Primary x-axis represents the dose tested in µg per arena, and secondary x-axis represents the dose tested in µg per ml. There are two to five experimental repetitions per treatment. Probit regression (red colored-line), linear regression (black colored-line), and logarithmic regression (black colored-dashed line) fit to dose-response and presented if significant at $p < 0.05$ (*s.); n.s. non-significant regression and no line is therefore presented.

3.2 Laboratory efficacy of commercial insecticides against *D.v.virgifera* larvae.

All seven insecticides tested caused some mortality of larvae of *D. v. virgifera* (Fig. 3, Table 2, 3). The doses required to kill larvae varied widely between the active ingredients. Highest toxicity to larvae had imidacloprid (refer to lowest ED_{50} value in Table 2, 3). In contrast, unrealistically high doses were needed for novaluron to have any effect on larvae. The best, i.e. least variable dose-efficacy response curves with regard to larval mortality were found for imidacloprid (refer to highest and significant X^2 and R^2 in Table 2, 3).

All insecticides tested caused some stunting among the surviving larvae. In conclusoin, imidacloprid was the only insecticide that showed a good dose-response in causing larval mortality, and some stunting, and is therefore proposed as the most optimal positive control.


Active ingredients
Larvae mortality within 3 days compared to untreated control
Larvae stunting within 3 days compared to untreated control
Larvae mortality within 5 days compared to untreated control
Larvae stunting within 5 days compared to untreated control
Imidacloprid
Novaluron
*s
n.s


Cypermethrin
Chlorpyrifos
-methyl
Acetamiprid
*s
n.s

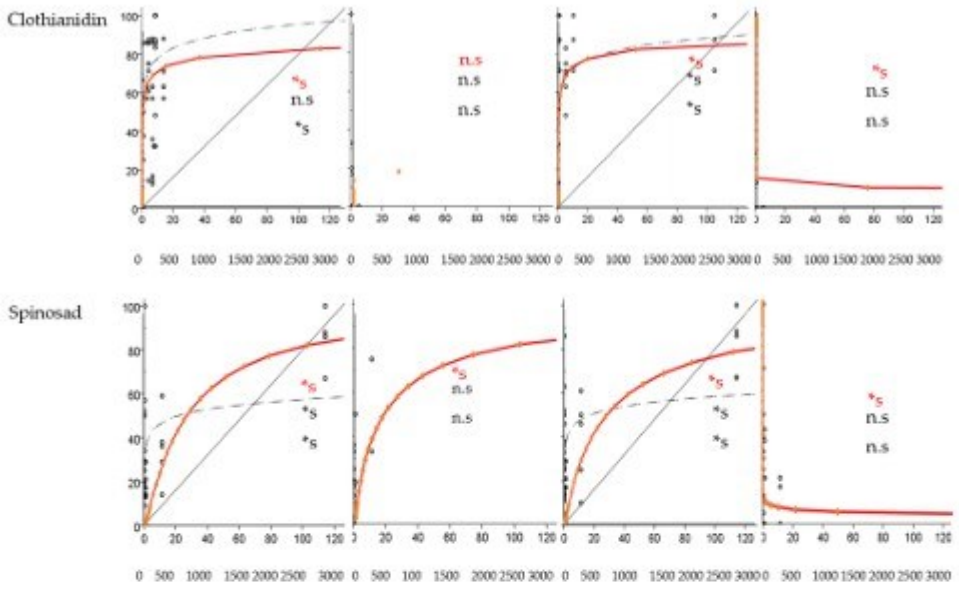
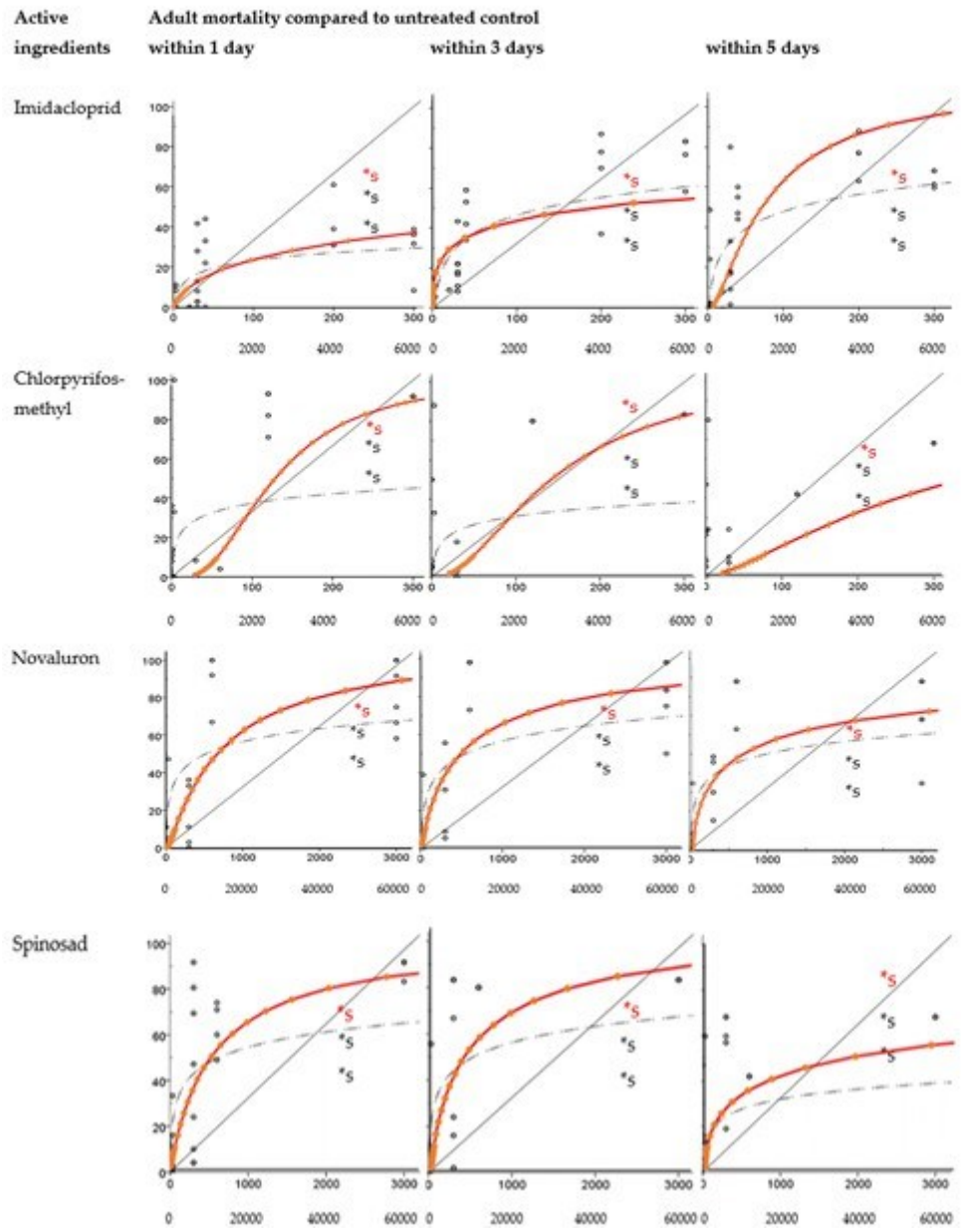


Figure 3. Mortality and stunting of *D. v. virgifera* larvae when exposed to artificial diet treated with different concentrations of active ingredients of insecticides in diet-overlay experiments under standardized laboratory conditions (23-25°C, 50-90% r.h.) using 96-well plates. The y-axis represents % larvae mortality and % stunting within 3 and 5 days compared to the untreated control. Primary x-axis represents the dose tested in µg per arena, and secondary x-axis represents the dose tested in µg per ml. Eight wells with one neonate per each of 7 plates per each of 2 to 5 experimental repetitions per treatment. Probit regression (red colored-line), linear regression (black colored-line), and logarithmic regression (black colored-dashed line) fit to dose-response and presented if significant at $p < 0.05$ (*s.); n.s non-significant regression and no line is therefore presented.

3.3. Laboratory efficacy of commercial insecticides against D.v.virgifera adults

All tested insecticides caused some mortality of adult *D. v. virgifera*, and a dose response was detectable (Fig. 4, Table 2, 3). The doses needed to kill adults varied widely the active ingredients. Highest toxicity to adults had acetamiprid (refer to lowest ED_{50} value in Table 2, 3). In contrast, unrealistically high doses were for example needed for novaluron, spinosad, and cypermethrin.


Active ingredients
Adult mortality compared to untreated control
within 1 day
within 3 days
within 5 days
Imidacloprid
Chlorpyrifos-methyl
Novaluron
Spinosad

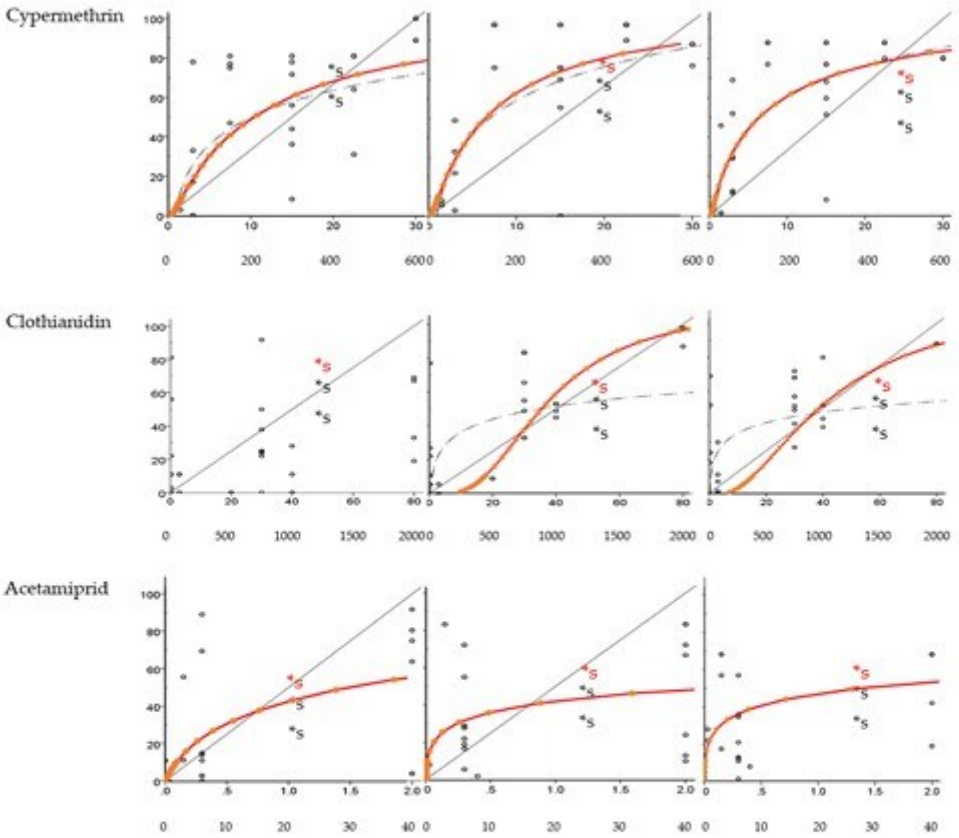


Figure 4. Mortality of *Diabrotica v. virgifera* adults when exposed to artificial diet cores treated with different concentrations active ingredients of insecticides under standardized laboratory conditions (23-25ºC, 50-90% r.h.). Each treatment has a total of 12 replicates with about 3 beetles each on 4 of 6-well plates which per each has three experimental repetitions per treatment. Primary x-axis represents the dose tested in µg per arena, and secondary x-axis represents the dose tested in µg per ml. Probit regression (red colored-line), linear regression (black colored-line), and logarithmic regression (black colored-dashed line) fit to dose-response presented if significant at $p < 0.05$ (*s.); n.s non-significant regression and no line is therefore presented.

Table 2. Effective doses (ED) (µg ml^{-1}) of common insecticides against *Diabrotica v. virgifera* eggs, larvae, and adults in standard bioassays under laboratory conditions. Efficacy data standardized to data from untreated controls. Egg dipping method (Eppendorf tubes) applied for addressing effects on eggs subsequently placed onto filter paper in petri dishes; artificial diet - overlay bioassays for larvae (96-well plates); artificial diet core-overlay bioassay for adults (6-well plates). Equations only shown in case of significant relationships at $p < 0.05$; ED $_{50}$ and ED $_{80}$ values limited to a ceiling of a max of 1000 µg ml $^{-1}$.

			Probit						Linear						Logarithmic					
Active ingredients	**Effects**	**df**	**Z value**	**P value**	**X^2**	**Probit equation**	**ED_{50} (95% fiducial limit) µg ml^{-1}**	**ED_{80} (95% fiducial limit) µg ml^{-1}**	**F value**	**P value**	**R^2**	**Linear equation**	**ED_{50} µg ml^{-1}**	**ED_{80} µg ml^{-1}**	**F value**	**P value**	**R^2**	**Logarithmic equation**	**ED_{50} µg ml^{-1}**	**ED_{80} µg ml^{-1}**
	Eggs																			
imidacloprid	Hatching of eggs within 5 days (%)	52	-28.2	< 0.001	1517	p = 0.9 - 0.5*log$_{10}$ x	95 (8-1000)	2 (1-29)	30.3	< 0.001	0.4	y= -0.009* x+84		492	38.5	< 0.001	0.4	y= -9.4*ln(x)+106	418	17
	Hatching of eggs within 7 days (%)	52	-32.7	< 0.001	1112	p = 1.6 - 0.7 *log$_{10}$ x	176 (42-1000)	12 (2-49)	50.6	< 0.001	0.5	y= -0.01* x+85		546	97.7	< 0.002	0.7	y= -10.4*ln(x)+111	366	21
	Mortality of hatching larvae within 3 days (%)	52	6.4	< 0.001	582				12.7	0.001	0.2	y= 0.01* x			5.3	0.03	0.1	y= -3.3*ln(x)+3	0.003	2.96*10 $^{-7}$
	Mortality of hatching larvae within 5 days (%)	52	4.2	< 0.001	507				35.3	< 0.001	0.4	y= 0.01* x			9.8	0.003	0.2	y= -4.9*ln(x)+219	0.002	3.92*10 $^{-6}$
	Delay in egg hatching (days)	52	1.1	0.29	6				5.1	0.03	0.09				0.06	0.8	0.001			
novaluron	Hatching of eggs within 5 days (%)	50	-2.5	0.01	675	p = 0.5 - 1*log$_{10}$ x		0.000031	5.9	0.02	0.1	y= -0.004* x+93			5.8	0.02	0.1	y= -3.4*ln(x)+100		364
	Hatching of eggs within 7 days (%)	50	-7.6	< 0.001	458	p = 0.9 - 0.2 *log$_{10}$ x		2	19.9	< 0.001	0.3	y= -0.006* x+102			11.8	0.001	0.2	y= -4.2*ln(x)+108		778
	Mortality of hatching larvae within 3 days (%)	50	-2.6	0.009	528				0.00007	0.99	0.000001				0.2	0.6	0.01			

	Mortality of hatching lar- vae within 5 days (%)	50	0.3	0.79	214				0.05	0.8	0.001			0.05	0.8	0.001			
	Delay in egg hatching (days)	50	[a]						0.3	0.6	0.01			0.01	0.9	0.002			
cypermethrin	Hatching of eggs within 5 days (%)	52	-30.4	< 0.001	1160	p = 1 -0.6 *$\log_{10}$ x	66 (6-1000)	2 (0.088-18)	27.2	< 0.001	0.4	y= -0.013* x+86	507	40.6	< 0.001	0.5	y= -0.013*ln(x)+91		
	Hatching of eggs within 7 days (%)	52	-31.2	< 0.001	1089	p = 18 - 0.6 *$\log_{10}$ x	179 (18-1000)	6 (0.4-63)	44.6	< 0.001	0.5	y= -0.013* x+91	852	55.1	< 0.001	0.5	y= -10.3*ln(x)+115	577	32
	Mortality of hatching lar- vae within 3 days (%)	52	2.2	0.03	537				9.2	0.004	0.2	y= 0.01* x		2.8	0.09	0.05			
	Mortality of hatching lar- vae within 5 days (%)	52	-2.5	0.01	172	p = -0.5 - 0.2 *$\log_{10}$ x	0.004	8 *10^{-8}	20.8	< 0.001	0.3	y= 0.01* x		13.1	0.001	0.2	y= -5.85*ln(x)+17	0.004	2.2*10^{-5}
	Delay in egg hatching (days)	52	0.7	0.47	4				3.1	0.084	0.06			0.03	0.9	0.001			
clothianidin	Hatching of eggs within 5 days (%)	52	14.9	< 0.001	1139	p = 1 - 0.3 *$\log_{10}$ x		2.5 (5*10^{-5}-1000)	27.1	< 0.001	0.4	y= -0.01* x+107		14.9	< 0.001	0.2	y= -8.9*ln(x)+130		312
	Hatching of eggs within 7 days (%)	52	-21.2	< 0.001	991	p = 1.4 - 0.5 *$\log_{10}$ x		19 (0.2-1000)	42.1	< 0.001	0.5	y= -0.01* x+107		17.3	< 0.001	0.2	y= -8.2*ln(x)+126		300
	Mortality of hatching lar- vae within 3 days (%)	52	-1.3	0.18	432	p = -0.4 - 0.1*$\log_{10}$ x	0.000003	5*10^{-19}	1.6	0.2	0.03			0.08	0.8	0.001			
	Mortality of hatching lar- vae within 5 days (%)	52	-0.9	0.34	139				18.5	< 0.001	0.3	y= 0.01* x		6.5	0.01	0.1	y= -3.9*ln(x)+23	0.001	5*10^{-7}
	Delay in egg hatching (days)	52	-0.1	0.90	4	p = -2.2 - 0.03*$\log_{10}$ x	3.3*10^{-89}	6.810^{-123}	11.6	0.001	0.2			3.5	0.07	0.06			
chlorpyrifos-methyl	Hatching of eggs within 5 days (%)	59	-4.4	< 0.001	607	p = 0.7 - 0.3*$\log_{10}$ x	405	0.19	17.8	< 0.001	0.2	y= -0.36* x+109	165	81	21.9	< 0.001	0.3	y= -9.9*ln(x)+113	648	31
	Hatching of eggs within 7 days (%)	59	-6.1	< 0.001	430	p = 1- 0.4*$\log_{10}$ x	387	2	20.2	< 0.001	0.3	y= -0.3* x+105	183	84	28.7	< 0.001	0.3	y= -8.7*ln(x)+109	1000	31

	Mortality of hatching larvae within 3 days (%)	59	2.1	0.04	700				2.1	0.2	0.03				2.2	0.2	0.04		
	Mortality of hatching larvae within 5 days (%)	59	3.1	0.002	316				1.2	0.3	0.02				1.5	0.2	0.03		
	Delay in egg hatching (days)	59	0.2	0.840	6				2.3	0.1	0.04				2.3	0.1	0.04		
acetamiprid	Hatching of eggs within 5 days (%)	56	-3.0	0.002	1274	$p = 0.4 - 0.2 * \log_{10} x$	273	0.001	2	0.2	0.04				7.4	0.009	0.1	$y = -4.7 * \ln(x) + 88$	6.5
	Hatching of eggs within 7 days (%)	56	-8.2	< 0.001	814	$p = 0.6 - 0.3 * \log_{10} x$	150	0.2	4.4	0.04	0.07				21.2	< 0.001	0.3	$y = -6.1 * \ln(x) + 93$	9
	Mortality of hatching larvae within 3 days (%)	56	5.0	< 0.001	1206	$p = -0.4 + 0.1 * \log_{10} x$	404		0.07	0.8	0.001				0.73	0.4	0.01		
	Mortality of hatching larvae within 5 days (%)	56	-1.9	0.06	839				2.5	0.1	0.04				0.09	0.7	0.002		
	Delay in egg hatching (days)	56	-0.3	0.77	12	$p = -2 - 0.09 * \log_{10} x$	$2.2 * 10^{-23}$	$1.2 * 10^{-32}$	3.4	0.07	0.06				2.3	0.1	0.04		
spinosad	Hatching of eggs within 5 days (%)	38	0.6	0.52	283				1.2	0.3	0.03				0.02	0.9	0.0004		
	Hatching of eggs within 7 days (%)	38	2.0	0.051	161				1.2	0.3	0.03				0.4	0.5	0.01		
	Mortality of hatching larvae within 3 days (%)	38	-8.8	< 0.001	513	$p = -0.4 - 1 * \log_{10} x$	0.42	0.06	3.9	0.05	0.1				0.4	0.5	0.01		
	Mortality of hatching larvae within 5 days (%)	38	-1.2	0.22	197	$p = -0.6 + -0.05 * \log_{10} x$	$3.2 * 10^{-14}$	$2.4 * 10^{-33}$	5.6	0.02	0.1	$y = 0.1 * x$	500	800	0.2	0.6	0.01		
	Delay in egg hatching (days)	38	0.1	0.90	2				8.4	0.006					1.6	0.2	0.04		
	First instar larvae																		

imidacloprid	Larval mortality within 3 days (%)	29	21.7	< 0.001	513	$p = 1.3 + 1*\log_{10} x$	0.06	0.4	15.9	< 0.001	0.4	y= 50* x	1	2	50.4	< 0.001	0.6	y= 13.1*ln(x)+86	0.06	0.7
	Larval mortality within 5 days (%)	29	14.8	< 0.001	466	$p = 0.7 + 0.9*\log_{10} x$	0.2	1.4	29.1	< 0.001	0.5	y= 50* x	1	2	67.9	< 0.001	0.006	y= 15.4*ln(x)+74	0.2	1.5
	Larval stunting within 3 days (%)	7	-1.1	0.27	269				0.3	0.6	0.05				0.2	0.7	0.03			
	Larval stunting within 5 days (%)	5	-0.4	0.67	49				19.9	< 0.001	0.8	y= 50* x	1	2	19.9	0.01	0.8	y= -16.2*ln(x)+11	0.09	0.02
novaluron	Larval mortality within 3 days (%)	42	15.2	< 0.001	1392				7.3	< 0.001	0.1	y= 0.002* x			18.9	< 0.001	0.3	y= 7.5*ln(x)-10		
	Larval mortality within 5 days (%)	42	13.6	< 0.001	1291				5.9	0.02	0.1	y= 0.002* x			14.2	0.001	0.3	y= 6.3*ln(x)-2		
	Larval stunting within 3 days (%)	27	a						0.3	0.6	0.01				0.01	0.9	0.0002			
	Larval stunting within 5 days (%)	25	4.1	< 0.001	670				0.2	0.7	0.01				0.6	0.4	0.03			
cypermethrin	Larval mortality within 3 days (%)	72	20	< 0.001	2578				9.6	0.003	0.1	y= 0.0125* x			12.5	0.001	0.2	y= 4.3*ln(x)+28	135	
	Larval mortality within 5 days (%)	71	14.2	< 0.001	1774				8.1	< 0.001	0.1	y= 0.013* x			8.9	0.004	0.1	y= 4.2*ln(x)+243	597	
	Larval stunting within 3 days (%)	34	-2.1	0.04	1	$p = -1.3 - 0.2*\log_{10} x$	0.000001	$1.9*10^{-10}$	0.4	0.5	0.01				0	0.9	0.00005			
	Larval stunting within 5 days (%)	34	-0.9	0.359	917				0.2	0.7	0.01				0.01	0.9	0.0004			
clothianidin	Larval mortality within 3 days (%)	44	17.1	< 0.001	1637	$p = -0.4 + 0.5*\log_{10} x$	7 ($3*10^{-7}$-140)	354 (1.4-1000)	3.5	0.06	0.08				18.4	< 0.001	0.3	y= 7.8*ln(x)+28	16	770
	Larval mortality within 5 days (%)	44	20.2	< 0.001	1228	$p = -0.4 + 0.5*\log_{10} x$	6 (6.2-162)	456 (7.5 - 1000)	4.1	0.04	0.09	y= 0.017* x			14.6	< 0.001	0.2	y= 7.1*ln(x)+27	25	

	Larval stunting within 3 days (%)	26	1.4	0.15	1830				0.2	0.7	0.01			0.02	0.8	0.001		
	Larval stunting within 5 days (%)	24	-2.3	0.02	1530	$p = -1 -1*\log_{10} x$	$7.7*10^{-12}$	$6*10^{-23}$	0.3	0.6	0.01			0.1	0.8	0.01		
chlorpyrifos-methyl	Larval mortality within 3 days (%)	60	19.8	< 0.001	1594	$p = -2.6 + 1.1*\log_{10} x$	242 (69-521)		24.3	< 0.001	0.3	y= 0.13* x	400	640	22.8	< 0.001	0.3	y= 9.6*ln(x)+1.3	161
	Larval mortality within 5 days (%)	60	7.6	< 0.001	1260	$p = -3 + 1*\log_{10} x$	935		20.6	< 0.001	0.3	y= 0.13* x	400	640	20.9	< 0.001	0.3	y= 8.3*ln(x)+6.5	192
	Larval stunting within 3 days (%)	37	7.4	< 0.001	1105				0.8	0.4					0.05	0.8	0.002		
	Larval stunting within 5 days (%)	33	4.1	< 0.001	1880				0.08	0.8					0.1	0.8	0.003		
acetamiprid	Larval mortality within 3 days (%)	48	18.5	< 0.001	881	$p = -1.7 + 0.9*\log_{10} x$	80 (9 -292)	793 (218-1000)	21.6	< 0.001	0.3	y= 0.05* x	1000		21.1	< 0.001	0.3	y= 4.8*ln(x)+28	80
	Larval mortality within 5 days (%)	48	14.6	< 0.001	506	$p = -1.8 + 0.8*\log_{10} x$	190 (28-744)		17.5	< 0.001	0.3	y= 0.05* x	1000		22.8	< 0.001	0	y= 5.1*ln(x)+26	115
	Larval stunting within 3 days (%)	38	6.2	< 0.001	286	$p = -3.4 + 2.2*\log_{10} x$	37	93	1.4	0.2	0.04				0.7	0.4	0.02		
	Larval stunting within 5 days (%)	36	a						0.7	0.2	0.004				0.2	0.6	0.01		
spinosad	Larval mortality within 3 days (%)	27	11	< 0.001	980	$p = -5 + 1.7*\log_{10} x$	606 (5-1000)		6	0.02	0.2	y= 0.05* x	1000		26.5	< 0.001	0.4	y= 5.55*ln(x)+16.6	414
	Larval mortality within 5 days (%)	27	8	< 0.001	787	$p = -3.9 + 1.4 *\log_{10} x$	647		7	0.01	0.2	y= 0.05* x	1000		30.9	< 0.001	0.4	y= 5.46*ln(x)+18	337
	Larval stunting within 3 days (%)	41	8	< 0.001	465	$p = -3.5 + 1.4 *\log_{10} x$	416		1.6	0.2	0.04				0.05	0.8	0.001		
	Larval stunting within 5 days (%)	39	-2.7	< 0.001	1195	$p = -0.9 - 0.3*\log_{10} x$	4.1×10^{-4}	$2*10^{-7}$	0.5	0.5	0.01				1.2	0.3	0.03		

	Adults																			
imidacloprid	Adult mortality within 1 days (%)	35	8.4	< 0.001	410				22.1	< 0.001	0.4	y= 0.013* x			24.3	< 0.001	0.4	y= 5.5*ln(x)-20		
	Adult mortality within 3 days (%)	35	17	< 0.001	233				83.8	< 0.001	0.7	y= 0.013* x			72.8	< 0.001	0.7	y= 12.9*ln(x)-54		
	Adult mortality within 5 days (%)	35	13	< 0.001	430				37.4	< 0.001	0.5	y= 0.013* x			48.3	< 0.001	0.6	y= 12.46*ln(x)-50		
novaluron	Adult mortality within 1 days (%)	35	15	< 0.001	1145				50	< 0.001	0.6	y= 0.0012* x			34.1	< 0.001	0.5	y= 9.98*ln(x)-44		
	Adult mortality within 3 days (%)	35	13	< 0.001	580				46.9	< 0.001	0.6	y= 0.0012* x			44.1	< 0.001	0.6	y= 11.37*ln(x)-58.6		
	Adult mortality within 5 days (%)	35	18	< 0.001	381				37	< 0.001	0.5	y= 0.0012* x			33.8	< 0.001	0.5	y= 9.31*ln(x)-44		
cypermethrin	Adult mortality within 1 days (%)	35	20	< 0.001	756	p = -3.7+ 1.6*$\log_{10}$ x	206 (44-450)	697 (302-1000)	40.5	< 0.001	0.5	y= 0.13* x	400	640	36.4	< 0.001	0.5	y= 19.94*ln(x)-60	253	
	Adult mortality within 3 days (%)	35	16	< 0.001	1040	p = -3.6 + 1.7*$\log_{10}$ x	145 (0.052-444)	463 (22-1000)	37.5	< 0.001	0.5	y= 0.13* x	400	640	52.1	< 0.001	0.7	y= 25.08*ln(x)-79	173	573
	Adult mortality within 5 days (%)	35	13	< 0.001	609	p = -2.9+ 1.4*$\log_{10}$ x	131(0.42-424)	549 (48-1000)	33.4	< 0.001	0.5	y= 0.13* x	400	640	64.5	< 0.001	0.7	y= 22.9*ln(x)-66	159	589
clothianidin	Adult mortality within 1 days (%)	35	Δ						7.3	< 0.001	0.2	y= 0.05* x	1000		1.8	0.2	0.05			
	Adult mortality within 3 days (%)	35	16.	< 0.00	561	p = -12+ 4*$\log_{10}$ x	898 (620-1000)		60.7	< 0.001	0.6	y= 0.05* x	1000		18.7	< 0.001	0.4	y= 11.1*ln(x)-25	859	
	Adult mortality within 5 days (%)	35	12.4	< 0.001	370	p = -9 + 3.03*$\log_{10}$ x	997 (612-1000)		43.2	< 0.001	0.5	y= 0.05* x	1000		12.8	0.001	0.3	y= 8.99*ln(x)-14		
chlorpyrifos-methyl	Adult mortality within 1 days (%)	35	22.7	< 0.001	1036	p = -12.6 + 3.7*$\log_{10}$ x			40.3	< 0.001	0.5	y= 0.013* x			7.1	0.01	0.2	y= 6.96*ln(x)-17		

	Adult mortality within 3 days (%)	35	18.6	< 0.001	526	p = -9.7 + 2.8*log10 x			33.4	< 0.001	0.5	y= 0.013* x			5.8	0.02	0.2	y= 6.38*ln(x)-18
	Adult mortality within 5 days (%)	35	11.6	< 0.001	322	p = -7.4 + 1.9*log10 x			15.9	< 0.001	0.3	y= 0.013* x			0.8	0.4	0.02	
acetamiprid	Adult mortality within 1 days (%)	35	15.4	< 0.001	928	p = -1.6 + 1*log10 x	38 (12-1000)	262 (54-1000)	4.5	0.04	0.1	y= 2* x	25	40	2.6	0.1	0.07	
	Adult mortality within 3 days (%)	35	6.1	< 0.001	628	p = -0.9 + 0.6*log10 x	55		5.5	0.02	0.1	y= 2* x	25	40	3.4	0.07	0.09	
	Adult mortality within 5 days (%)	35	6.7	< 0.001	473	p = -0.9+ 0.5*log10 x	46		0.8	0.4	0.02				0.09	0.7	0.003	
spinosad	Adult mortality within 1 days (%)	35	24.	< 0.001	633				38.2	< 0.001	0.5	y= 0.0012* x			35.1	< 0.001	0.5	y= 9.6*ln(x)-42
	Adult mortality within 3 days (%)	35	18	< 0.001	619				21.9	< 0.001	0.3	y= 0.0012* x			28.1	< 0.001	0.4	y= 10.1*ln(x)-46
	Adult mortality within 5 days (%)	35	8.	< 0.001	247				16.5	< 0.001	0.3	y= 0.0012* x			7.8	0.008	0.2	y= 6.36*ln(x)-32

Δ Probit analysis cannot be computed due to linear dependence among covariates or a zero slope.

4. Discussion

We have assessed the activity and dose-response of commonly used insecticides against eggs, first instar larvae, and adults of the major maize pest *D. v. virgifera*. Our detailed findings, as presented in Figs 1 to 4 as well as in Table 2 and 3, are hoped to aid researchers in choosing an appropriate positive control for screening novel crop protection agents depending on the to-be-assessed mode of activity, the targeted pest stage, the type of planned bioassay type and the to-be-assessed parameter. It will also help researchers in assessing levels of resistance of the different life stages of *D.v.virgifera* to certain insecticides [72,73,74,25,75,76].

We applied probit, linear, and logarithmic regression to accurately select the appropriate positive control, filtered from the best fit among the three regression results. Such regression models are useful methods to understand the relationship between dose and response and are a pre-requisite for evaluating reliable ED_{50} and ED_{80} of insecticides on insects.

A positive control is defined as a thoroughly validated reference substance that consistently elicits a predetermined reaction in a test protocol, confirming the method's fidelity to anticipated outcomes [35]. Scientists evaluate agents as positive control considering primarily the reliability of effects and the dose–response relationships, but may also include other aspects such as reversibility and persistence, cost effectiveness, commercial availability, low experimental hazard, specificity or generality of an effect [38,39,40]. [38] reported that detailed dose-response data can be extremely important in interpreting results from agents with unknown activities, or for assessing reduced activities due to potential resistance. The scientist selected an agent as positive control based on the shape of the dose-response function (linear or nonlinear). Selection of a suitable dose for the positive control while screening agents or substance is equally important [37]. Thus, in our study, we based the decision for appropriated positive control on the level of significance of effects (lowest p-value), the highest toxicity (lowest ED_{50}) [41] and most robust dose-response relationships (highest X^2 or R^2).

We successfully assessed the dose-response of seven insecticides to *D. v.virgifera*. Naturally, all our tested common insecticides had some effects on this maize pest. Most mortality was caused to the adult stage, some mortality to the larvae, and least and sometimes no mortality to the eggs stage. However, it is hard to compare efficacy among eggs, larvae and adults. Therefore, we tried to standardize the doses to body weight (Table 1) and followed experiences and results presented in the literature.

The egg stage of *D.v.virgifera* is rarely targeted by plant protection companies because (a) eggs are non-feeding and usually well protected insect stages, (b) the eggs are concealed and widely distributed in the soil and therefore difficult to reach, and (c) because it is not a damaging pest stage and therefore of less interest to farmers. Nevertheless, a reduction of egg numbers would, if feasible, reduce the initial pest pressure. Our standard dipping assays with ready-to-hatch eggs subsequently incubated on filter paper showed that several insecticides caused some, but not high egg mortality except spinosad; and novaluron only at unrealistically high doses. Imidacloprid and clothianidin were the insecticides that seem most suitable for egg standard bioassay considering the significance of their effects and their high X^2 and R^2; to the three parameters recorded (hatching rates, delay in egg hatching, and mortality of hatching larvae).

There are not too many comparative studies on insect eggs with regard to our here-assessed insecticides. For example, a low dose of 0.06 g of active ingredient imidacloprid per liter was reported to cause completely prevent hatching (100%) in *Aphis gossypii* compared to untreated control [42]. Imidacloprid at a dose of 2 g L^{-1} was reported to reduce the larval hatching rate of *Hippodamia convergens* (Coleoptera: Coccinellidae) [43], and caused 56% egg mortality on *Trichogramma chilonis* when applied at 25 g active ingredients ha^{-1} [71].

However, in our study, least variable dose-efficacy response curves with regard to egg mortality of *D.v.virgifera* were found for imidacloprid, novaluron, cypermethrin, clothianidin, chlorpyrifos-methyl and acetamiprid; but not for spinosad. All insecticides also caused some mortality to the neonates hatching from treated eggs; with the best response for are imidacloprid, cypermethrin, clothianidin and spinosad. Our results are comparable with the results reported by [46] who treated 3 to 4 days old eggs of *Cnaphalocrocis medinalis* (Lepidoptera: Crambidae) with imidacloprid at a dose of 2 mg L^{-1} inhibiting 20% to 92% of egg hatching. Novaluron, clorpyrifos-methyl, or acetamiprid seem less or not suitable due to their low toxicity to eggs, not delaying egg hatching, and only affecting hatching larvae are high dose. In conclusion, there are two options proposed to use common insecticides as positive control in comparative screenings (referring to the significance of effects, high X^2 and R^2, and highest toxicity) against eggs of *D.v.virgifera*, this is imidacloprid and clothianidin (Table 3). Both also cause mortality of hatching larvae as well as the delay in egg hatching.

The larval stage *D.v.virgifera* is the most important pest management target for growers as the three larval instars are the most damaging stages through their feeding on the maize roots. Therefore, a number of insecticidal products have been developed over nearly a century, and have been widely used [10,46,47,49]. Nevertheless, larval control is not easy because of their concealment below ground, and their relatively long presence in the soil for at least a month, their ability to develop resistance, and the ban of several products [21,48,50,51]. Many researchers are conducting intense research and development for new ingredients and products. One of the first activities to find new agents is high-throughput screening on the target pest under standardized conditions. Our artificial diet overlay assays with larvae in 96-well plates revealed that all tested standard insecticides caused, as expected, some mortality to larvae. Highest toxicity to larvae had imidacloprid and least toxicity had novaluron. Imidacloprid has systemic properties with broad-spectrum and relatively long-lasting activity [52]. Our bioassay results are consistent with [53,54] showing that imidacloprid was more toxic to larvae of *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) with an LC_{50} of 287 ppm than other insecticides such as bychlorpyrifos or nano-imidacloprid. It was also toxic to larvae of *Bradysia odoriphaga* (Diptera: Sciaridae) [55]. Despite some advantages of imidacloprid, there are, according to our results several options to use common insecticides as positive controls in comparative screenings against larvae, such as imidacloprid, cypermethrin, clothianidin, chlorpyrifos-methyl, acetamiprid or spinosad (Table 3). Novaluron seems less suitable due to the need of unrealistically high doses. However, the best dose-efficacy response curves were found for imidacloprid making it most suitable as positive controls.

The adult stage of *D.v. virgifera* is, next to the larvae, another important stage in the management of this pest. As adults are easily exposed to insecticide sprays due to their feeding habits on the maize plants, several products have been developed for nearly a century. Nevertheless, the ban of a number of such insecticides requires the search for new agents. Our artificial diet-core overlay assays with adults in 6-well plates revealed that all tested insecticides caused, as expected, some mortality in adults. However, the adults were slightly more difficult to kill than the insect larvae. Highest toxicity to adults had acetamiprid and least had clothianidin and cypermethrin. All insecticides with activity caused good dose-efficacy responses (Fig.4), and many confirm existing studies. For example, acetamiprid at a high concentration of 1 ppm caused 93% of adult mortality of *Tribolium confusum* (Coleoptera: Tenebrionidae [56, 57 58]. Acetamiprid at 8 ppm was also reported to cause 78 to 97% mortality to adult of *Trogoderma granarium* (Coleoptera: Dermestidae) [59]. Cypermethrin caused 100% mortality on darkling beetles (*Alphitobius diaperinus*) [60] and Khapra beetles (*Trogoderma granarium*) [61] at doses from 0.5% to 4%. [62] stated that clothianidin showed high toxicity to adults of *Bemisia tabaci* (Hemiptera: Aleyrodidae) with an LC_{50} of 6 mg L^{-1}. Clothianidin was also reported toxic to adults of *Atractomorpha lata* (Orthoptera: Pyrgomorphidae) [63] and *Aphidius gifuensis* (Hymenoptera: Braconidae) with mortalities ranging from 30 to 99% [64].

In conclusion, according to our results and the literature, acetamiprid might be proposed as positive controls in adult bioassays, as it had a good dose-response in killing adults, and this with high toxicity.

In general, it needs to be noted that our suggestions for positive controls are propositions only, as the choice depends on the study aim. Our provided details on ED_{50} and ED_{80} values, the different dose-response equations (Table 2) as well as our summary in table 3 may aid this decision process. These results can be helpful for researchers in the future because the non-diapause of *D. v. virgifera* is acceptable when evaluating the development of resistance to insecticides.

Table 3. Suitability of common insecticides against *Diabrotica v. virgifera* eggs, larvae, and adults as positive controls in standard bioassays under laboratory conditions, discussed with regard to their significance of effects (+ at $p < 0.05$), their least variable dose-response (+ if $X^2 > 300$ and $R^2 > 0.3$), and their highest toxicity (+ if lowest ED_{50}). Cells are framed an insecticide is considered most suitable for a specific bioassay type against a particular pest stage and assessed parameter.

	Significant effects / High X^2 and R^2 / Highest toxicity						
	Imidacloprid	Novaluron	Cypermethrin	Clothianidin	Chlorpyrifos-methyl	Acetamiprid	Spinosad
Eggs	Screening assay type: Egg dipping method with eggs subsequently placed onto filter paper in petri dishes						
Hatching rate of eggs within 5 or 7 days (%)	+ /+ / -	+ / + / -	+ / + / -	+ / + / -	+ / + / -	+ / + / -	- / - / -
Delay in egg hatching (days)	+ / - / -	- /- / -	- /- / -	+ /- / -	- /- / -	- /- / -	+ /- / -
Mortality of hatching larvae within 3 or 5 days (%)	+ / + / +	+ / + / -	+ / + / +	+ / + / +	+ / + / -	+/ + / -	+ / + / +
First instar larvae	Screening assay type: Artificial diet - overlay bioassays for neonates (96-well plates, 1 neonate per well)						
Larval mortality within 3 or 5 days (%)	+ / + / +	+ / + / -	+ / - / -	+ / + / -	+ / + / -	+ / + / -	+ / + / -
Larval stunting	+ / + / -	+ / + / -	+ / + / +	+/ + / -	+/ + / -	+ / + / -	+ / + / -

within 3 or 5 days (%)							
Adults	Screening assay type: diet core- overlay bioassay for adults (6-well plates, 3 adults per well).						
Adult mortality within 1, 3 or 5 days (%)	+ / + / -	+ / + / -	+ / + / -	+ / + / -	+ / + / -	+ / + / +	+ / + / -

5. Conclusions

In conclusion, for the here applied bioassay methods on the different life stages of *D. v. virgifera* under standardized laboratory conditions, we may propose imidacloprid or clothianidin as positive control in egg dipping bioassay. We may also propose imidacloprid in artificial diet overlay larval bioassays, because of its robust dose-response curves, its multiple types of effects, and its relatively high toxicity. In contrast, acetamiprid might be proposed as positive controls in adult bioassays, as it had a good dose-response in killing adults, and this with relatively high toxicity. Those, as well as potentially other choices for appropriate positive controls with reference to our provided details and depending on the study target and aim, are hoped to aid researchers in their screening of new crop protection agents against the eggs, larvae or adults of this important invasive pest.

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6 Chapter IV.

Effect of microbial bio stimulants on maize and its pest, the western corn rootworm, *Diabrotica virgifera virgifera*

Agronomy (under revision)

6.1 Abstract and Introduction

agronomy

MDPI

Article

Effects of microbial bio stimulants on maize and its pest, the western corn rootworm, *Diabrotica virgifera virgifera*

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Abstract: The western corn rootworm (WCR), *Diabrotica virgifera virgifera*, (Coleoptera: Chrysomelidae) is a serious pest of maize in the USA and Europe. Microbial plant bio stimulants, such as bacteria, fungi, or algae, are designed to stimulate plant nutrition and growth, with some hypothesized to possess insecticidal properties. We tested 10 bio stimulants (4 bacteria, 5 fungi, 1 alga) under laboratory and greenhouse conditions. In the laboratory, 10% of bio stimulants had a positive effect on WCR eggs with 40% of the bio stimulants having insecticidal effects on WCR larvae including *Beauveria bassiana, Rhizophagus irregularis, Trichoderma asperellum* (all fungi) and *B. japonicum* (bacterium). None affected WCR adults. In the greenhouse, 20% of bio stimulants (50% of the bacterial) promoted maize growth without WCR larvae infestation, particularly *B. japonicum* and *Ensifer meliloti*. Furthermore, 30% of bio stimulants positively enhanced plant defence against WCR larvae, assessed by the IOWA root damage scale. These included *Bacillus amyloliquefaciens, Bacillus subtilis,* and *E. meliloti*. These results suggest that *B. amyloliquefaciens, B. subtilis, E. meliloti* could be promising candidates for increasing maize resilience against WCR, thereby contributing to sustainable and integrated pest management strategies. Further research into their modes of action and field trials are necessary to optimize their use in sustainable agriculture.

Keywords: biocontrol agent; pest management strategiesbio stimulants; insecticidal effects; egg; larva; adult

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1. Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte is a chrysomelid beetle, which is one of the most important pests of maize (*Zea mays* L.) in the USA and Europe [1,2]. Its larvae feed on maize roots which can lead to plant instability, reduced growth and yield loss [1]. This pest has 7 developmental stages: egg, three larval instars, pre-pupa, pupa, and adult. Adult females lay 300-400 eggs in the top 5-20 cm of soil among the roots of maize [3], and these eggs then go through a period of diapause. The first instar larvae (L1) burrow through the soil to search for and feed on the maize roots before metamorphosing into the second larval stage (L2) and finally into the third larval stage (L3). In order to control *Diabrotica v. virgifera* larvae, farmers primarily use crop rotation, GMO in the USA, entomopathogenic nematodes (*Heterorhabditis bacteriophora)* [42], and also usually utilize insecticides spraying approaches.

Farmers apply either granular or occasionally liquid soil insecticides in the seed furrow or insecticide-coated seeds to control the insect larvae. Additionally, farmers also occasionally spray insecticides over large areas of the leaves against the adult insects in order to reduce egg laying and thus minimize the damage caused by the larvae in the following season. As a result, farmers in many maize-growing areas are struggling to control soil insects such as the corn rootworm due to the fact that some insecticides are banned to

* Larger figures are available in the appendices

6.2 Material and methods

use because of its toxicity to bees population and also some insecticides such as organophosphates, carbamates, pyrethroids and neonicotinoids have encountered resistance in populations of *Diabroticaw. virgifera* [4,5,6,7,1,8]. Microbial bio stimulants consist of microbes like bacteria, fungi, or algae. They improve plant nutritional processes without relying on their nutrient content, with the primary goal of enhancing plant agronomic performance. They are usually applied as planting materials or growing substrates and sometimes as foliar treatments. Furthermore, they are claimed to have the capacity of modifying physiological processes of a plant in a way that provides benefits to nutrient uptake or efficiency, and/or to plant growth or stress tolerance [9]. Commercial microbial bio stimulants are available on the market and have been used in agriculture for decades. Many ingredients of microbial bio stimulants have been reported to have insecticidal properties [10].

Assessing their potential insecticidal effects against *Diabrotica v. virgifera* is needed to better understand the multiple effects of microbial bio stimulants on maize plants. Our finding will be able to identify candidate of microbial bio stimulants that can enhance maize growth and also possess insecticidal properties against *Diabrotica v. virgifera*. By determining which bio stimulants are effective in controlling different life stages of this pest, we can provide farmers with more sustainable and integrated pest management strategies. This could potentially reduce the reliance on chemical insecticides, lower the risk of resistance development, and mitigate the negative environmental impacts associated with conventional pest control methods. Furthermore, our research will contribute to the broader understanding of the multifaceted roles that microbial bio stimulants can play in modern agriculture, paving the way for more resilient and productive cropping systems.

2. Materials and Methods

2.1. Tested commercial microbial bio stimulants under laboratory condition

In this study, ten commercially available microbial bio stimulants were tested to determine their effects on *Diabrotica v. virgifera* life stages (eggs, larvae, and adults) under laboratory standard condition. These microbial bio stimulants were categorized as bacterial group (*Bacillus amyloquafaciens, Bradyrhizobium japonicum, Bacillus subtilis, Ensifer meliloti,* and *Rhizobium leguminosarum*), fungal group (*Trichoderma asperellum, Beauveria bassiana, Trichoderma harzianum,* and *Rhizophagus irregularis*) and algal group (*Chlorella vulgaris*). Three to six concentrations of each microbial bio stimulants were tested against eggs, larvae, and adults. All agents were commercial products and diluted in sterile tap water to the required doses. Imidacloprid was used as positive control and sterilized tap water was served as negative control. The dosages tested were based on the unit of the product indicated on the labels (Table 1).

Table 1. Specifications of common microbial bio stimulants tested for their insecticidal effects against different life stages of *Diabrotica virgifera virgifera* under standardized laboratory conditions. Six experimental replicates per treatment and dose were tested for egg bioassay, three to five experimental replicates per treatment and dose were used for the larvae and adult bioassays.

Active ingredients	Trade name	Active ingredient concentration in product	Formulation	Tested dosage range			
				eggs	larvae	adults	unit
Bacillus amyloliquefaciens	CAP ITO BIO	$5x10^9$ spore/ml	liquid	10^2 - 10^8	10^3-10^8	10^4 -10^9	spores/ml
Bradyrhizobium japonicum	Phylazonit NG	$2x10^8$ cfu/ml	liquid	10^2 - 10^8	10^3-10^8	10^5 - 10^9	cfu/ml
Bacillus subtilis	AmazoN	$5x10^8$ cfu/g	granule	$2x10^3$ - $2x10^7$	10^3-10^8	10^3 - 10^9	cfu/ml
Ensifer meliloti	RhizoFix® RF-50	$1x10^8$ cfu/ml	liquid	10^2-10^8	10^4-10^8	10^5 - 10^9	cfu/ml
Rhizobium leguminosarum	RhizoFix® RF-40	$1x10^8$ cfu/ml	liquid	$2x10^2$- $2x10^6$	10^5-10^8	10^5- $2x10^8$	cfu/ml
Trichoderma asperellum	Hi-SPore	$3.5x10^7$ cfu/g	liquid	10^2 - 10^7	1.10^3- $2x10^7$	10^5 - 10^7	cfu/ml
Beauveria bassiana	Bora R	5 m/m %	powder	10^3 - 10^7	10^3-10^6	10^3 - 10^7	cfu/g
Trichoderma harzianum	Tricho immun	$2x10^8$ cfu/g	powder	10^2 - 10^8	10^3-10^7	10^3 -10^7	cfu/g
Rhizophagus irregularis	LALRISE® MAX	2000 spore/g	powder	$4x10^2$ - $2x10^5$	2.10^1- 2.10^7	$2x10^1$ - $2x10^3$	spore/g
Chlorella vulgaris	Bioplasm algatragya	$2x10^7$ cell/ml	liquid	10^2-10^7	10^3-10^7	10^5 - $2x10^7$	cell/ml
Imidacloprid	Confidor 200SL	200 mg/ml	liquid	0.1-10000	0.02-20	7.5-7500	µg/ml
Untreated control (unsterilized tap water)							

2.2. Egg bioassay under laboratory

For egg bioassay, to assess the effect of microbial plant bio stimulants on eggs, we applied standard screening methods under controlled semi-sterile conditions [11]. In detail, ready-to-hatch eggs were washed and placed onto a 100 µm sieve. Treatments were prepared based on the concentrations that was decided based on the label on the ingredient products. Eggs were first washed, then transferred to the tube treatments using a stainless-steel spoon (2 cm long). Stainless spoon was dipped into 70% ethanol and sterilized tap water for 3 seconds while being transferred to another treatment tube. Eggs were transferred to the treatment tubes and then soaked in treatments for 1 hour, then 20µl eggs were pipetted and then placed in the petri dish treatment. Eggs were placed in a moist filter paper of petri dish (150 mm×25 mm) and then 100 µl of sterilized tap water was added. The pipette tip was replaced between treatments. The number of eggs transferred was counted per filter paper and dish (1998 ± 325). Eggs were then incubated in the dishes at 24-25 °C for 7 days, at which point the experiment was terminated. Egg hatching and mortality of newly hatched larvae were recorded. Data were collected at 1,3, 5 and 7 days after treatments.

2.3. Larvae bioassay under laboratory

For larvae bioassay, to assess the effect of microbial plant bio stimulants on neonates of *D. v. virgifera*, we applied artificial diet-overlay bioassays under controlled semi-sterile conditions. These are standard screening methods for novel agents used by many researchers [12,13,11,14]. Sterilized tap water was used as the untreated control. Each bioassay consisted of 3 to 6 96-well polystyrene plates (07-6096 of Biologix Ltd., USA, or Costar 3917 of Corning Inc., USA). Each well had a 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.

In details, 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 [15, 16,17,18,11]. This diet formulation consisted of grinded maize roots and food color, 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 grinded maize roots were grinded and added to 88 ml fluid 60 to 70°C agar. After mixing and cooling to 55 to 60°C, 0.75 g grinded lyophilized maize roots was added as well as 0.1 g green food color for better larvae observation. Thereafter, 1.7 to 1.8 ml 10% w/v KOH were added to reach a pH between 6.2 and 6.5. This mixture was mixed 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. Plates with diet were allowed to dry in a laminar flow cabinet during 45 min, and then stored overnight at 3 to 5°C. Treatments were applied the following day. This is, 17 µl of a treatment was applied to the 0.34 cm^2 diet surface reaching good coverage and therefore forcing larvae to feed through (10 to 100 µl pipette). The order of treatments was reversed every other plate to avoid edge effects. Plates were dried for 1 to 1.5 hours, and then cooled in a 3 to 5°C fridge for 1 hour. One neonate larva was placed onto the diet surface 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 of the brush onto the diet. Larvae were not placed in treatment column order but in a rectangular arrangement to avoid systemic errors. After every 12 individual larvae, the brush then was cleaned in 70% ethanol followed by sterile tap water. The filled plate was sealed with an optically clear adhesive qPCR seal sheet (#AB-1170, Termo scientific, USA or #BS3017000, Bioleader, USA) allowing data assessments without opening the plate. Four to five holes were made with famed 00-insect pins into the seal per well to allow aeration. Plates containing larvae were incubated at 24±2°C and 50 to 70% r.h. in dark in a ventilated incubator for 5 days. We assessed mortality and stunting of larvae within 3 and 5 days. These parameters were visually assessed through the clear seals of the bioassays plates using a stereomicroscope (10× magnification, SMZ-B4, Optec, Chongqing, China). Data from a plate were only accepted if the natural mortality threshold of 37.5% in the untreated control was not reached, i.e., no more than 3 dead of 8 larvae per column of wells per treatment. This is contrary to common practices with other insects in bioassays where the quality acceptance is <10% natural background mortality [18]. However, this is rarely achievable for rootworm larvae as the artificial diets known to date remain suboptimal [19]. For adult bioassay, to assess the effect of microbial plant bio stimulants on adult *D. v. virgifera*, artificial diet-overlay bioassays with different dosage were conducted under controlled semi-sterile conditions. These are standard screening methods for novel agents used by many researchers [20,8]. Sterilized tap water was served as the untreated control.

In detail, each bioassay consisted of 6 polystyrene plates of 6 wells each. Each treatment was applied to 3 wells of each plate per bioassay. The adult diet for a bioassay had been prepared 1-7 days before treatment and adult infestation. The diet was prepared under semi-sterile conditions following methods of [21,16].

2.4. Adult bioassay under laboratory

For adult diet: wheat germ, soya flower was used. For example, for 200 ml of diet, 16.5 g sucrose, 9 g cellulose, 8 g casein, 6 gr soy flour, 2.5 g yeast, 0.6 g Wesson salt mic and 0.15 g cholesterol diet were grinded and added to 165 ml fluid 60 to 70°C agar. After mixing and cooling to 55 to 60°C, 6 g grinded wheat germ was added as well as 0.0064 g chlortetracycline and 0.0064 g streptomycin sulphate were added. Thereafter, 5.5 ml glycerol were added to reach a pH 5 with temperature between 50-55°C. Then, the diet was poured out to 5-6 sterile 11 mm petri dishes. The plates with diet were allowed to dry for up to 15 minutes under laminar flow cabinet then stored at 3 to 5°C overnight. The following day, a core of diet was first transferred using screw iron. A core diet was placed to all 6-well plates.

Treatments (40 µl) were then applied above the surface of the diet core (0.34 cm^3). Adult were then transferred from the rearing cage into the wells of the 6-well plates containing diet and treatments using a handheld tube aspirator. For easiness of transfer, adults were cooled down in a fridge for 4 to 7 minutes. Each well plate received 3 to 4 adults. Adults were then incubated in the plates at 24-25°C for 7 days until the end of the experiment. Adult mortality was recorded at 1, 3, and 5 days after treatments.

2.5. Tested commercial microbial plant bio stimulants under greenhouse

In this study, ten commercials microbial bio stimulants were tested under greenhouse conditions to determine their effects on *Diabrotica v. virgifera* larvae. These were microbial bio stimulants from a group of bacteria (*Bacillus amyloquafaciens, Bradyrhizobium japonicum, Bacillus subtilis, Ensifer meliloti, and Rhizobium leguminosarum*), a group of fungi (*Trichoderma asperellum, Beauveria bassiana, Trichoderma harzianum, and Rhizophagus irregularis*) and a group of algae (*Chorella vulgaris*). As a positive control, NPK was applied to maize seeds, both infested and uninfested, with *Diabrotica virgifera virgifera* larvae. For the negative control, unsterilized tap water was used, both infested and uninfested, with *Diabrotica virgifera virgifera* larvae (Table 2).

We examined the effects of ten microbial bio stimulants at recommended dosages as written in the label, with 1-3 dosages of each bio stimulants products. All agents diluted in unsterile tap water to the required doses. The dosages were based on the unit of the products indicated on the labels (Table 2). The experiment was conducted in a greenhouse of Plant Protection and Soil Conservation Directorate of Csongrad-Csanad County in Southern Hungary, rented by CABI. The glass greenhouse consisted of closed-system compartments with ventilation capacity.

Table 2. Specifications of commercial microbial bio stimulants tested for their insecticidal effects on *Diabrotica v. virgifera* larvae under greenhouse conditions.

Active ingredients	Trade name	Treatment code	Active ingredient concentration in product	Formulation	Dose tested
Bacillus amyloliquefaciens	CAP ITO BIO	ba	$5x10^9$ spore/ml	liquid	10^4; 10^6; 10^8 spore/ml
Bradyrhizobium japonicum	Phylazonit NG	bj	$2x10^9$ cfu/ml	liquid	$2x10^9$ cfu/ml
Bacillus subtilis	AmazoN	bs	$5x10^9$ cfu/g	granule	$5x10^6$;10^7;10^9 cfu/g
Ensifer meliloti	RhizoFix® RF-50	em	$1x10^9$ cfu/ml	liquid	10^8 cfu/ml
Rhizobium leguminosarum	RhizoFix® RF-40	rl	$1x10^9$ cfu/ml	liquid	10^4;10^6;10^8 cfu/ml

Trichoderma asperellum	Hi-Spore	ta	$3.5x10^7$ cfu/g	liquid	10^8 cfu/g
Beauveria bassiana	Bora R	bb	5 m/m %	powder	5 m/m % (5000 µl/seed)
Trichoderma harzianum	Tricho immun	th	$2x10^8$ cfu/g	powder	$2x10^8$ cfu/g (5000 µl/seed)
Rhizophagus irregularis	LALRISE® MAX	ri	2000 spore/g	powder	2000 spore/g (7350 µl/seed)
Chlorella vulgaris	Bioplasm algatragya	cv	$2x10^7$ cell/ml	liquid	10^3;10^5;10^7 cell/ml
Untreated control (unsterilized tap water)		uc			
NPK	BIONOVA	npk	2 ml/L	liquid	2 ml/L

2.5. Preparation and storage of Diabrotica v.virgifera eggs

Diabrotica virgifera virgifera (Coleoptera: Chrysomelidae) a western corn rootworm of a non-diapause colony was used. The beetles were mass reared in the laboratory and the eggs (ready to hatch or 7 days old) were used to infest into the plastic cup maize plants in the greenhouse experiment. Beetle rearing and handling followed procedures of [23,24,25]. Briefly, soil dishes with laid eggs were removed from the adult rearing cages when the eggs were one week old and ready to hatch. Eggs were then washed with cool tap water with 0.01% NaOCI through 300 micrometer- mesh and transferred to semi sterile, cool and slightly moist river sand (<200 micrometer grains) in petri dishes, which were stored at 24°C until use.

2.6. Greenhouse experiment set-up

To assess the insecticidal effect of microbial bio stimulants, against *Diabrotica v. virgifera* larvae, one systematic controlled trial (SCT) was conducted using plastic cups and plants under semi natural conditions in a glass greenhouse. The experiment was conducted in roughly 41 days. NPK was served as positive control both infested and un infested larvae. Unsterilized tap water was served as negative control both infested and un infested larvae. Each of the treatment has 20 plastic cups representing 10 plastic cups treated and infested with larvae and 10 other plastic cups representing treated and un infested larvae. Thus, a total of 20 data points per treatment (=sample size) had to be recorded. Two replicate of the experiment was performed. The maize was sown individually into 0.5-liter soil in 1 liter plastic cup (8 cm inner diameter 14 cm height). Briefly, the plastic cup was first filled with 0.5-liter soil. One maize seed were placed per plastic cup then 20-40 ml water was applied. Treatments were applied as liquid pipette directly to the surface of the maize seed onto the soil, then ½ liter of soil was added, burying the treatment and seeds 3 cm deep into the soil, and leading to soil surface of 9 cm diameter in the plastic cup.

The soil used in this study was a black clay loam field soil without added garden soil (pure soil only). Temperature and relative humidity were measured using an ordinary temperature tool (21-25 °C and 55% relative humidity in the greenhouse). Plants were watered with 90-100 ml of water per week (average 20-30 ml per week, total 0.3 liter over experimental period). Eggs ready to hatch (roughly 100 eggs) were transferred to the plants (3 or 4 leaf stages) 3 weeks after sowing. Eggs then were transferred by making a 5-10 cm hole in the soil. Firstly, eggs were prepared in the form of a dilution by dissolving 0.2 g agar powder in 1 L water. A 100 µl of egg dilution was pipetted then placed in onto the filter paper on Petri dish. The eggs then were counted under stereomicroscope. The

filter paper then was folded and placed over the hole previously made next to the hole of maize seed then water is slowly dripped onto the filter paper until all the eggs had entered the hole. The hole was then slowly covered until no eggs appeared on the surface of soil.

Seed germination rate was recorded 3 days after sowing the maize seed. Plant height, leaf number and shoot length were recorded 3 weeks after planting. Root length, fresh root weight, root damage, root volume, above ground biomass, and number of living larvae were recorded at week 5 or 28 days after maize seed sowing. The effects of microbial bio stimulants were assessed at the expected second and early third instar stages at around 5 to 7 leaf stage of maize. The number of living larvae, root damage and above-ground biomass were assessed. In detail, each maize plant was lifted from the soil, and gently shaken to remove loosely adhering soil particles from the roots. Each maize plant was cut 1 cm above the roots and fresh weight, leaf number and plant height were measured. Then the soil of each plastic cup was placed onto a plastic screen to dry out and allow living larvae to emerge and drop onto the wet tissue paper in the tray below, following the Berlese approach (Figure 1) [26,27]. The number of living larvae was counted after 1, and 3 days. The untreated control aimed to have a minimum of 20% infestation with second or third instar larvae to validate the results on the effect of the bio stimulants. In this experiment, larvae were recovered from 100% of the infested pots in the untreated control.

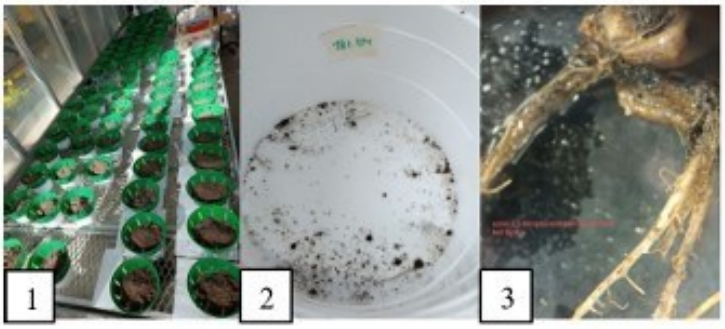


Figure 1. The assessment of microbial bio stimulants on *Diabrotica v. virgifera* larvae 28 days after sowing maize and 2 weeks after infestation of approximately 100 eggs in plastic cups. The assessment included: 1) assessing surviving larvae after 3 weeks of treatment using the Berlese method; 2) recording the number of living larvae observed on filter paper; 3) assessing root damage under a stereomicroscope using the IOWA scale.

2.7. Data analysis

For data collected under laboratory, to allow comparisons between experimental replicates, all data were standardized to the data of corresponding negative control which treated with sterilized tap water One-way ANOVA was then used to analyze the effect of the treatments on eggs, first instar larvae, or adults. For data collected under greenhouse conditions, mean value and the standard deviation (SD) were used to see the effects of microbial bio stimulants on plant height, leaf number, shoot length, root length, root weight, root volume, above ground biomass, root damage, and the number of living larvae. All the results were assessed then presented in histogram. One-way ANOVA Dunnet test was used to see the significant effect ($p < 0.5$) among the treatments. IPM SPSS statistic version 22 statistical software was used [22].

6.2 Results and Discussion

3. Results

3.1. Effect of microbial bio stimulants on Diabrotica virgiferavirgifera life stages under laboratory

3.1.1. Effects of microbial bio stimulants on *Diabrotica virgiferavirgifera* eggs

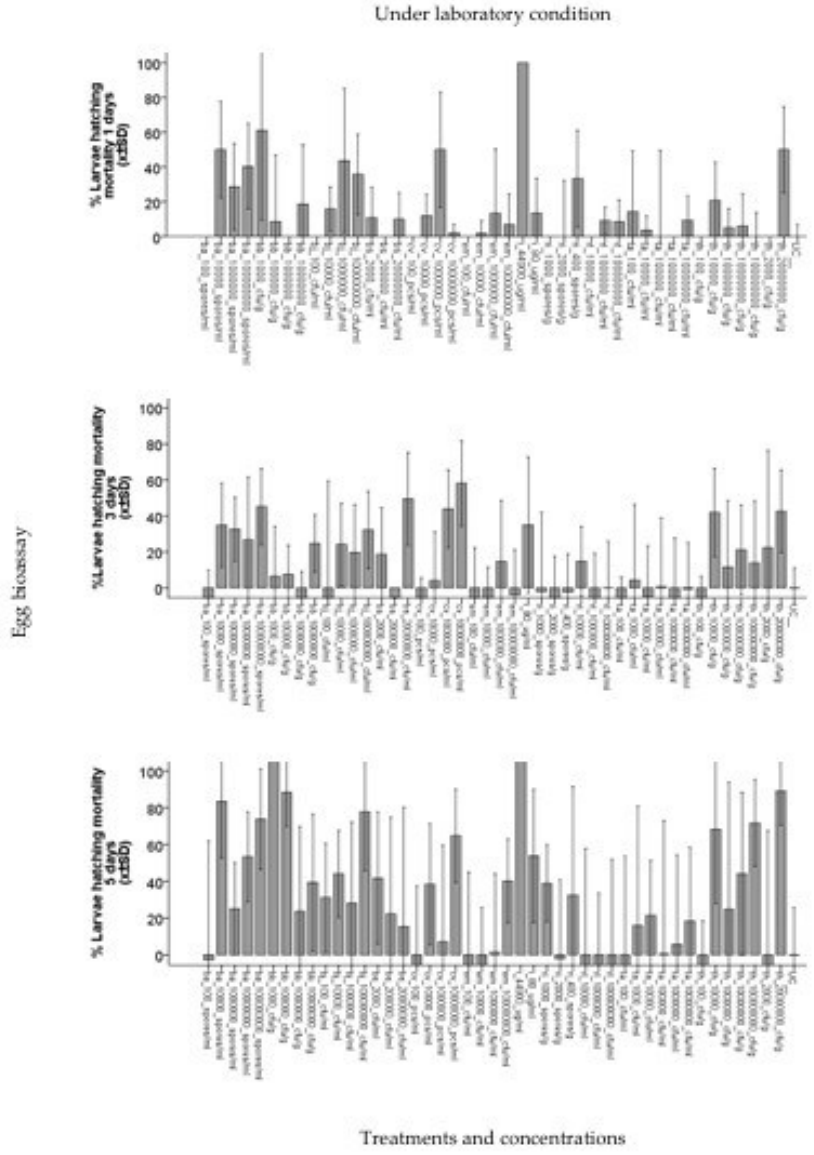


Figure 1. Effect of microbial bio stimulants on hatching larval mortality of *Diabrotica virgiferavirgifera* (treatment name on x-axis followed by the treatment code, referred to Table 2).

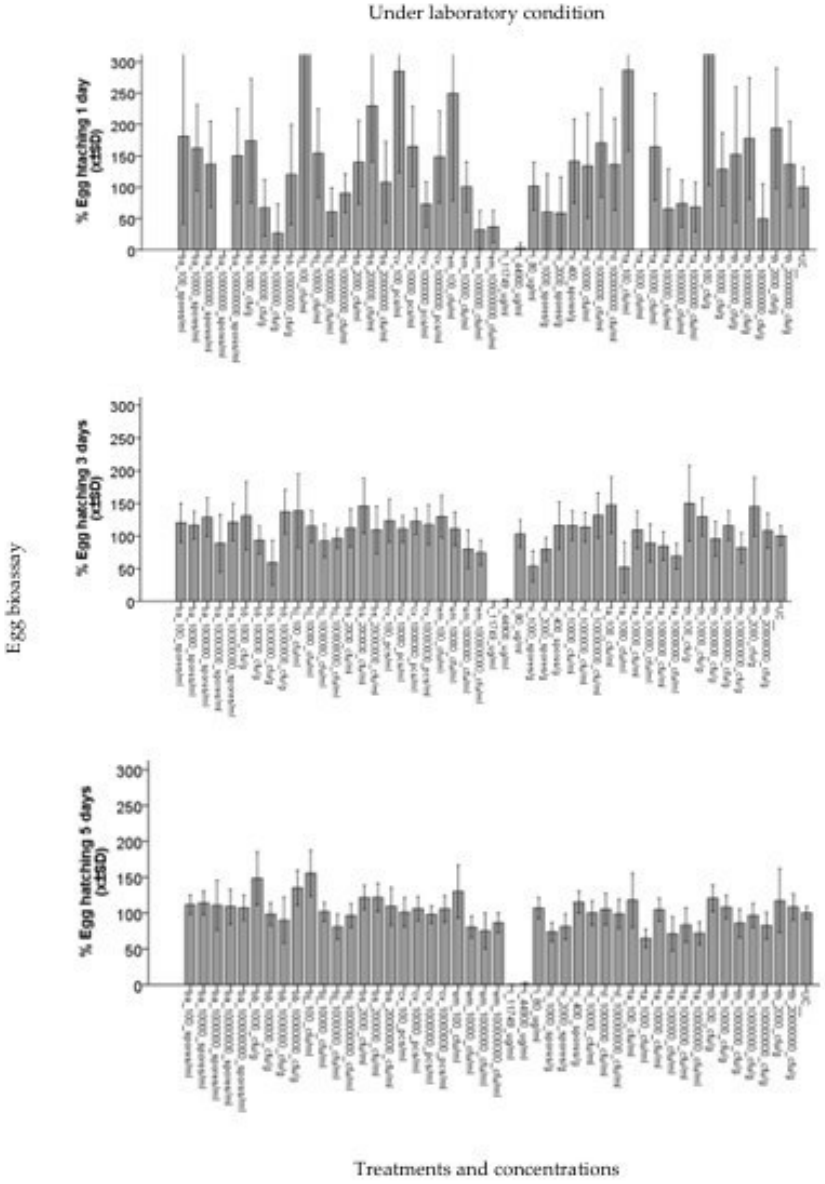


Figure 2. Effects of microbial biostimulant on egg hatching rate of *Diabrotica v. virgifera.* (treatment name on x-axis followed by the treatment code, referred to Table 2).

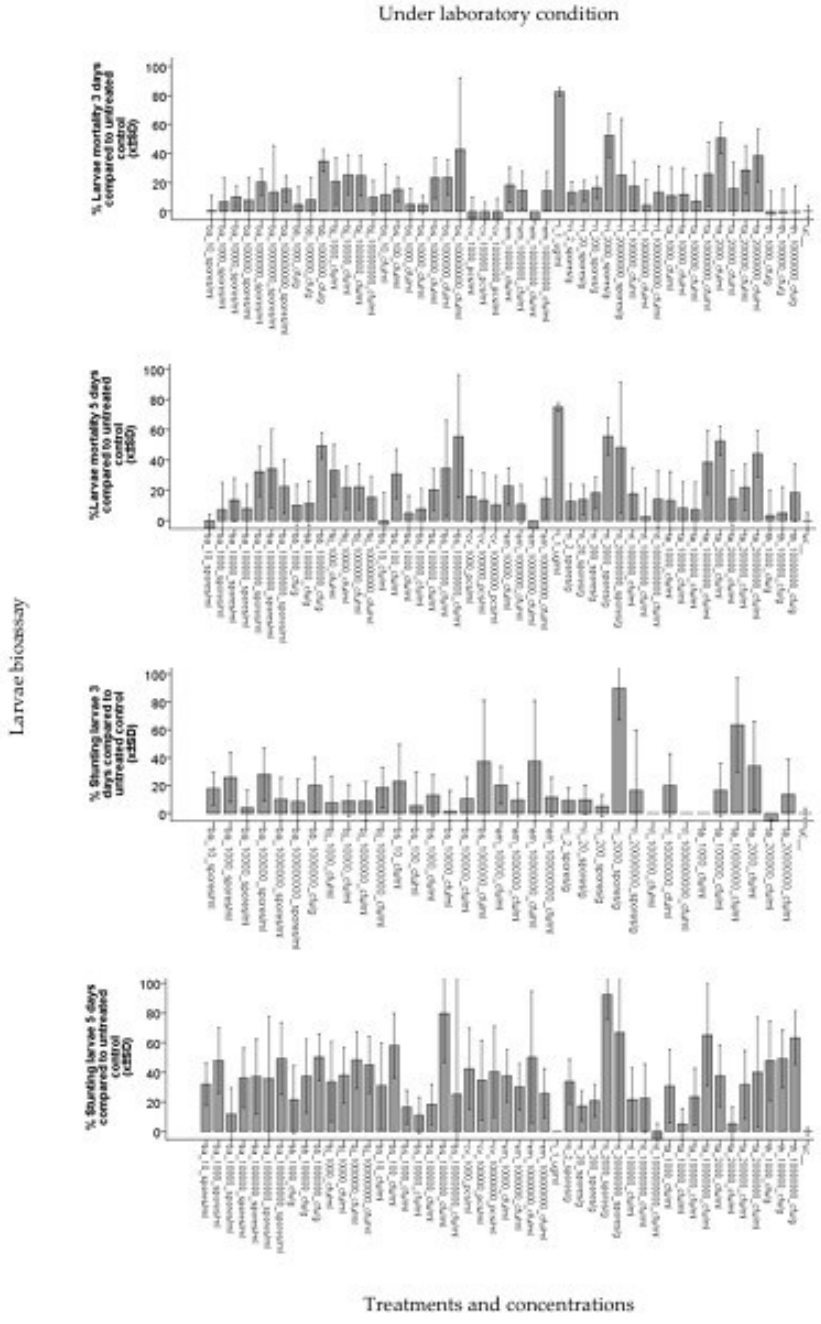

Under laboratory condition
Larvae bioassay
% Larvae mortality 3 days compared to untreated control (≥350)
%Larvae mortality 5 days compared to untreated control (≥350)
% Stunting larvae 3 days compared to untreated control (≥350)
% Stunting larvae 5 days compared to untreated control (≥350)
Treatments and concentrations

Figure 3. Effect of microbial bio stimulants on *Diabrotica v. virgifera* larvae (treatment name on x-axis followed by the treatment code, referred to Table 2).

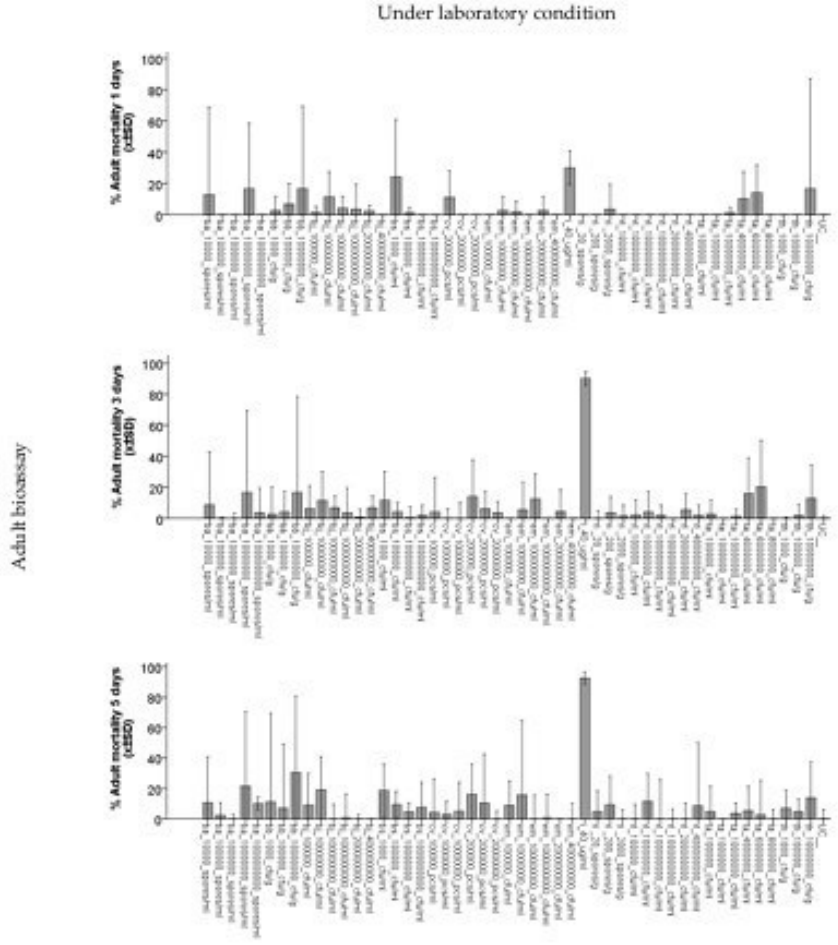


Figure 4. Effects of microbial bio stimulants on *Diabrotica v. virgifera* adult (treatment name on x-axis followed by the treatment code, referred to Table 2).

Our results from the screening of bio stimulants tested on *Diabroticav. virgifera* eggs in the laboratory showed that none were able to kill larvae hatching from eggs (df=44, Fvalue=3.03, Pvalue= $p < 0.001$, $R^2 = 0.17$). *Bradyrhizobium ja ponicum* at 100 cfu/ml increased the hatching rate of *Diabrotica v. virgifera*eggs within 1 day.

3.1.2. Effects of microbial bio stimulants on *Diabrotica virgiferavirgifera* larvae

Beauveria bassiana at dose 10^6 cfu/g, *Ensifer meliloti* at 10^7 cfu/ml, *Rhizophagus irregularis* at 2000 spore/g and Trichoderma asperellum at dose 2000 cfu/ml were caused mortality of *Diabrotica v. virgifera* first instar larvae in diet overlay assays within 3 days (df=48, Fvalue=14.8, Pvalue= $p < 0.001$, R^2=0.20) (Figure 3). *B. japonicum* at dose 1000 cfu/ml, *R. irregularis* at 2000 spores/g, *B.bassiana* at dose 10^6 cfu/g, and *Trichoderma asperellum* at dose 2000 cfu/ml were able to cause mortality in *Diabroticav. virgifera* first instar larvae post treatments 5 days.

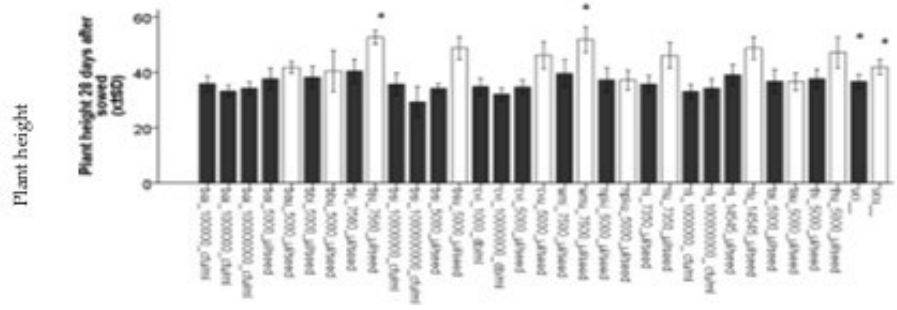
3.1.3. Assessing the effects of microbial bio stimulants on *Diabrotica virgiferavirgifera* adults

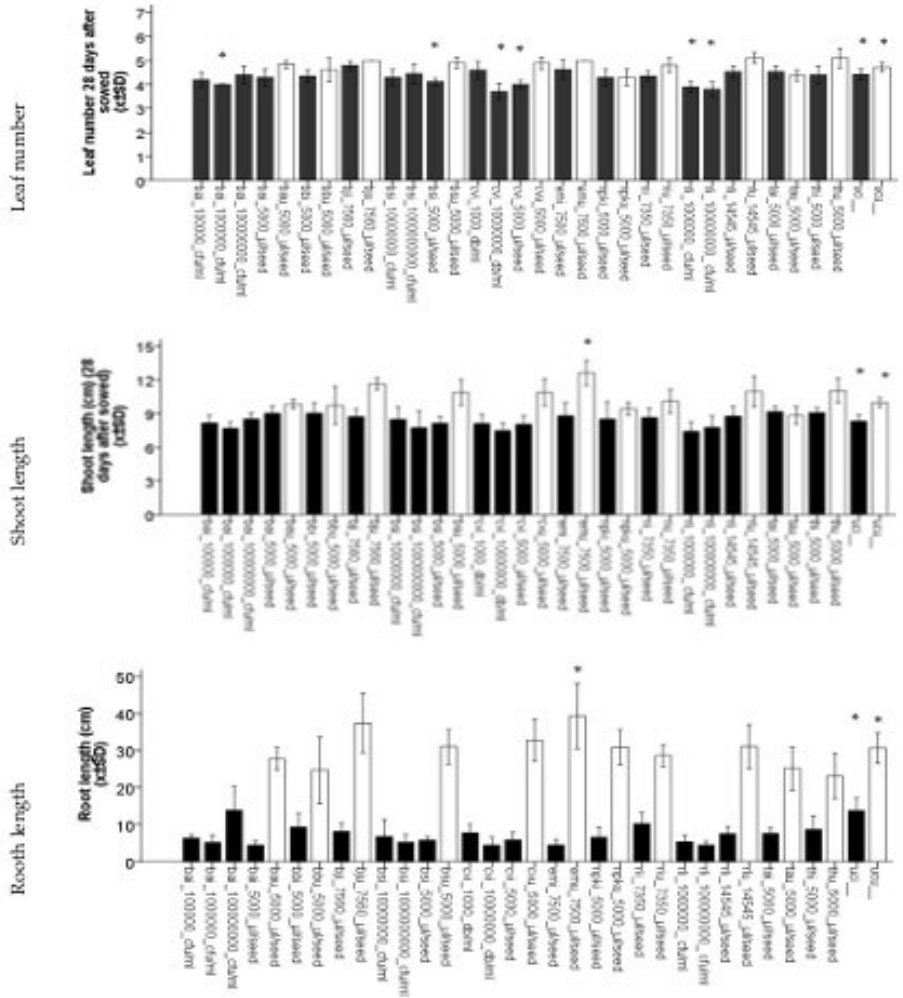
The microbial bio stimulants tested showed a significant effect on adult mortality within 1 day, as indicated by ANOVA (df=46, Fvalue= 2.61, Pvalue= $p < 0.001$, R^2= 0.38). However, Tukey's HSD tests revealed that this significant effect was primarily due to the positive control group. No statistically significant differences were found among the individual bio stimulant treatments themselves. The microbial bio stimulants tested had a significant effect on adult mortality within 3 days as shown by ANOVA (df=49, Fvalue= 11.20, Pvalue= $p < 0.001$, R^2=0.68). However, post-hoc analyses revealed that the significant differences were also primarily due to the positive control, with no significant differences observed among the bio stimulant treatments themselves. The microbial bio stimulants tested had a significant effect on adult mortality within 5 days as indicated by ANOVA (df= 49, Fvalue= 10.42, Pvalue= $p < 0.001$, R^2=0.60). Again, post-hoc analyses also showed that this significant effect was primarily due to the positive control group, with no significant differences observed among the bio stimulant treatments.

3.2. Effects of microbial bio stimulants on Diabrotica virgiferavirgifera second instar larvae under greenhouse conditions

3.2.1. Effect of microbial bio stimulants on maize crop

Under greenhouse condition




Leaf number
Leaf number 28 days after sowed (d28)
Shoot length
Shoot length (cm) (28 days after sowed) (d28)
Rooth length
Root length (cm) (d28)

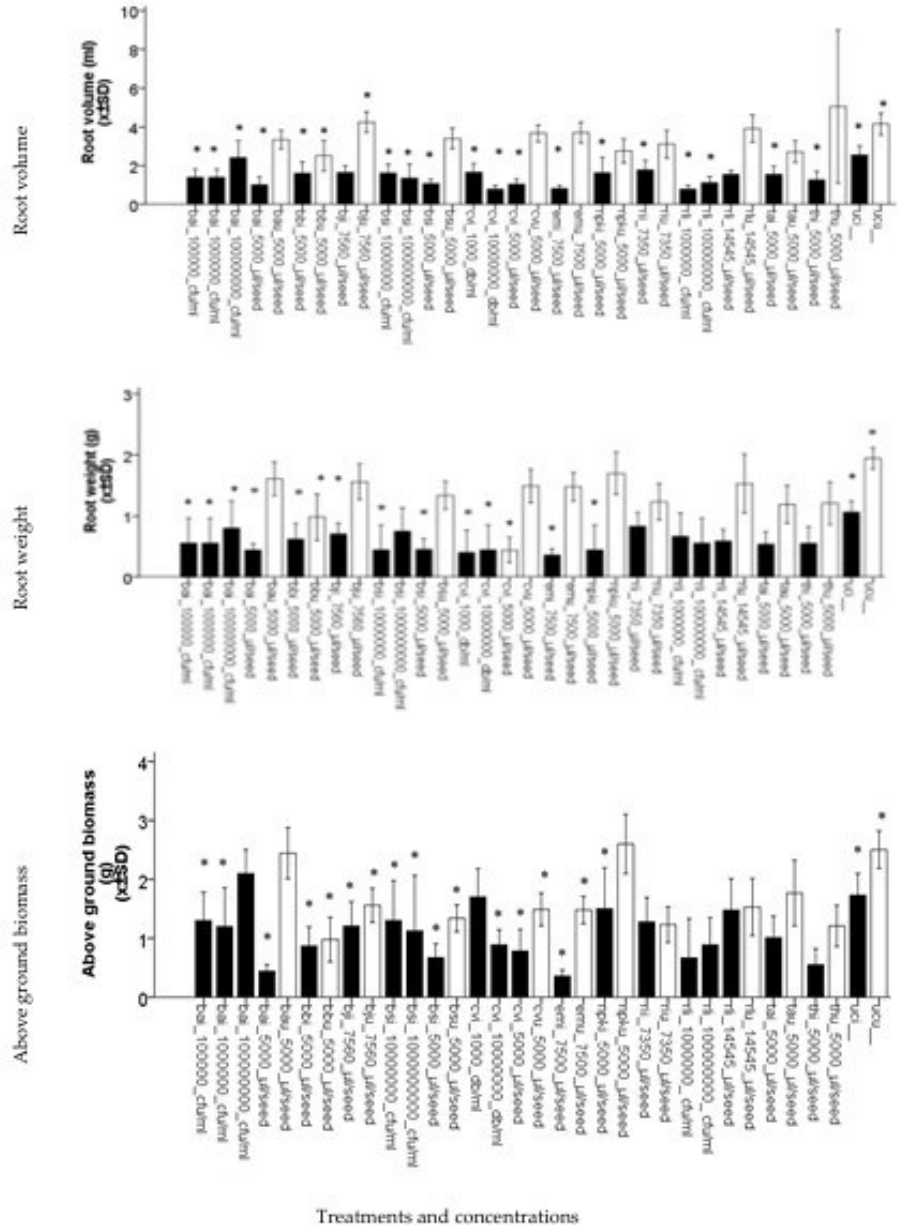

Root volume
Root weight
Above ground biomass
Root volume (ml) (±SD)
Root weight (g) (±SD)
Above ground biomass (g) (±SD)
Treatments and concentrations

Figure 5. Effect of microbial bio stimulants on the maize crop. (The name of the treatments on x-axis has been followed by the treatment code referred to in Table 2; the bar chart with white colour represents maize crops treated with bio stimulants and un infested with *Diabrotica virgifera-virgifera* larvae, while the bar chart with black colour represents maize crops treated with bio stimulants and infested with *Diabrotica virgiferavirgifera* larvae).

Our study revealed that the microbial bio stimulants tested had a significant effect on increasing the plant height of maize plants (df=32, F=9.28, $p < 0.01$, R^2=0.41). *B. japonicum* at a dose of 7560 µl/seed and *Ensifer meliloti* at a dose of 7500 µl/seed were found to significantly increase plant height compared to the untreated control, both un infested and infested with *Diabrotica v. virgifera* larvae.

Microbial bio stimulants had a significant effect on reducing the number of leaves in maize plants (df=32, Fvalue=7.47, Pvalue=$p < 0.01$, R^2=0.31). Some bio stimulants, such as *Bacillus amyloliquefaciens* at dose of 10^6 cfu/ml, *Bacillus subtilis* at dose of 5000 µl/seed, *Chlorella vulgaris* at dose of 10^7 cell /ml and 5000 µl/seed, *Rhizobium leguminosarum* at dose of 10^6 and 10^8 cfu/ml, significantly reduced the number of leaves in maize seedlings compared to the untreated control, both infested and un infested with larvae.

Around 90% of the microbial bio stimulants tested significantly reduced the shoot length of maize plants (df=32, Fvalue=8.60, Pvalue=$p < 0.01$, R^2= 0.39). *B. amyloliquefaciens* at doses of 10^5 and 10^6 cfu/ml, *B. subtilis* at doses 10^9 and 5000 µl/seed, *R. leguminosarum* at doses of 10^6 and 10^8 cfu/ml, *C. vulgaris* at doses of 1000 cell/ml and 5000 µl/seed significantly reduced the shoot length of maize seedlings compared to the untreated control, both infested and un infested with larvae. However, we found that *Ensifer meliloti* at a dose of 7500 µl/seed significantly increased the shoot length compared to the untreated control, both infested and un infested with larvae.

Microbial bio stimulants have a significant effect on increasing the root length of maize seedlings (df=32, Fvalue=34.62, Pvalue=$p<0.01$, R^2=0.73). *E. meliloti* at dose of 7500 µl/seed increased the root length of maize seedlings compared to untreated control infested and un infested with *D. v. virgifera* larvae. Microbial bio stimulants including NPK significantly reduced the root length of maize seedlings (df=32, Fvalue=11.52, Pvalue=$p<0.01$, R^2=0.4). Treatments that reduced the root length of maize seedlings compared to the untreated control, both infested and un infested with larvae, were *B. amyloliquefaciens* at doses of 10^5, 10^6 and 10^8 cfu/ml, and 5000 µl/seed, *Beauveria bassiana* at dose 5000 µl/seed, *B. japonicum* at dose of 7560 µl/seed, *B. subtilis* at doses of 10^7, 10^8 cfu/ml and 5000 µl/seed, *C. vulgaris* 10^3,10^7 cell/ml and 5000 µl/seed, NPK at dose 5000 µl/seed, *Rhizophagus irregularis* at dose 7350 µl/seed, *R. leguminosarum* at doses 10^6, 10^8, and *Trichoderma asperellum* at dose of 5000 µl/seed and *Trichoderma harzianum* at dose of 5000 µl/seed.

Microbial bio stimulants, including NPK, had a significant effect on reducing the root weight of maize plants (df=32, Fvalue=13.24, Pvalue=$P<0.01$, R^2=0.51). *B. amyloliquefaciens* at doses of 10^5, 10^6 and 10^8 cfu/ml and 5000 µl/seed, *B. bassiana* at dose 5000 µl/seed, *B. japonicum* at dose of 7560 µl/seed, *B. subtilis* at dose 10^7 cfu/ml and 5000 µl/seed, *C. vulgaris* at dose 1000 and 10^7 cell/ml and 5000 µl/seed, *E. meliloti* at dose of 7500 µl/seed and NPK at dose 5000 µl/seed significantly reduced the root weight of maize seedlings compared to the untreated control, both infested and un infested with larvae.

Microbial bio stimulants, including NPK, had a significant effect on reducing the above-ground biomass of maize seedlings (df=32, Fvalue=7.99, Pvalue=$p<0.01$, R^2=0.38). *B. amyloliquefaciens* at dose of 10^5, 10^6 cfu/ml and 5000 µl/seed, *B. bassiana* at dose 5000 µl/seed, *B. japonicum* at dose of 7560 µl/seed, *B. subtilis* at dose 10^7, 10^9 and 5000 µl/seed, *C. vulgaris* at dose of 5000 µl/seed, *E. meliloti* at dose of 7500 µl/seed, NPK at dose of 5000 µl/seed significantly reduced the above-ground biomass of maize seedlings compared to the untreated control, both infested and un infested with larvae.

3.2.2. Effect of microbial bio stimulants on Diabrotica v. virgifera larvae

Under greenhouse condition

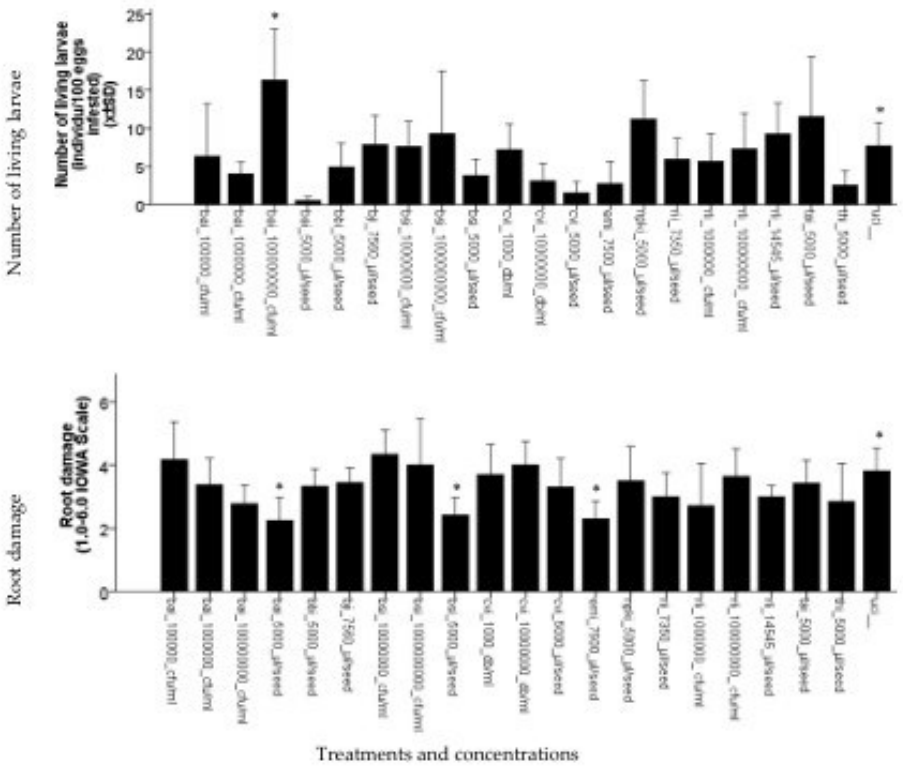


Figure 6. Effect of microbial bio stimulants on *Diabrotica v. virgifera* larvae. (The name of the treatments on x-axis has been followed by the treatment code referred to in Table 2; the bar chart with black colour represents maize crops treated with bio stimulants and infested with *Diabrotica v. virgifera* larvae).

Among the ten bio stimulants tested, only one bio stimulants showed a significant effect on the number of alive larvae (df=20, Fvalue=3.15, Pvalue=p < 0.01, R^2=0.19). *B. amyloliquefaciens* at a dose of 10^8 cfu/ml significantly increased the number of alive larvae compared to the untreated control, both infested and un infested with larvae.

Among the ten bio stimulants, we found three microbial bio stimulants that significantly reduced root damage caused by *Diabrotica v. virgifera* larvae on maize plants (df=20, Fvalue=2.34, Pvalue=P < 0.01, R^2=0.16). *Bacillus amyloliquefaciens* at dose of 5000 µl/seed, *Bacillus subtilis* at 5000 µl/seed, and *Ensifer meliloti* at 5000 µl/seed significantly prevented root damage compared to the untreated control, both infested and un infested with larvae.

4. Discussion

In this study, we successfully assessed the insecticidal effect of microbial bio stimulants on *D. v. virgifera* larvae under laboratory and greenhouse conditions. Under laboratory conditions, our study revealed that among the ten bio stimulants tested, *Beauveria bassiana*, *Ensifer meliloti*, *Rhizophagus irregularis*, and *Trichoderma asperellum* cause mortality on *D. v. virgifera* larvae. *Beauveria bassiana* killed the *D. v.virgifera* neonate larvae. *B. bassiana* is well known as a biopesticide [28]. [29] reported that *B. bassiana* at dose of $5x10^7$

conidia/ml reduced the total productive capacity (mean number of eggs/female), declined egg viability and the total of egg production of *D. v.virgifera*. Our result is consistent with the finding of [30] that *B. bassiana* killed the African malaria mosquito, *Anopheles gambiae* larvae. *Beauveria bassiana* infects mosquito larvae by attaching to the larval body at the head and perispiracular lobes of the siphon [32,33,34] and by ingestion of fungal spores [34]. Infection of mosquito larvae with *B. bassiana* causes histological changes in the larval body, including disintegration and deformation of the larval cuticle, epidermis, and adipose tissue [35,33]. *Rhizophagus irregularis* cause mortality to *D. v.virgifera* neonate larvae, which is consistent with finding from [36], which reported that inoculation *R. irregularis* on maize plants significantly reduced the larval development of *D. v. virgifera*. The mechanisms behind the interaction between *R. irregularis* and *D. v. virgifera* remain unknown. *Trichoderma asperellum* killed *D. v.virgifera* larvae in our laboratory study. This is also consistent with the results done by [37] reported that inoculation *T. asperellum* to Anopheles spp. with LD_{50}= 2.68×10^{7} conidia/ml caused 85% mortality after 72 hours of treatment. Another example is the study done by [38] which also showed that treating poplar seedlings with *T. asperellum* significantly decreased the survival, body weight, body length, and head capsule width of the Asian gypsy moth larvae, *Lymantria dispar*. According to them, the mechanism by which the *T. asperellum* killed the larvae was that through the fungal spores attached Anopheles spp. on the larval surface by using specific carbohydrate-containing adhesives. The spores then grew and secreted specific enzymes that degraded the cuticle, the outer layer of the larvae. Thus, this allowed the spores to puncture the cuticle and enter the larval body, ultimately causing death.

In our study, we evaluated the effects of microbial bio stimulants on maize plants and *D. v. virgifera* larvae under semi-field conditions. Our results revealed that *B. japonicum* and *E. meliloti* significantly increased maize plant height. The observed increase in plant height with *B. japonicum* aligns with the findings of [39], who reported a 22% increase in soybean plant height following inoculation with these bio stimulants. This suggests that *B. japonicum*, while traditionally used for nitrogen fixation in legumes, can also improve growth parameters in maize. The growth enhancement observed may be attributed to improved nutrient availability and enhanced plant physiological processes facilitated by the biostimulant.

Similarly, *E. meliloti* significantly increased maize plant height in our study. This finding is consistent with research conducted by [40], which demonstrated that *E. meliloti* improved plant growth, including plant height, in *Arabidopsis thaliana* under nitrogen-deficient conditions. This supports the notion that *E. meliloti* may have beneficial effects on plant growth beyond its primary association with legumes, probably through mechanisms such as enhanced nutrient uptake or stress tolerance.

Our semi field conditions demonstrated that three bio stimulants such as *B. japonicum*, *B. subtilis*, and *E. meliloti* were effective in preventing root damage caused by *D. v. virgifera* larvae. This finding highlights the potential of these bio stimulants as viable options for integrated pest management (IPM) strategies aimed at mitigating the impact of *D. v. virgifera* on maize plants. *B. japonicum*, known primarily for its role in nitrogen fixation, also showed significant efficacy in protecting maize roots from damage. This result suggests that the beneficial effects of the bio stimulants may extend beyond nitrogen fixation, potentially enhancing plant resistance against *D. v. virgifera* attacks. The specific mechanisms through which *B. japonicum* exerts its protective effects are worth further investigation, but it is possible that it induces systemic resistance in maize plants or creates a less favourable environment for rootworm larvae. Similarly, *B. subtilis* and *E. meliloti* are well-documented for their biocontrol properties. *B. subtilis* is known for its ability to produce a range of antimicrobial compounds, which could contribute to its efficacy in reducing root damage [41]. *E. meliloti*, although traditionally recognized for its symbiotic relationship with legumes, also appears to provide protection to maize as well, possibly through similar mechanisms of plant growth promotion and stress resistance. The effectiveness of these bio stimulants in preventing root damage from *D. v. virgifera* larvae

highlights their potential role in improving maize health and productivity. Integrating these bio stimulants into crop management practices could reduce the reliance on chemical insecticides and promote more sustainable agricultural practices.

Our results also suggest that the application of bio stimulants might not only help in reducing root damage but could also improve overall plant health and yield. Future research should focus on elucidating the exact modes of action of these bio stimulants and optimizing application methods, and evaluating their performance under different environmental conditions and pest pressures. Additionally, exploring the potential synergistic effects of combining bio stimulants with other IPM tools could offer further benefits in the management of *D. v. virgifera*.

5. Conclusions

In conclusion, our study suggests several effects of microbial bio stimulants on both the maize crop and its main pest, *D.v.virgifera*. In particular, the positive effect of *B. japonicum*, *B. subtilis*, and *E. meliloti* on reducing root damage from *D. v. virgifera* larvae highlights their potential as effective components of integrated pest management strategies. Further studies are essentially needed to gain a better understanding of plant-microbes interactions and their underlying mechanisms. Continued research and development in this area could contribute to more sustainable and effective solutions for *D. v. virgifera* control in maize crop production.

Author Contributions: ST (Stefan Toepfer), GT (Turóczi György), JK (Jozsef Kiss), NPY (Nhu Phuong Y Doan) and SIT (Sri Ita Tarigan) jointly develop the study. ST, JK and GT supervised the study. SIT conducted laboratory bioassay experiments. SIT. and NPY conducted data collections and analysis. SIT wrote the manuscript with support from all authors. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data is unavailable due to privacy or ethnical restrictions.

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Conflicts of Interest: No potential conflict of interest was reported by the authors.

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7. General discussion and conclusions

Chapter I. – Biological control properties of microbial plant bio stimulant. A review

Microbial bio stimulants are cutting-edge solutions that employ beneficial microbes, including bacteria and fungi, to boost plant growth, enhance crop productivity, and bolster stress resistance in farming practices. Our review highlighted a wide range of microbial-based bio stimulants that growers can utilize to boost their crop production. These commercial products seem to be derived from at least 245 distinct microorganism species, as examined from six different countries. Interestingly species of bacteria seem had more contribute on microbial bio stimulants products compared to fungi and algae. In our study, there are 82% of products contain bacteria, 63% contain fungi and 14% contain algae. In addition, 29% contained bacteria-fungal mixes, and some contain bacteria-algae mixes or fungi-algae mixes or even mixes of all three groups. We highlighted that 53% of products (36% species) reviewed were reported to have insecticidal properties and 67% of products (54% species) were reported to defend a plant from insects.

In conclusion, many commercial microorganism-based bio stimulants not only enhance crop yield but also offer plant protection benefits. Consequently, they play a role in the biological management of insect pests. These effects warrant further study, and it is important to inform growers about their multiple effects.

Chapter II. – Can microbial plant bio stimulants be useful for insect soil pest control? A review

Soil pests are organisms that live in the soil and cause damage to plants by feeding on their roots, stems, or other underground parts. We successfully reviewed 245 species of bio stimulants registered in 6 countries (Hungary, Switzerland, Spain, France, Indonesia and Canada) for their potential effects on soil pest using both CAB Direct and Web of Science.

In our study, bacteria group with reported effects to soil insects, are strains of *Bacillus thuringiensis* and *Pseudomonas fluorescens*. *Bacillus thuringiensis,* which is a well-known insect pathogen should probably not appear as an ingredient in bio stimulant. Also, some *P. fluorescens* strains have insecticidal effects, such as against *Leptinotarsa decemlineata*. Fungi group with reported effects to soil insects, are strains of *Rhizophagus irregularis* (syn. *Glomus intraradices, Rhizophagus intraradices, G. irregulare, Rhizoglomus irregulare, G. irregular*)*, Glomus mosseae* (syn. *Funneliformis mosseae)* and *Beauveria bassiana*. For example, *R. irregularis* inoculation is known to reduce infestation of wheat by *Mayetiola destructor. Beauveria bassiana* is a well-known insecticidal fungus that should not appear in bio stimulant.

At least 9% of microorganisms (6 ± 2 species), registered as bio stimulant, were reported in the literature to affect rootworms, most of them through indirect effects. This relates to about 20% of commercial products potentially affecting rootworms (41 ± 46 products). Most of those originate from the bacterial kingdom (3 ± 1, thus 6% of species; 16 ± 13, thus 11% of products) followed by fungi (3 ± 2, 15% of species; 29 ± 35, 33% of products). Among the bacteria with potential effects on rootworms are strains of *Bacillus pumilus*, *Azospirilium brasiliense*, *B. thuringiensis*, or *Pseudomonas chlororaphis.* For example, the *B. pumilus* strain INR-7 is known to repel rootworms (Disi et al. 2018). Another example is *Azospirilium brasiliense* where rootworm preferentially orient toward roots of non-inoculated plants versus inoculated roots which emit the repellent (E)-β-caryophyllene (Santos and Pen 2014). Among the fungi are strains of *Rhizophagus irregularis*, *Saccharomyces cerevisiae*, *Beauveria bassiana*, *Metarhizium brunneum* (syn. *Metarhizium anisopliae*), and *Myceliophthora thermophila.* For example, *R. irregularis* was reported to render rootworms prone to predation by natural enemies, probably through an indirect effect by a modified endorhiza microbial community (Dematheis et al. 2013).

It's important to note that the multiple effects of microorganisms in bio stimulants often hinge on the specific strain, which is frequently not mentioned on product labels or in scientific studies. Nonetheless, it is evident that many microbial plant bio stimulants have multiple effects, including insect control, and this information should be communicated to growers.

Chapter III. – What are suitable positive controls for laboratory screening of novel agents against the maize pest, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)?

We successfully evaluated seven common insecticides (imidacloprid, clothianidin, acetamiprid, novaluron, cypermethrin, chlorpyrifos-methyl, spinosad) against eggs, larvae, and adults as potential positive controls for each of the proposed assay methods.

In our study, standard egg bioassays conducted in the laboratory revealed that imidacloprid and clothianidin were the insecticides that suitable as a positive control due to a robust dose-response in reducing egg hatching and causing mortality of hatching neonates. Standard diet overlay assays for the larvae revealed that only imidacloprid was suitable as positive control due to exhibiting best dose-mortality response as well as sub-lethal effects. In contrast, acetamiprid might be proposed as positive control in adult bioassays, as it had a good dose-response in killing adults, and this with high toxicity.

These options, along with other potential choices for suitable positive controls based on our provided details and the specific study objectives, are intended to assist researchers in screening new crop protection agents against the eggs, larvae, or adults of this significant invasive pest.

Chapter IV. – Effect of microbial bio stimulants on maize and its pest, the western corn rootworm, *Diabrotica virgifera virgifera*

Diabrotica virgifera virgifera is a serious pest affecting maize plants both USA and Europe. Microbial plant bio stimulants are products that use beneficial living microorganisms to enhance plant growth and improve crop yields. Some microbial bio stimulants are hypothesized to have insecticidal effects on insect pests. We successfully assessed 10 microbial bio stimulants products under laboratory and semi field conditions. Our laboratory assessments of ten microbial bio stimulants tested revealed that four out of ten products (40%)—*Beauveria bassiana*, *Trichoderma asperellum*, *Rhizophagus irregularis*, and *Ensifer meliloti*—demonstrated larvicidal effects against *Diabrotica virgifera virgifera* neonate larvae. This suggests that these bio stimulants may play a role in managing the early stages of rootworm infestations, potentially reducing *Diabrotica virgifera virgifera* pressure on maize crops. This is interesting, as bio stimulants should, per definition and regulation, not affect pests themselves. Particularly *Beauveria bassiana* should probably not be registered as a bio stimulant, but as a biopesticides, as it is a well-known bioinsecticide. Interestingly, *Bradyrhizobium japonicum* was found to increase egg hatching, which could indicate a complex interaction with the *Diabrotica virgifera virgifera* reproductive cycle. However, none of the bio stimulants affected, as expected *Diabrotica virgifera virgifera* adults, although many of the bio stimulants are endophytic.

In our semi-field conditions, the efficacy of the bio stimulants varied. Among the ten tested, only two products (20%)—*Bradyrhizobium japonicum* and *Ensifer meliloti*—were effective in enhancing maize crop performance, particularly in terms of plant height and shoot length. This suggests that these bio stimulants can contribute to improved crop growth under semi-field conditions, potentially leading to increased yields. We found eight bio stimulants products that had no positive effect on maize crops.

Additionally, 30% of the bio stimulants tested—*Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Ensifer meliloti*—were effective in preventing some root damage caused by *Diabrotica virgifera virgifera* larvae. This indicates their potential for mitigating the negative impacts of *Diabrotica virgifera virgifera* infestations on maize root systems, something that warrants further investigation.

The differential performance of the bio stimulants across laboratory and semi-field conditions highlights the complexity of their effects and the importance of context in evaluating their efficacy. While some bio stimulants showed promising larvicidal activity in controlled settings, their ability to translate these effects into improved plant performance and pest

management in semi-field conditions varied. The effectiveness of *B. japonicum* and *E. meliloti* in enhancing plant growth, along with the root-damage mitigation provided by *B. amyloliquefaciens*, *B. subtilis*, and *E. meliloti*, underscores the potential of these bio stimulants to contribute to both crop productivity and pest management.

Our study provides valuable insights into the potential applications of microbial bio stimulants in sustainable agriculture. The observed benefits of specific bio stimulants in promoting plant growth and managing pest damage suggest that they could be integrated into broader pest management and crop enhancement strategies. However, the variability in efficacy across different conditions highlights the need for further research to optimize the application of these bio stimulants and to explore their interactions with different crop varieties and environmental conditions.

In conclusion, while only a subset of the tested bio stimulants demonstrated significant effects, the findings highlight their potential as components of integrated pest management strategies and crop performance enhancement. Continued research and field trials will be essential for refining the use of bio stimulants and maximizing their benefits for maize production and pest control.

8. New scientific results

- I have concluded based on reviewed papers that the most common bio stimulants microorganisms with reported insecticidal effects are certain strains of *Rhizophagus irregularis*, followed by *Bradyrhizobium japonicum*, *Rhizobium leguminosarum*, *Bacillus megaterium*, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *Penicillium bilaiae*, *B. pumilus*, and *Ascophylum nodosum*.
- I have clarified the details of dose responses of common insecticides that were tested against *Diabrotica virgifera virgifera* (egg, neonate larvae, and adult) under standardized laboratory conditions. Imidacloprid and clothianidin can be used as a positive control in egg dipping bioassays. Imidacloprid can be used as a positive control in artificial diet overlays larvae bioassays. Acetamiprid might be proposed as positive controls in adult bioassays for *Diabrotica virgifera virgifera*.
- I have discovered that four (40%) of 10 bio stimulants tested had larvicidal effect on *D.v.virgifera*. neonates under laboratory, such as *Beauveria bassiana*, *Trichoderma asperellum, Rhizophagus irregularis and Ensifer meliloti*.
- I have found that microbial bio stimulants products containing *B. japonicum* or *E. meliloti* were able to increase maize plant height, and those containing *E. meliloti* were also able to increase maize shoot length.
- I have found that bio stimulants of *B. amyloliquefaciens*, *B. subtilis* and *Ensifer meliloti* were capable of preventing some root damage caused by *Diabrotica virgifera virgifera* larvae in maize plants.

9. Summary

Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae) is a major pest affecting maize especially in North America and Europe. To combat this pest, farmers usually use a variety of strategies including crop rotation or use synthetic chemical insecticides to target the larvae in the soil and adults above ground. However, the use of synthetic insecticides has raised environmental concerns and led to pest resistance. Additionally, many insecticides have recently been banned in several countries because they are highly toxic to bees. Consequently, there is a need for alternative pest control method that focus on developing novel, less-disruptive control measures. Increasingly, microbial bio stimulants are entering the market as they are easier to register than plant protection products. This study aims to better understand natural source agents, such as microbial bio stimulants, for controlling the invasive *Diabrotica virgifera virgifera* in maize, thereby expanding the IPM toolbox for growers.

A comprehensive literature review revealed that only 52% of the 483 reviewed products contained a single microorganism species, while many contained multiple organisms, with some having up to 16 different microorganisms. This complexity makes it difficult to distinguish the effects, whether positive or negative. Scientific literature often focuses on single microorganisms, rarely addressing the synergistic or antagonistic effects of combinations. Although it is well-known that microbial bio stimulants have multiple effects, this complicates their targeted use in crop production. We recommend further studies on the effects of bio stimulants on insects under field conditions and appropriate labelling of products.

Our review found that at least 9% of microorganisms (6 ± 2 species) registered as bio stimulants are reported in the literature to affect rootworms (Diabrotica pest group), mostly through indirect effects. This corresponds to about 20% of commercial products potentially affecting rootworms (41 ± 46 products). Most of these microorganisms are from bacteria kingdom (3 ± 1 species, 6% of species; 16 ± 13 products, 11% of products) followed by fungi (3 ± 2 species, 15% of species; 29 ± 35 products, 33% of products). Bacterial strains with potential effects on rootworms include *Bacillus pumilus*, *Azospirillum brasiliense*, *B. thuringiensis*, and *Pseudomonas chlororaphis*. Fungal strains include *Rhizophagus irregularis*, *Saccharomyces cerevisiae*, *Beauveria bassiana*, *Metarhizium brunneum* (syn. *Metarhizium anisopliae*), and *Myceliophthora thermophila*. However, it needs to be noted that the diverse effects of microorganisms in bio stimulants often depend on the specific strain, which is frequently not specified on product labels

or in scientific studies. It is evident that many microbial plant bio stimulants have multiple effects, including the control of insects, which growers should be informed about.

Secondly, we developed standard bioassay methods to test all life stages of *Diabrotica virgifera virgifera* under laboratory conditions for high-throughput screening. Proposed methods include egg dipping assays, artificial diet overlay assays for larvae, and artificial diet-core overlay assays for adults. We tested seven insecticides to evaluate their effectiveness against eggs, larvae, and adults, and their practicality as positive controls for comparative bioassays. We concluded that imidacloprid and clothianidin are likely the most suitable positive controls for egg bioassays due to their robust dose-response in reducing egg hatching and causing mortality in hatching neonates. Imidacloprid is also suitable for larval bioassays, showing the best dose-mortality response and sublethal effects. Lastly, acetamiprid is the most suitable positive control for adult bioassays, demonstrating the best dose-mortality response.

As a significant part of this PhD research, microbial bio stimulants identified as potentially effective in the review were tested for their insecticidal properties against *Diabrotica virgifera virgifera* under laboratory and semi-field greenhouse conditions. Specifically, ten microbial bio stimulant agents were tested on eggs, larvae, and adults under laboratory, and on eggs (ready to hatch) on maize plants under semi-field conditions. We used eggs from a non-diapause population of *Diabrotica virgifera virgifera.* Data were collected on hatching larvae mortality, hatching rate, larvae mortality, percentage of stunted larvae, plant performance parameters (plant height, shoot length, leaf number, root length, fresh root weight, root volume, and above-ground biomass), and plant protection parameters (root damage and the number of livings L2 or L3 instar larvae).

The results indicated that eight microbial bio stimulants had no beneficial effect on maize, regardless of whether the plants were infested with *Diabrotica virgifera virgifera* larvae. However, *B. japonicum* increased the plant height of maize crops that were not infested with *Diabrotica virgifera virgifera* Additionally, *E. melliloti* enhanced both plant height and shoot length. Interestingly, *B. amyloquefaciens*, *B. subtilis*, and *E. melliloti* were able to prevent some root damage caused by *Diabrotica virgifera virgifera* larvae. This could be due to several factors including: 1) induced systemic resistance (ISR) - beneficial bacteria can trigger the plant's own defence mechanisms, similar to a vaccine, 2) nutrient competition – beneficial bacteria can outcompete harmful pathogens for nutrients and space in the rhizosphere (root zone), thereby reducing the chances of rootworm larvae establishing themselves, 3) production of antimicrobial compound- some beneficial bacteria produce antimicrobial substances that can directly inhibit or kill rootworm larvae and other pathogens, 4) improvement of plant health- by enhancing nutrient uptake and promoting overall plant health, these microorganisms can make plants more robust and

less susceptible to damage from pests, 5) alteration of soil microbiome - beneficial microorganisms can alter the soil microbiome in ways that create a less favourable environment for rootworm larvae.

This study suggests that these bio stimulants may enhance plant defence mechanisms, improve nutrient uptake, or alter the soil microbiome to deter pests. It emphasizes the importance of integrating microbial bio stimulants into pest management strategies and calls for further research to optimize their use for effective and sustainable agriculture. By leveraging the diverse effects of these microorganisms, including their potential insecticidal properties, we can develop more targeted and environmentally friendly approaches to controlling *Diabrotica virgifera virgifera.* Additionally, a deeper understanding of the interactions between various microbial strains and their combined effects on both crop health and pest management will be crucial.

Future research may focus on field-based evaluations and the development of precise guidelines for the use of microbial bio stimulants, ensuring that products are labelled with detailed strain information and usage instructions. Since the effectiveness of microbial bio stimulants can vary greatly depending on the specific strain, it is crucial that products clearly identify the strains to enable targeted applications and consistent results. This thorough approach could help develop Integrated Pest Management (IPM) systems, reducing dependence on chemical insecticides and promoting sustainable agricultural practices. Highlighting the specificity of microbial strains will not only improve the effectiveness of pest control measures but also support the broader goal of maintaining ecological balance and protecting beneficial organisms in agricultural ecosystems.

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11. Appendices

Appendices are collections of enlarged figures for better readability

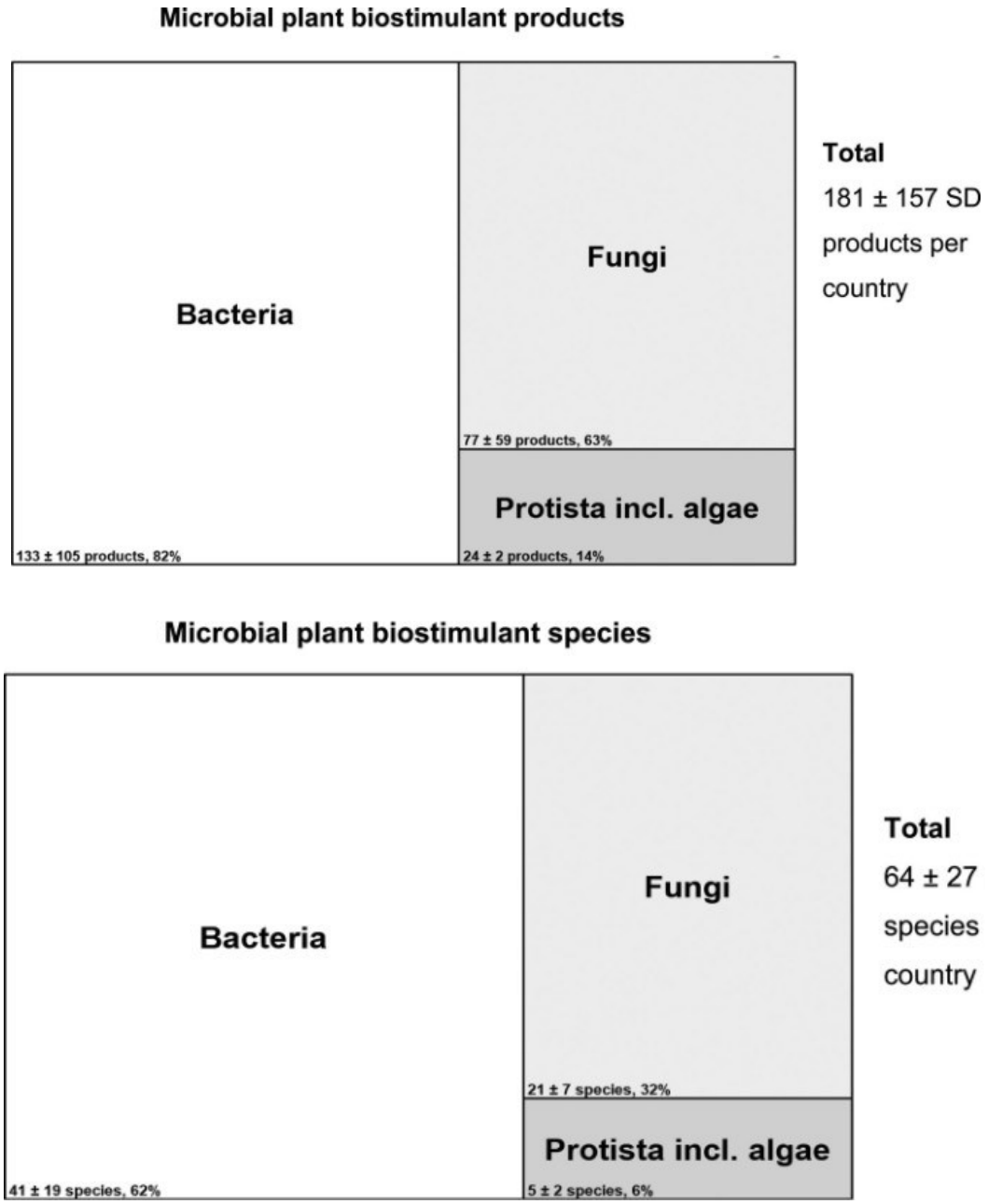


Figure 1. Commercial microbial plant biostimulants averaged per country. In total, 483 biostimulant products with 245 microorganism species from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada) by 2020. Some products contain several microorganisms from bacteria and fungi and/or protista.

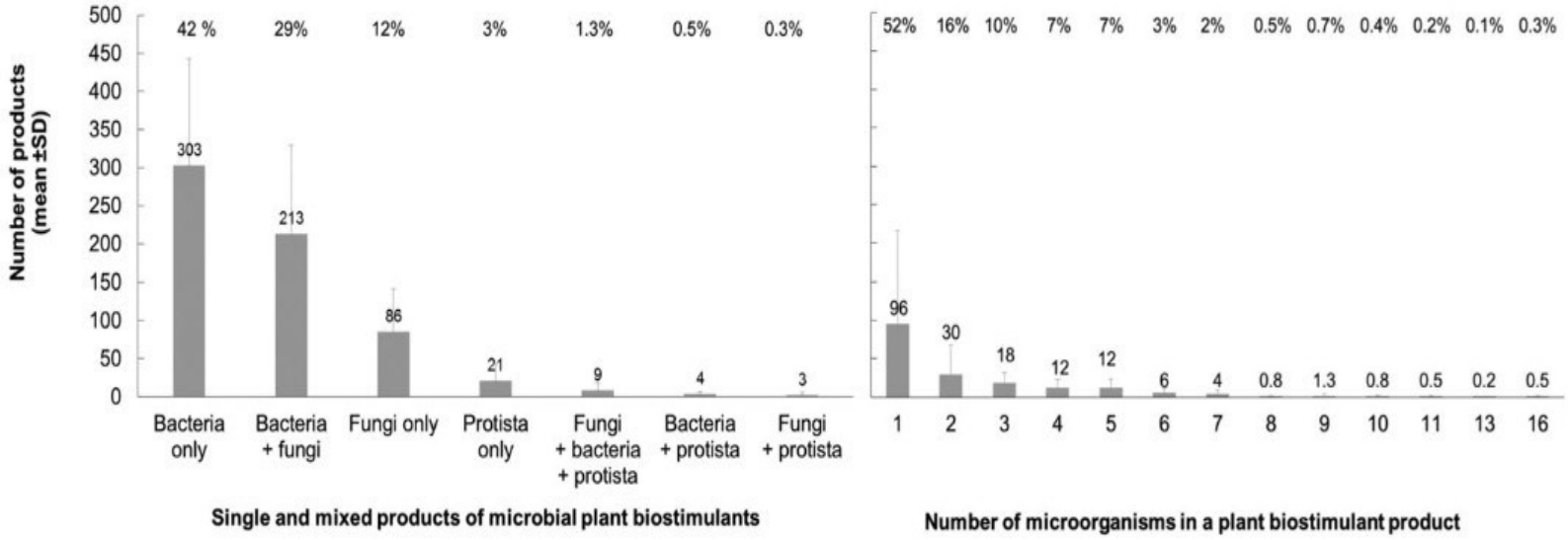


Figure 2. Average numbers and percentages of products of registered microbial plant biostimulant per country containing single or multiple microorganism groups and single or multiple species. 483 products with 245 microorganism species from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada) by 2020. Error bars = standard deviation.

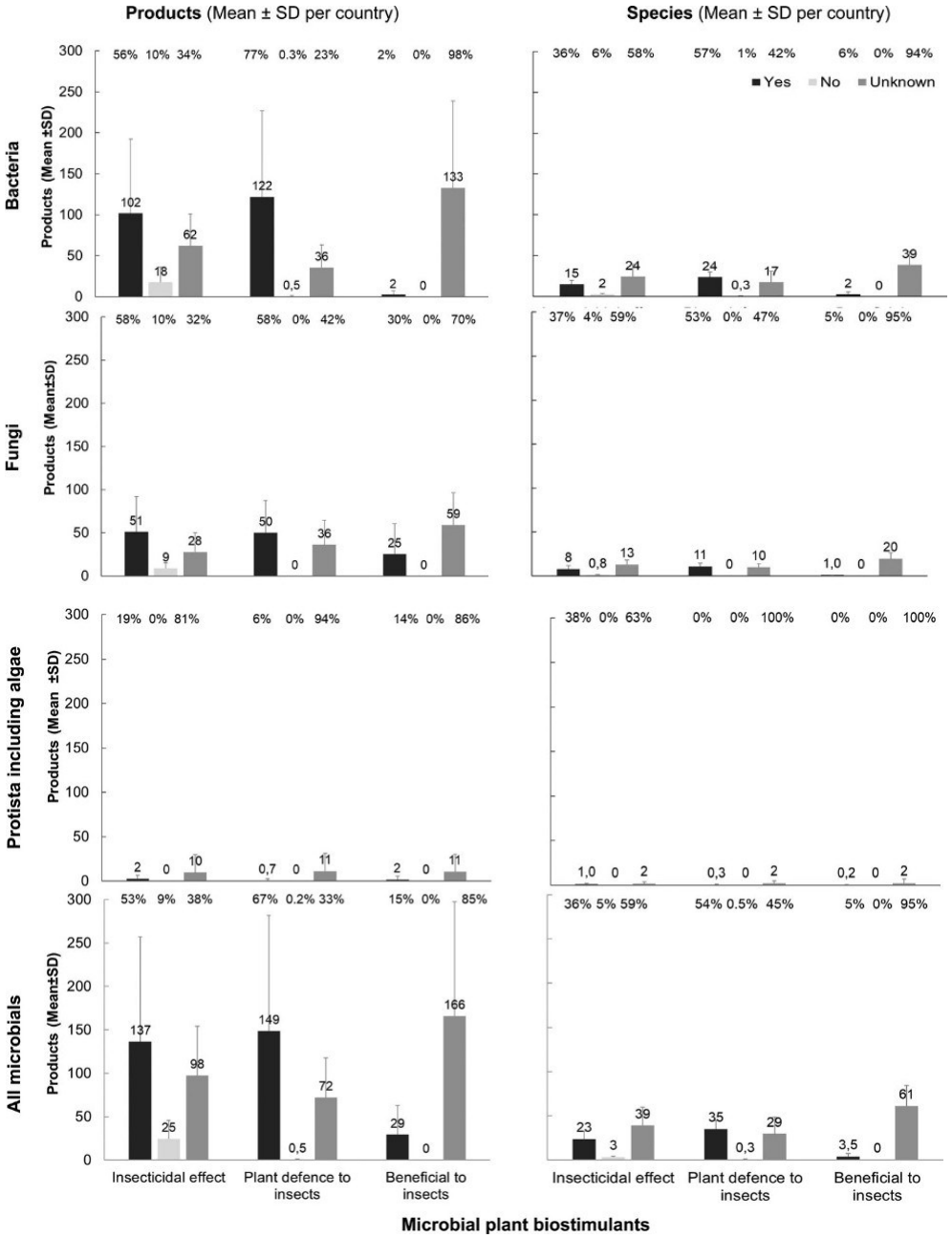
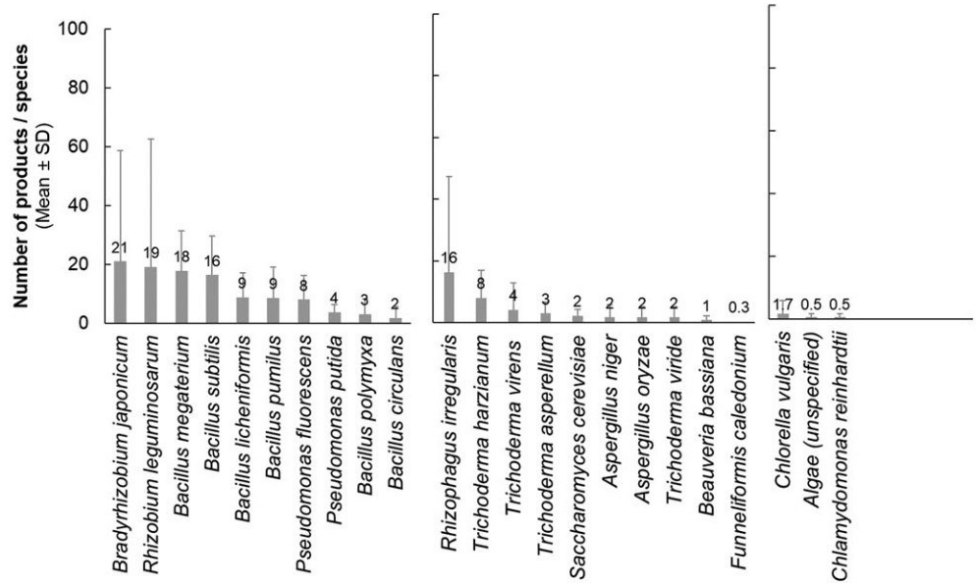
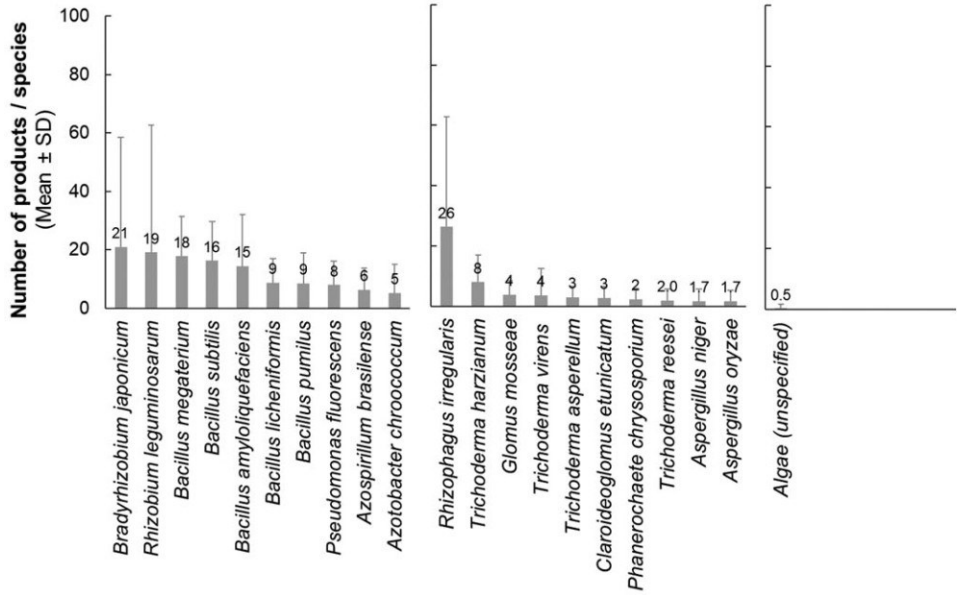


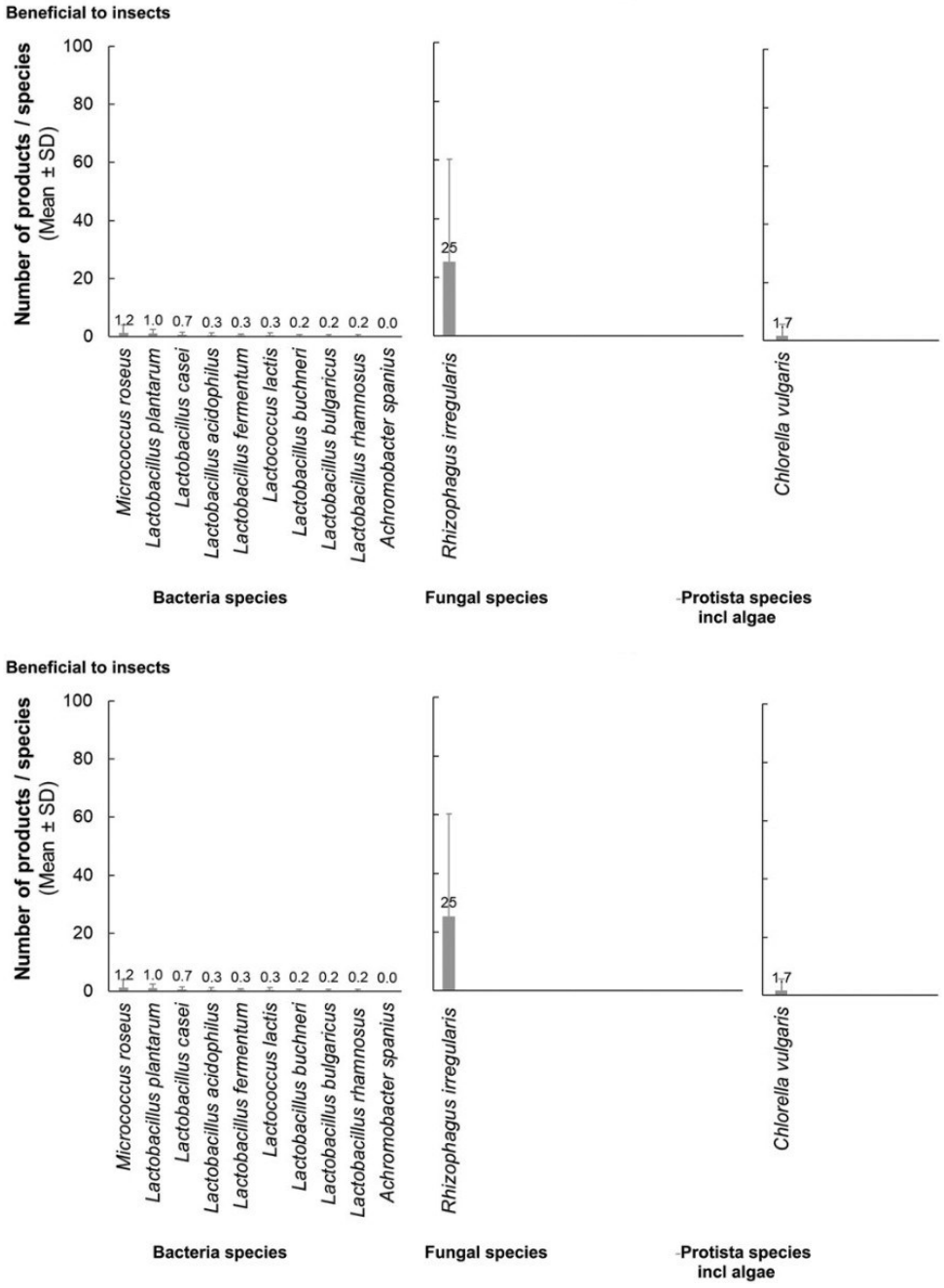
Figure 3. Average numbers and percentages of commercial microbial plant stimulants with potential effects on insects. Insecticidal, plant defence and beneficial effects of microorganism species with regard to insects reviewed in the literature as per Web of science (Clarivate 2022) and CAB Direct (CABI, 2022), but strain level information rarely available. In total, 483 products with 245 microorganism species reviewed from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada) by 2020. Averages per country shown with standard deviation as error bars.

Insecticidal properties



Plant defence properties to insects





Beneficial to insects
Number of products / species
(Mean ± SD)
100
80
60
40
20
0
1.2
1.0
0.7
0.3
0.3
0.3
0.2
0.2
0.2
0.0
25
1.7
Micrococcus roseus
Lactobacillus plantarum
Lactobacillus casei
Lactobacillus acidophilus
Lactobacillus fermentum
Lactococcus lactis
Lactobacillus buchneri
Lactobacillus bulgaricus
Lactobacillus rhamnosus
Achromobacter spanius
Rhizophagus irregularis
Chlorella vulgaris
Bacteria species
Fungal species
Protista species
incl algae

Figure 4. Most common microorganisms in commercial plant biostimulants with potential side effects on insects. Insecticidal, plant defence and beneficial effects of microorganism species with regard to insects reviewed in the literature as per Web of science (Clarivate 2022) and CAB Direct (CABI 2022), but strain level information rarely available. In total, 483 products with 245 microorganism species reviewed from six countries (Hungary, Switzerland, Spain, France, Indonesia, and Canada) by 2020. Averages per country shown with standard deviation as error bars.

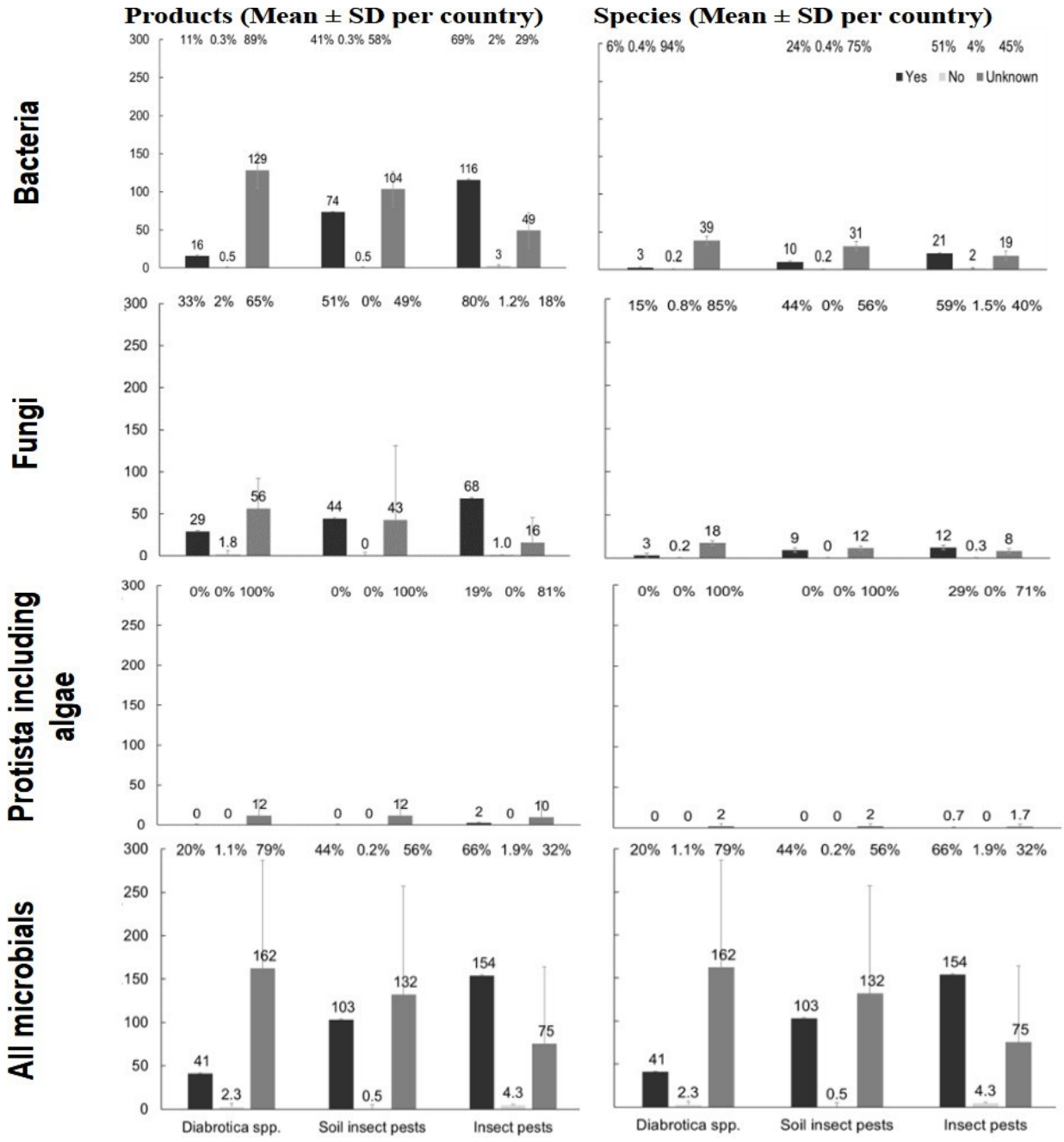
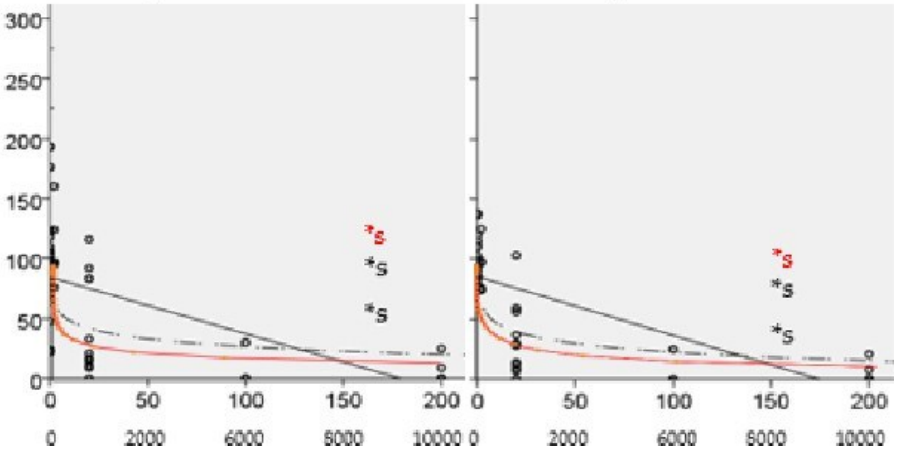
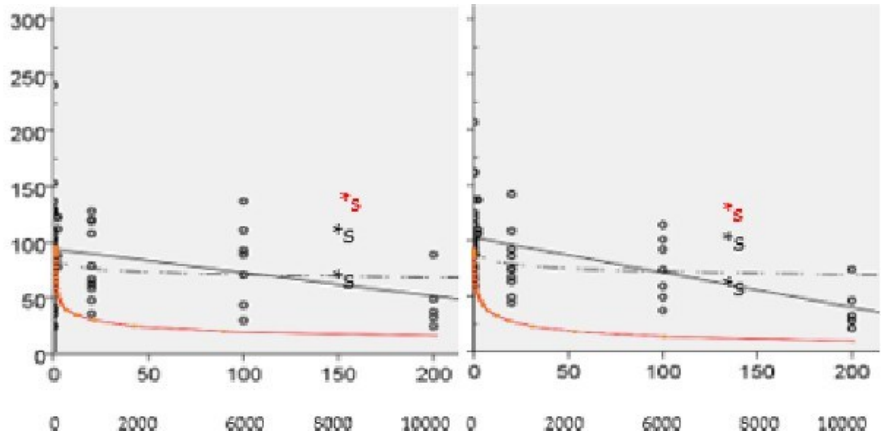
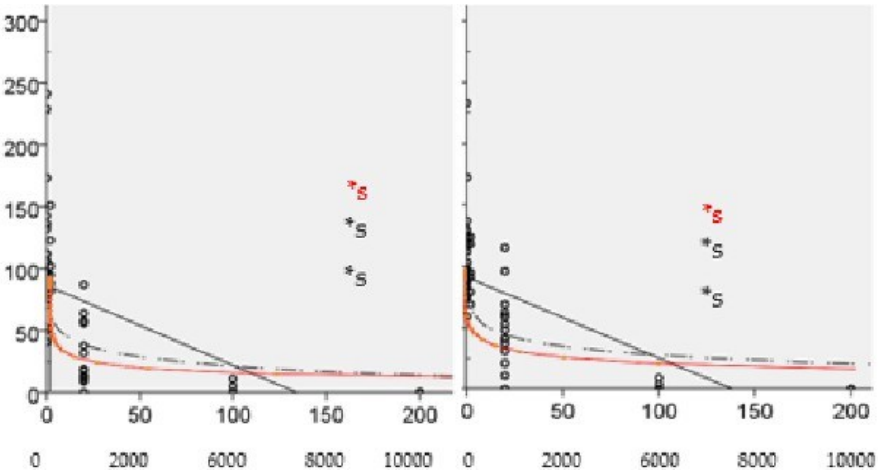
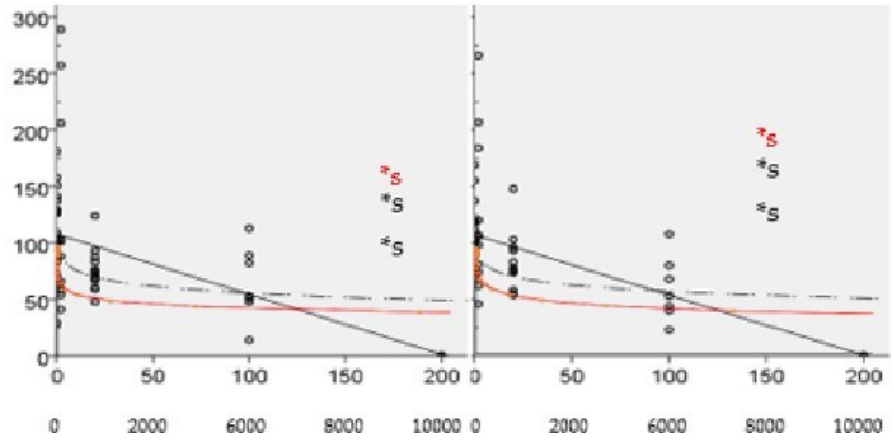


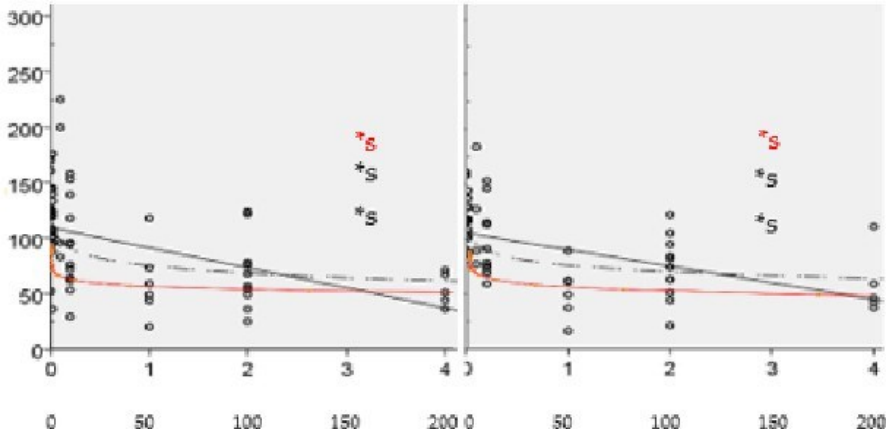
Figure 1. Average numbers and percentages of commercial microbial plant stimulants with potential effects on *Diabrotica* species (rootworms), soil insect pests, and insect pests in general, reviewed as per Web of Science (Clarivate, 2020) and CAB Direct (CABI, 2020). In total, 483 products reviewed with 245 microorganisms from Hungary, Switzerland, Spain, France, Indonesia, Canada, as per 2021.

Active ingredients	Percent egg hatching compared to untreated control	
	within 5 days	within 7 days
Imidacloprid		
Novaluron		
Cypermethrin		

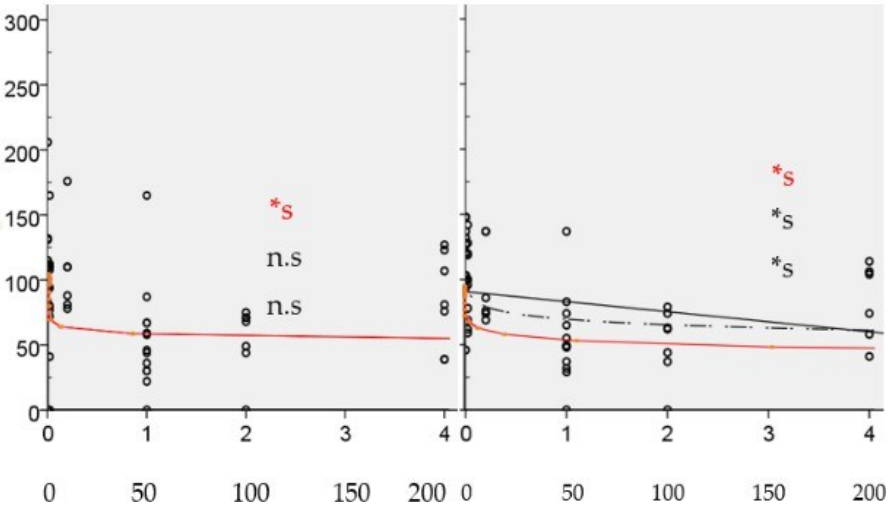
Clothianidin



Chlorpyrifos-methyl



Acetamiprid



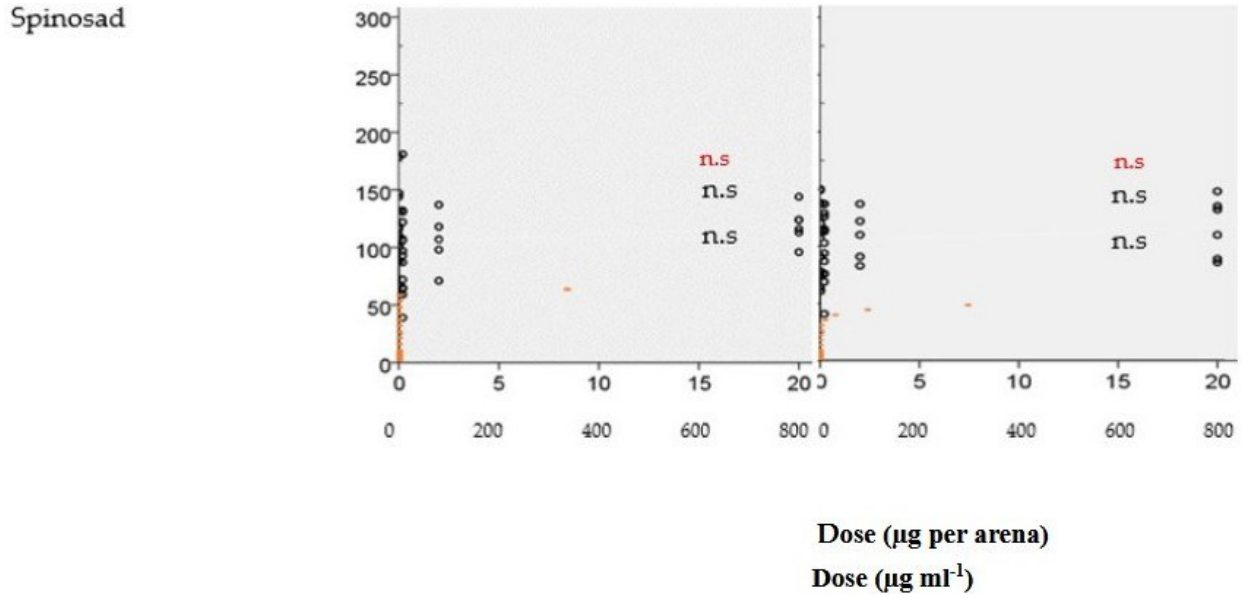
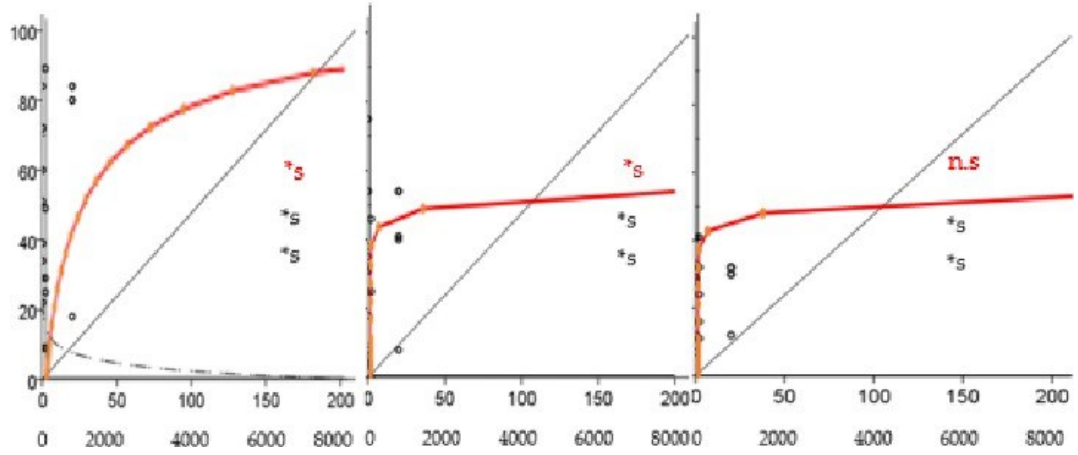
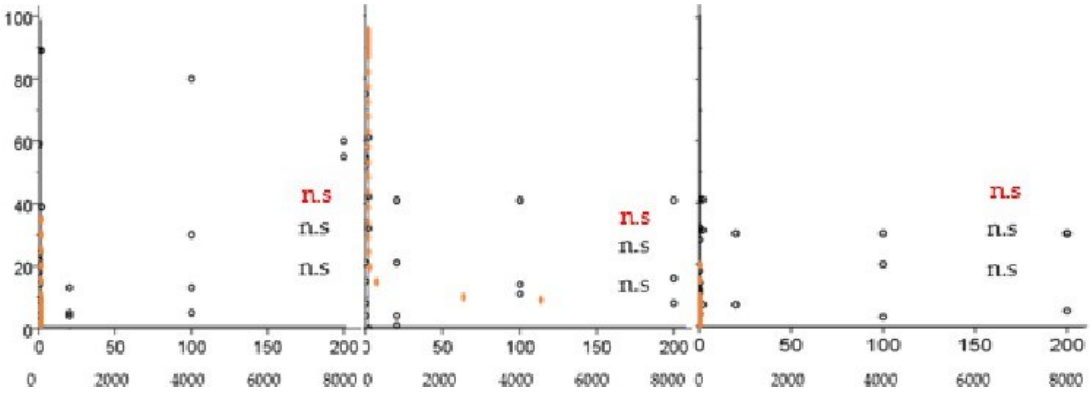
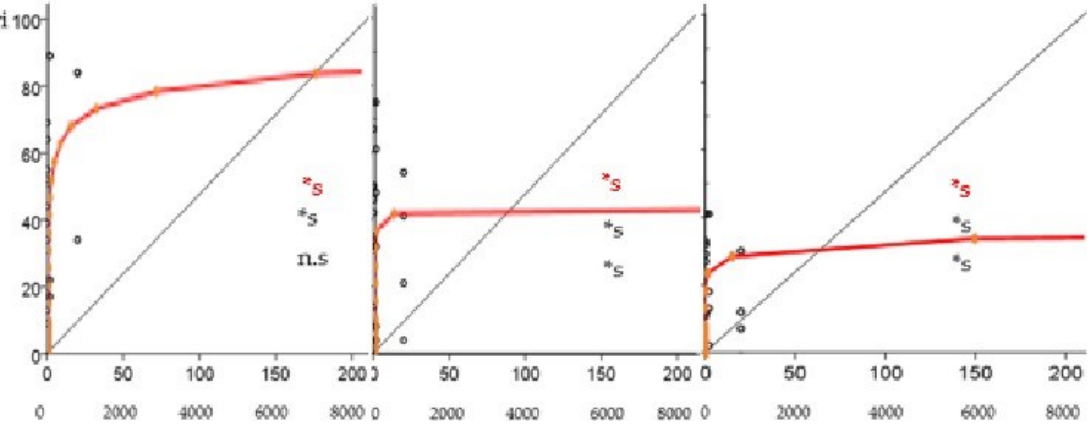
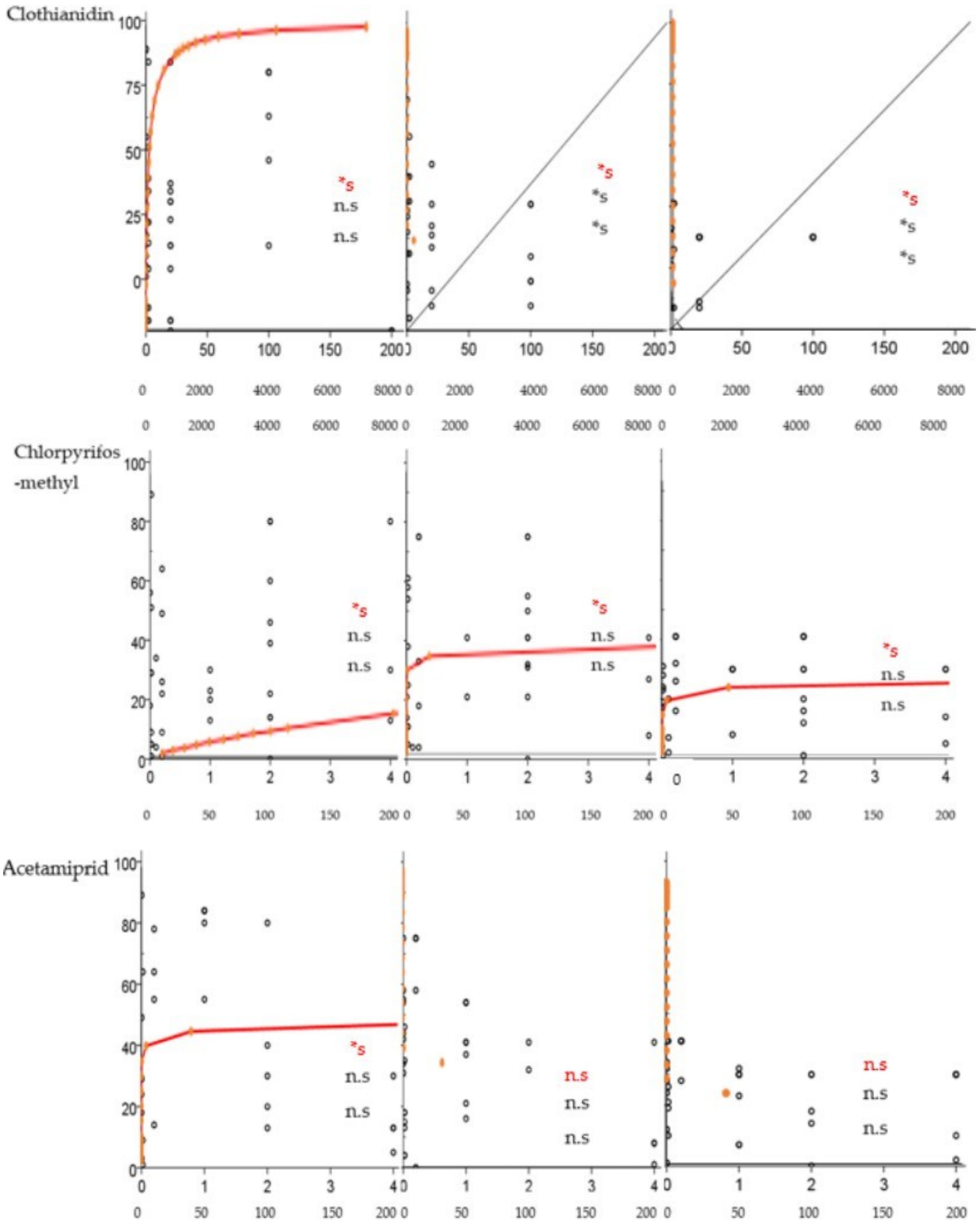


Figure 1. Hatching rate (%) of *Diabrotica v. virgifera* eggs treated with different concentrations of active ingredients of insecticides through dipping treatments. The bioassays were conducted by transferring the treated eggs to filter paper in petri dishes under laboratory conditions (23-25^{0}C, 50-90% r.h.). Reduced hatching reflects egg mortality. The y-axis represents the percent egg hatching compared to the untreated control. Primary x-axis represents the dose tested in µg per arena, and secondary x-axis represents the dose tested in µg per ml. There are three experimental repetitions per treatment and dose. Probit regression (red colored-line), linear regression (black colored-line), and logarithmic regression (black colored-dashed line) fit to dose-response and presented if significant at $p < 0.05$ (*s.); n.s. indicates a non-significant regression and no line is therefore presented.

Active ingredients	Percent daily mortality of neonates freshly hatched from eggs compared to untreated control		
	within 3 days	within 5 days	Within 7 days
Imidacloprid			
Novaluron			
Cypermethrin			


Clothianidin
Chlorpyrifos
-methyl
Acetamiprid
*s
n.s

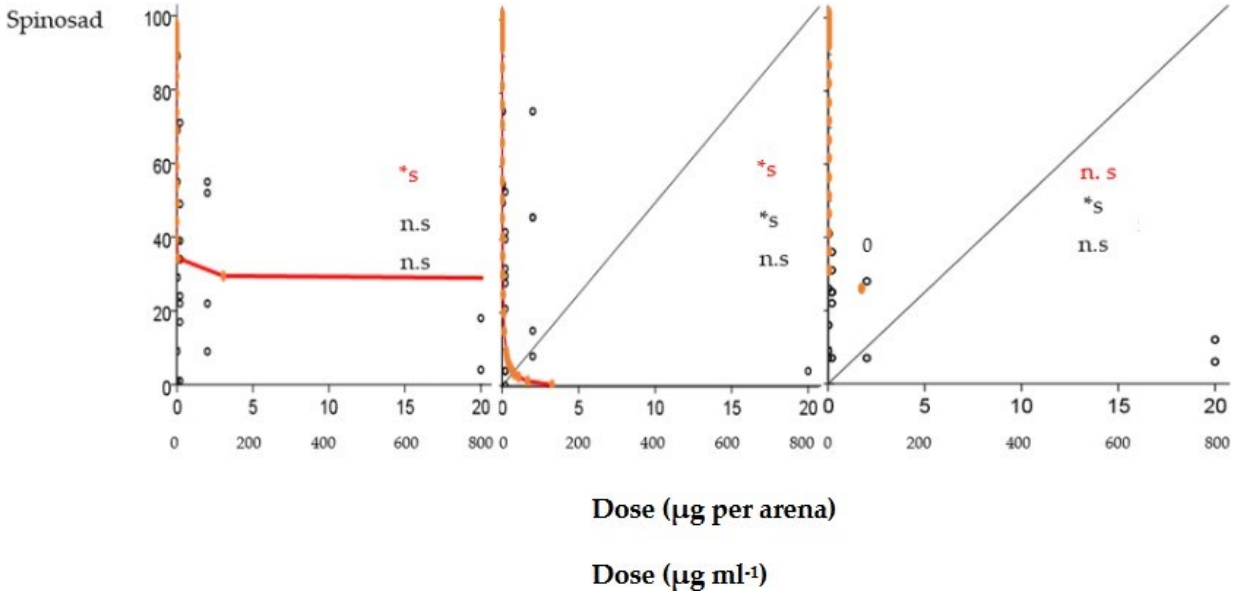
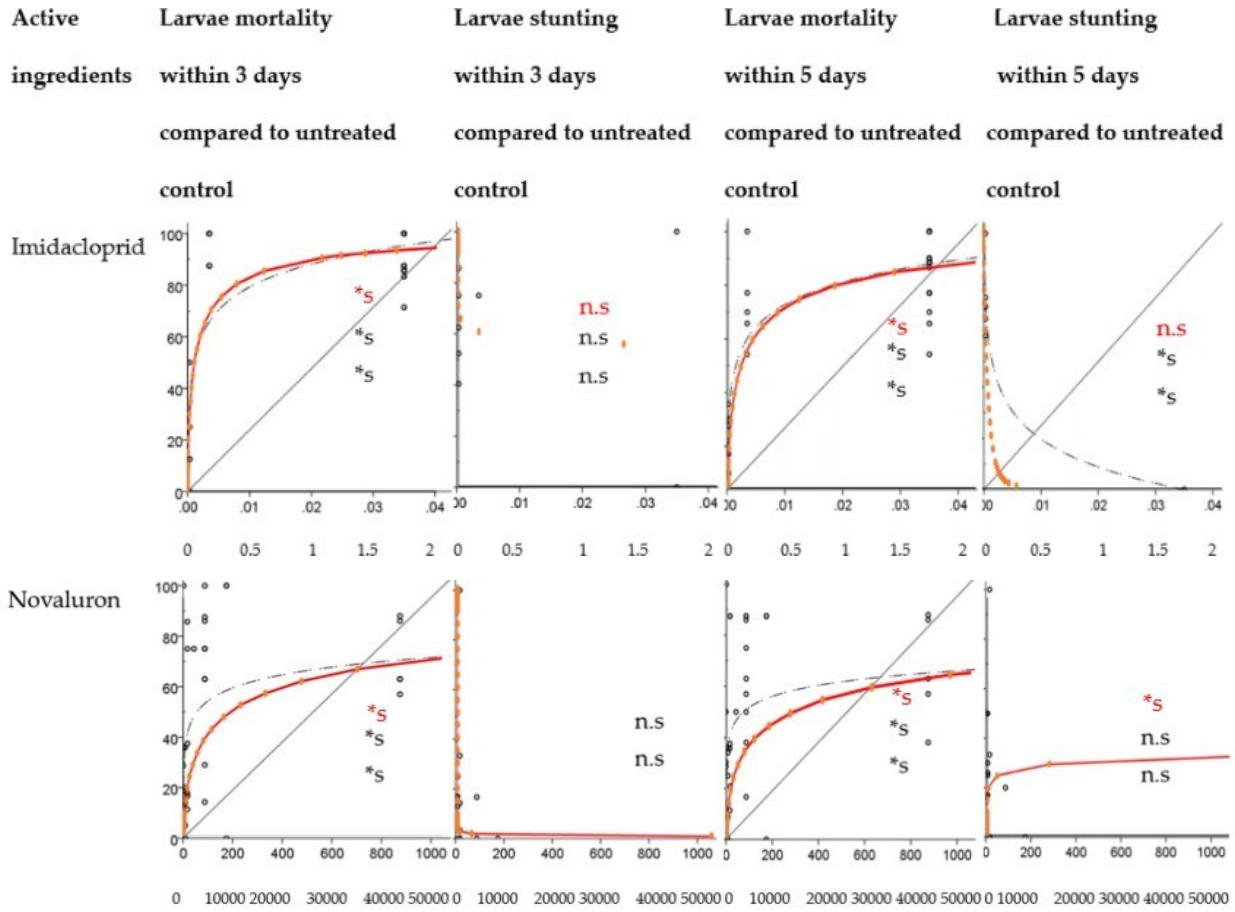
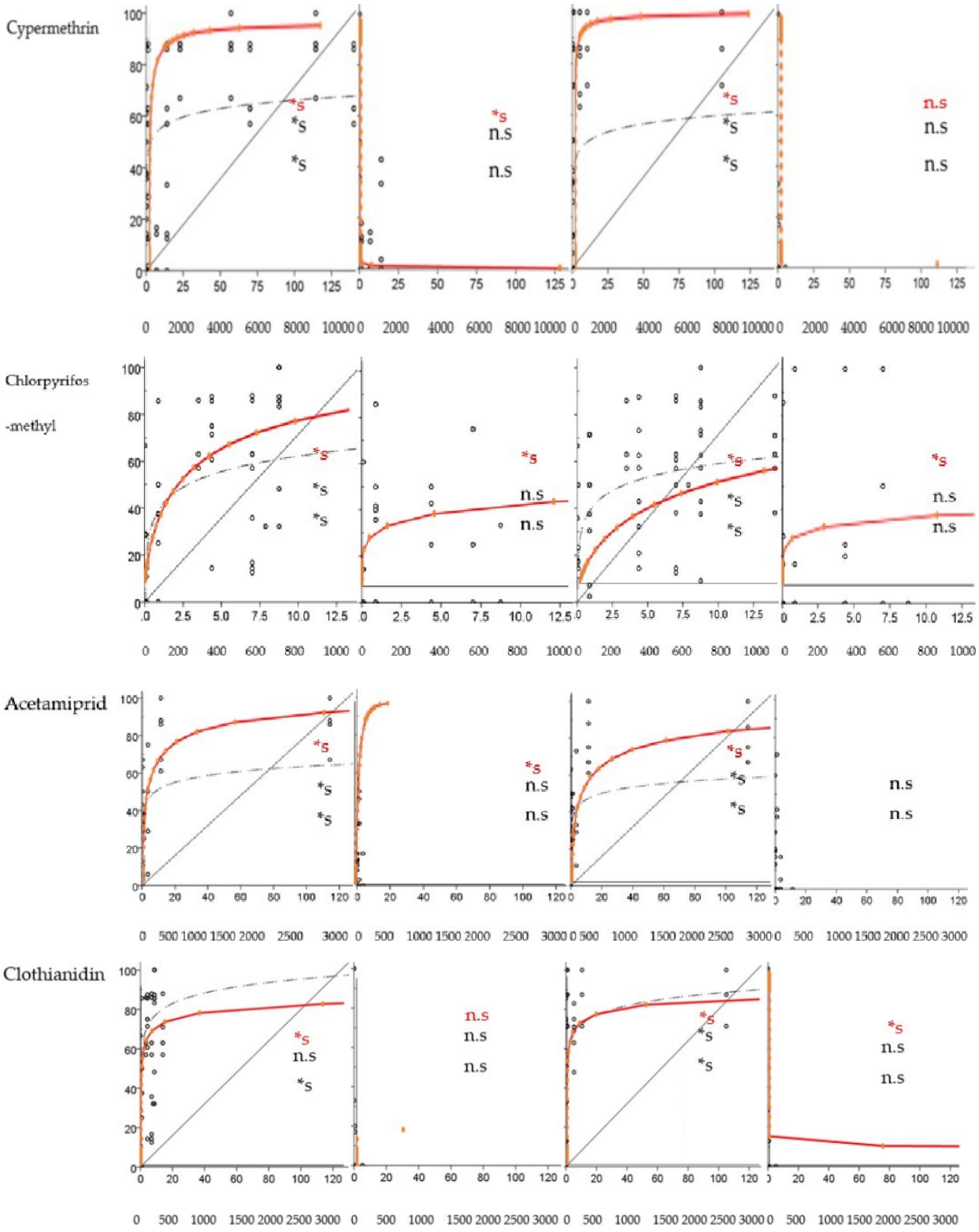


Figure 2. Mortality of *Diabrotica v. virgifera* larvae during hatching from eggs treated with different concentrations of active ingredients of insecticides through dipping treatments. The bioassays were conducted by transferring the treated eggs to filter paper in petri dishes under laboratory conditions (23-25ºC, 50 to 90% r.h.). Primary x-axis represents the dose tested in µg per arena, and secondary x-axis represents the dose tested in µg per ml. There are two to five experimental repetitions per treatment. Probit regression (red colored-line), linear regression (black colored-line), and logarithmic regression (black colored-dashed line) fit to dose-response and presented if significant at $p < 0.05$ (*s.); n.s. non-significant regression and no line is therefore presented.




Cypermethrin
Chlorpyrifos
-methyl
Acetamiprid
Clothianidin
*s
n.s

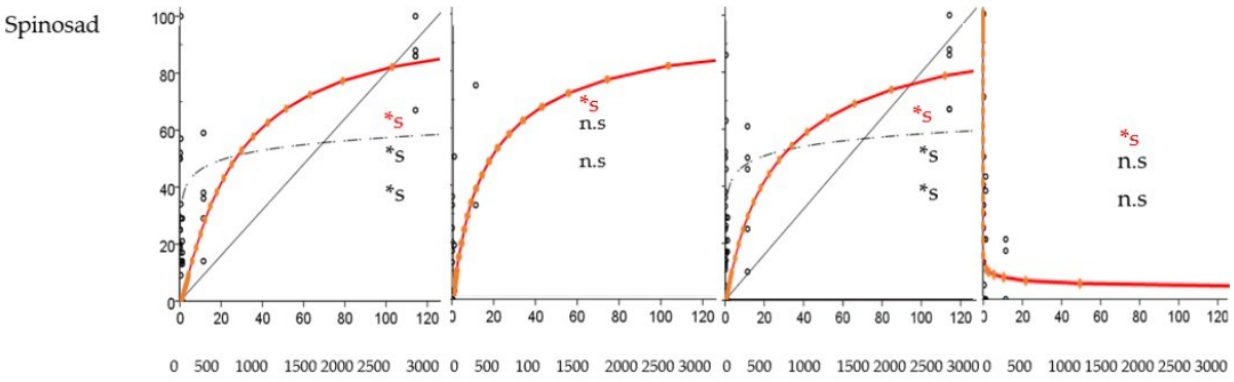
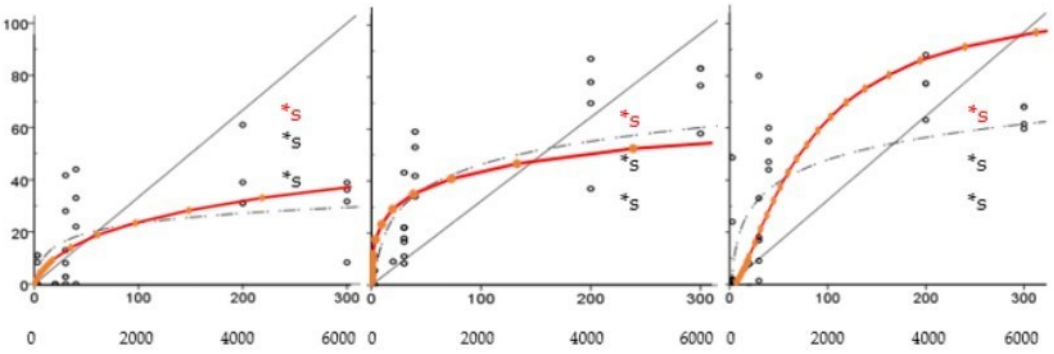
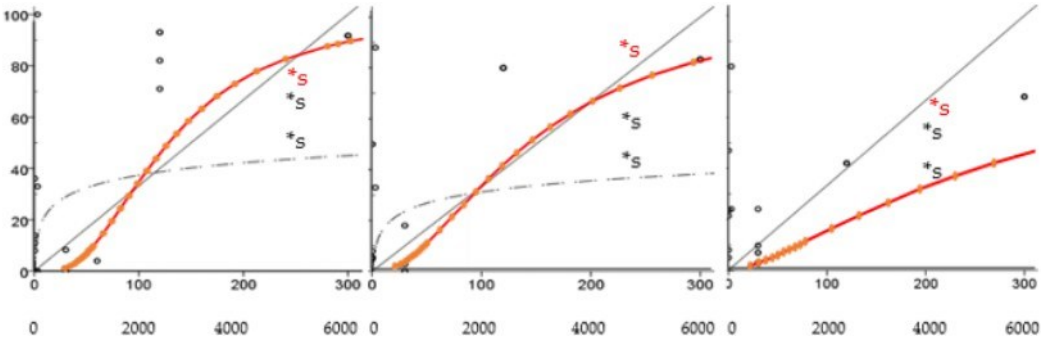
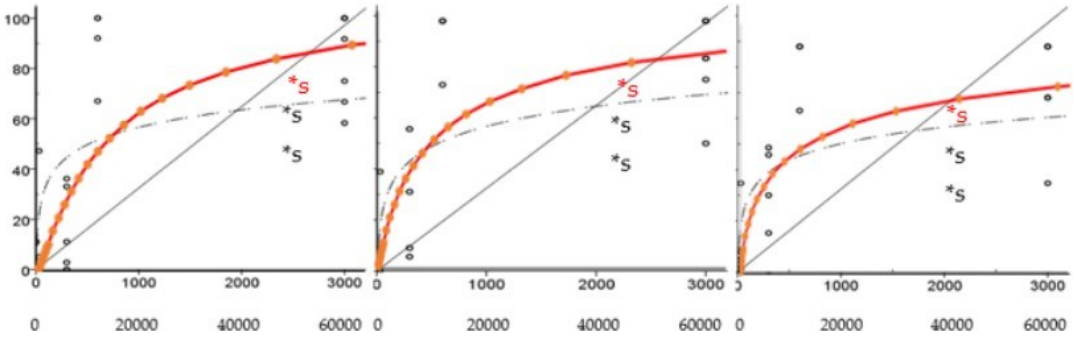
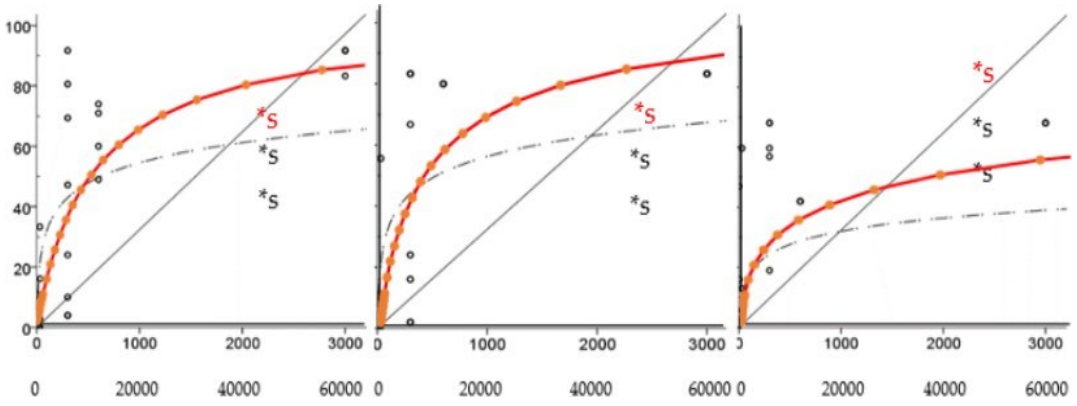


Figure 3. Mortality and stunting of *D. v. virgifera* larvae when exposed to artificial diet treated with different concentrations of active ingredients of insecticides in diet-overlay experiments under standardized laboratory conditions (23-25ºC, 50-90% r.h.) using 96-well plates. The y-axis represents % larvae mortality and % stunting within 3 and 5 days compared to the untreated control. Primary x-axis represents the dose tested in µg per arena, and secondary x-axis represents the dose tested in µg per ml. Eight wells with one neonate per each of 7 plates per each of 2 to 5 experimental repetitions per treatment. Probit regression (red colored-line), linear regression (black colored-line), and logarithmic regression (black colored-dashed line) fit to dose-response and presented if significant at $p < 0.05$ (*s.); n.s non-significant regression and no line is therefore presented.

Active ingredients	Adult mortality compared to untreated control within 1 day	within 3 days	within 5 days
Imidacloprid			
Chlorpyrifos-methyl			
Novaluron			
Spinosad			

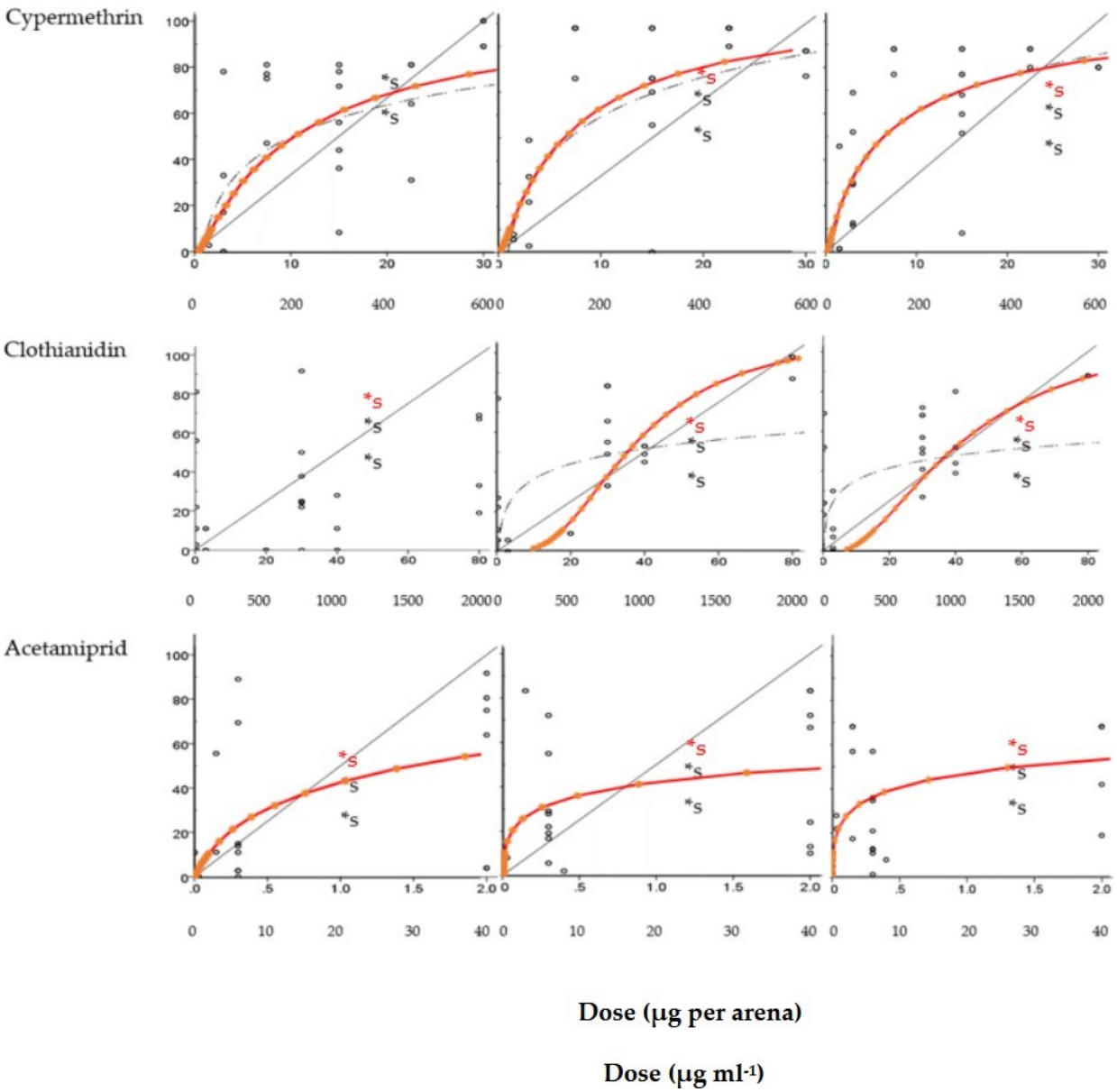
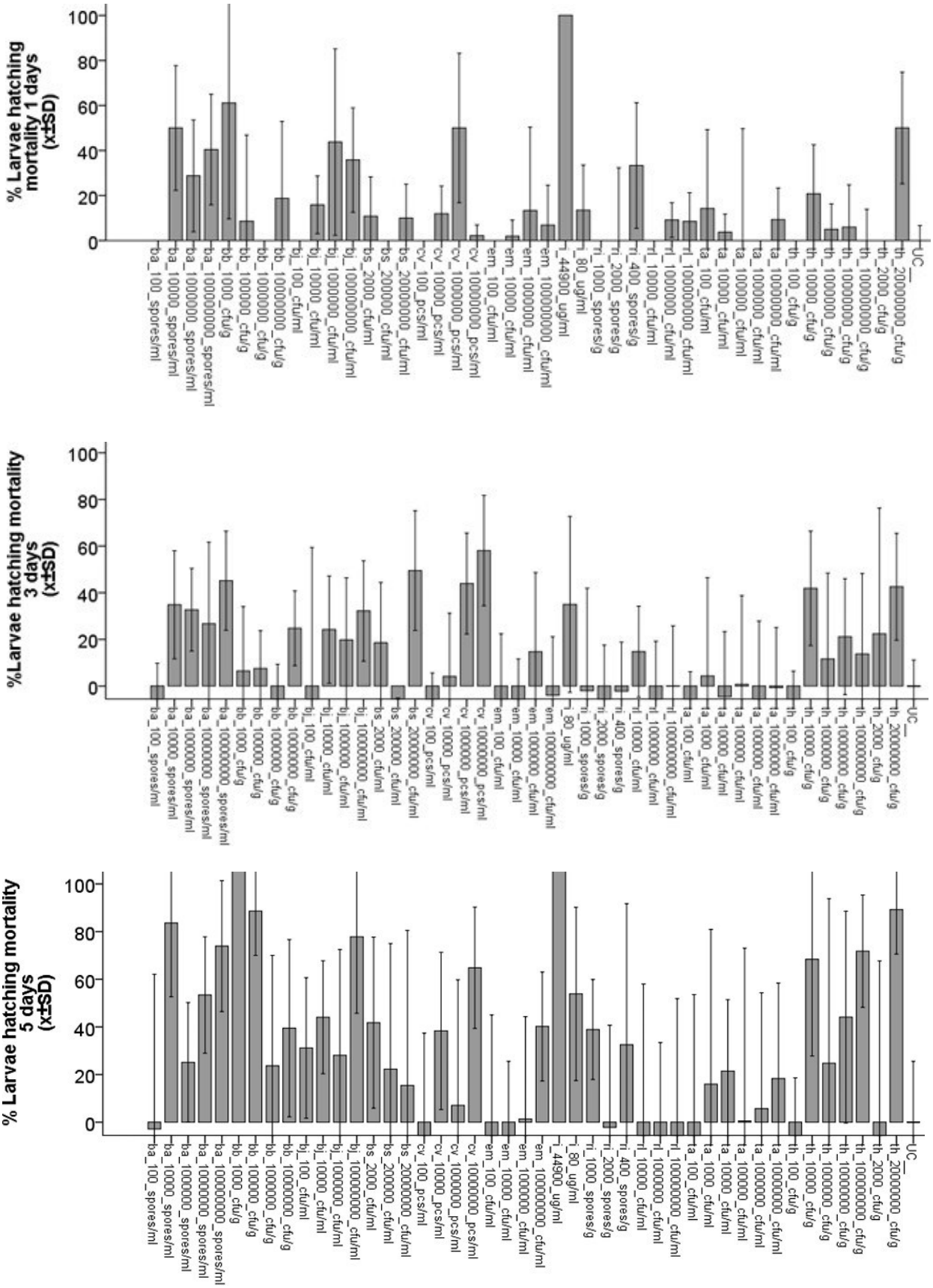
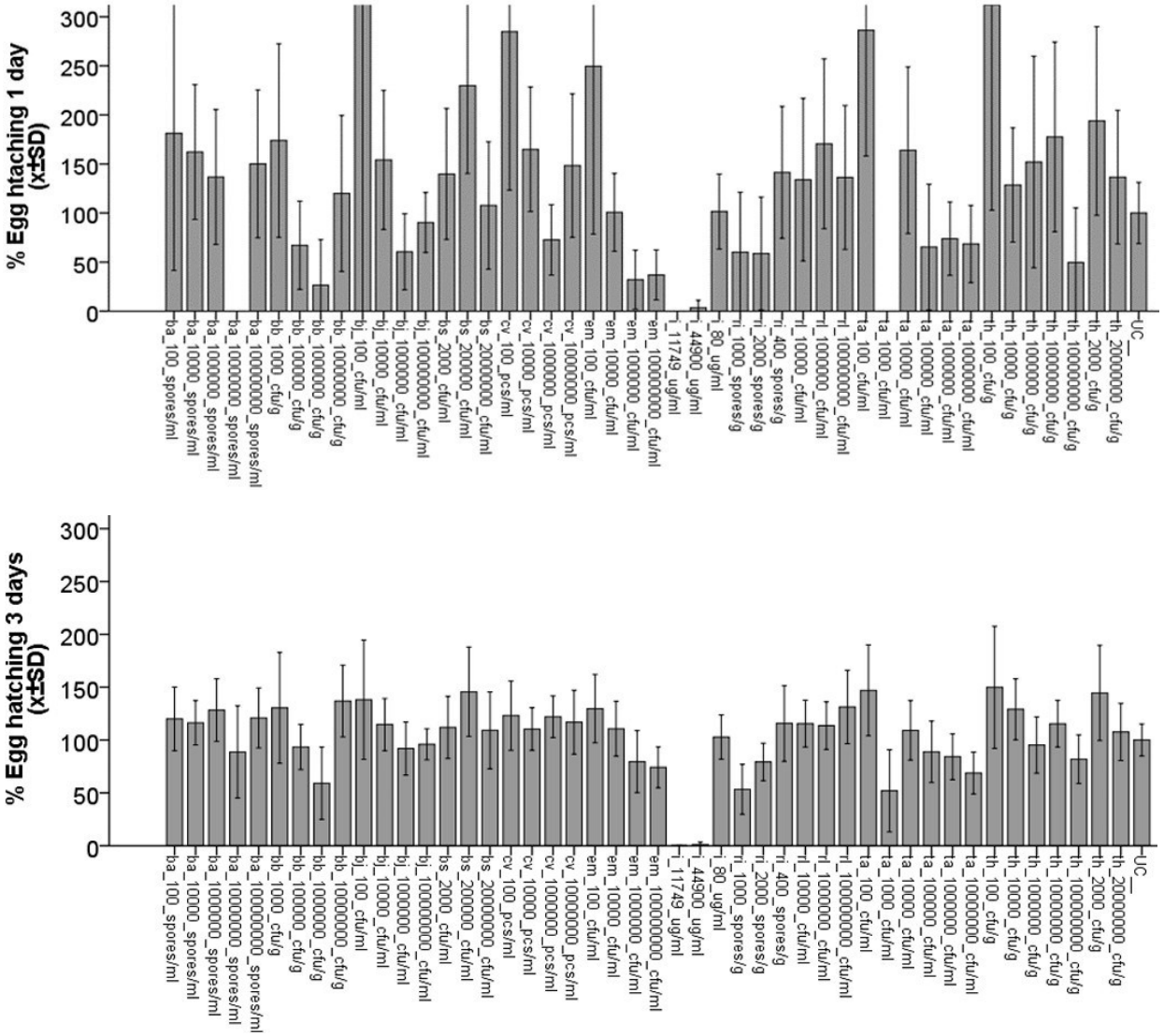


Figure 4. Mortality of *Diabrotica v. virgifera* adults when exposed to artificial diet cores treated with different concentrations active ingredients of insecticides under standardized laboratory conditions (23-25^{0}C, 50-90% r.h.). Each treatment has a total of 12 replicates with about 3 beetles each on 4 of 6-well plates which per each has three experimental repetitions per treatment. Primary x-axis represents the dose tested in µg per arena, and secondary x-axis represents the dose tested in µg per ml. Probit regression (red colored-line), linear regression (black colored-line), and logarithmic regression (black colored-dashed line) fit to dose-response presented if significant at $p < 0.05$ (*s.); n.s non-significant regression and no line is therefore presented.


% Larvae hatching mortality 1 days (x±SD)
%Larvae hatching mortality 3 days (x±SD)
% Larvae hatching mortality 5 days (x±SD)
0
20
40
60
80
100
ba_100_spores/ml
ba_10000_spores/ml
ba_1000000_spores/ml
ba_100000000_spores/ml
bb_1000_cfu/g
bb_100000_cfu/g
bb_10000000_cfu/g
bb_1000000000_cfu/g
bj_100_cfu/ml
bj_10000_cfu/ml
bj_1000000_cfu/ml
bj_100000000_cfu/ml
bs_2000_cfu/ml
bs_200000_cfu/ml
bs_20000000_cfu/ml
cv_100_pcs/ml
cv_10000_pcs/ml
cv_1000000_pcs/ml
cv_100000000_pcs/ml
em_100_cfu/ml
em_10000_cfu/ml
em_1000000_cfu/ml
em_100000000_cfu/ml
i_44900_ug/ml
i_80_ug/ml
ri_1000_spores/g
ri_2000_spores/g
ri_400_spores/g
rl_10000_cfu/ml
rl_1000000_cfu/ml
rl_100000000_cfu/ml
ta_100_cfu/ml
ta_1000_cfu/ml
ta_10000_cfu/ml
ta_100000_cfu/ml
ta_1000000_cfu/ml
ta_10000000_cfu/ml
th_100_cfu/g
th_10000_cfu/g
th_1000000_cfu/g
th_100000000_cfu/g
th_2000_cfu/g
th_20000000_cfu/g
UC

Treatments and concentrations

Figure 1. Effect of microbial bio stimulants on hatching larval mortality of *Diabrotica virgiferavirgifera* (treatment name on x-axis followed by the treatment code, referred to Table 2).



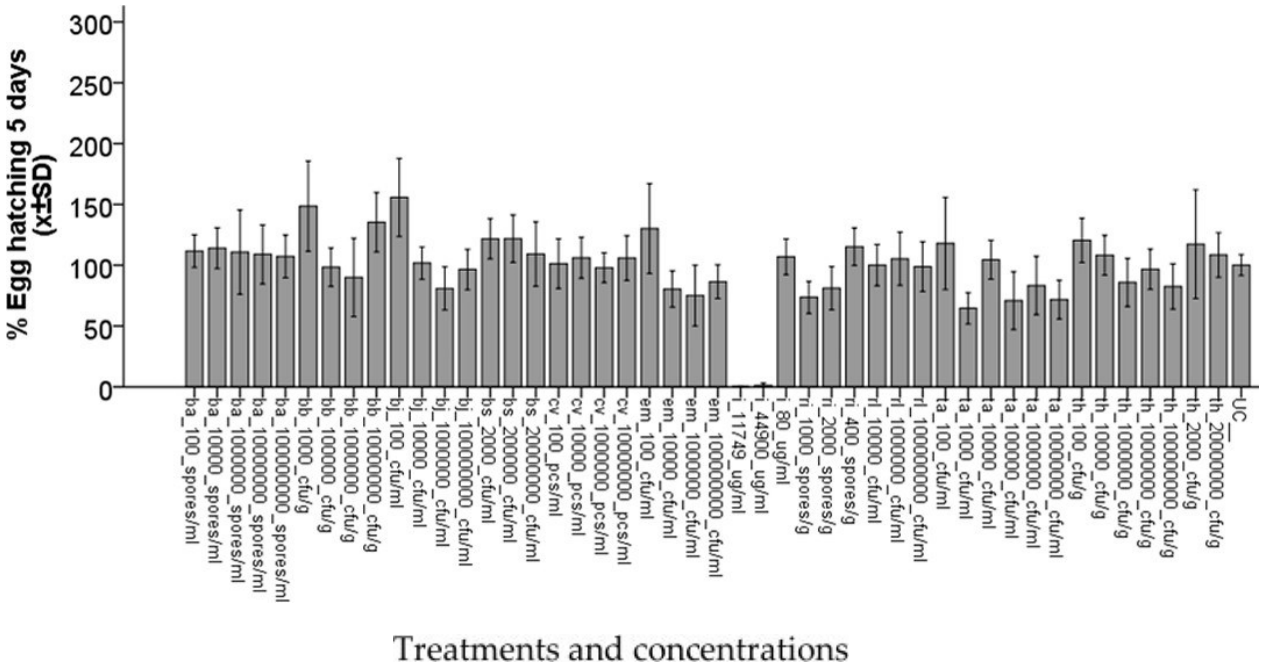
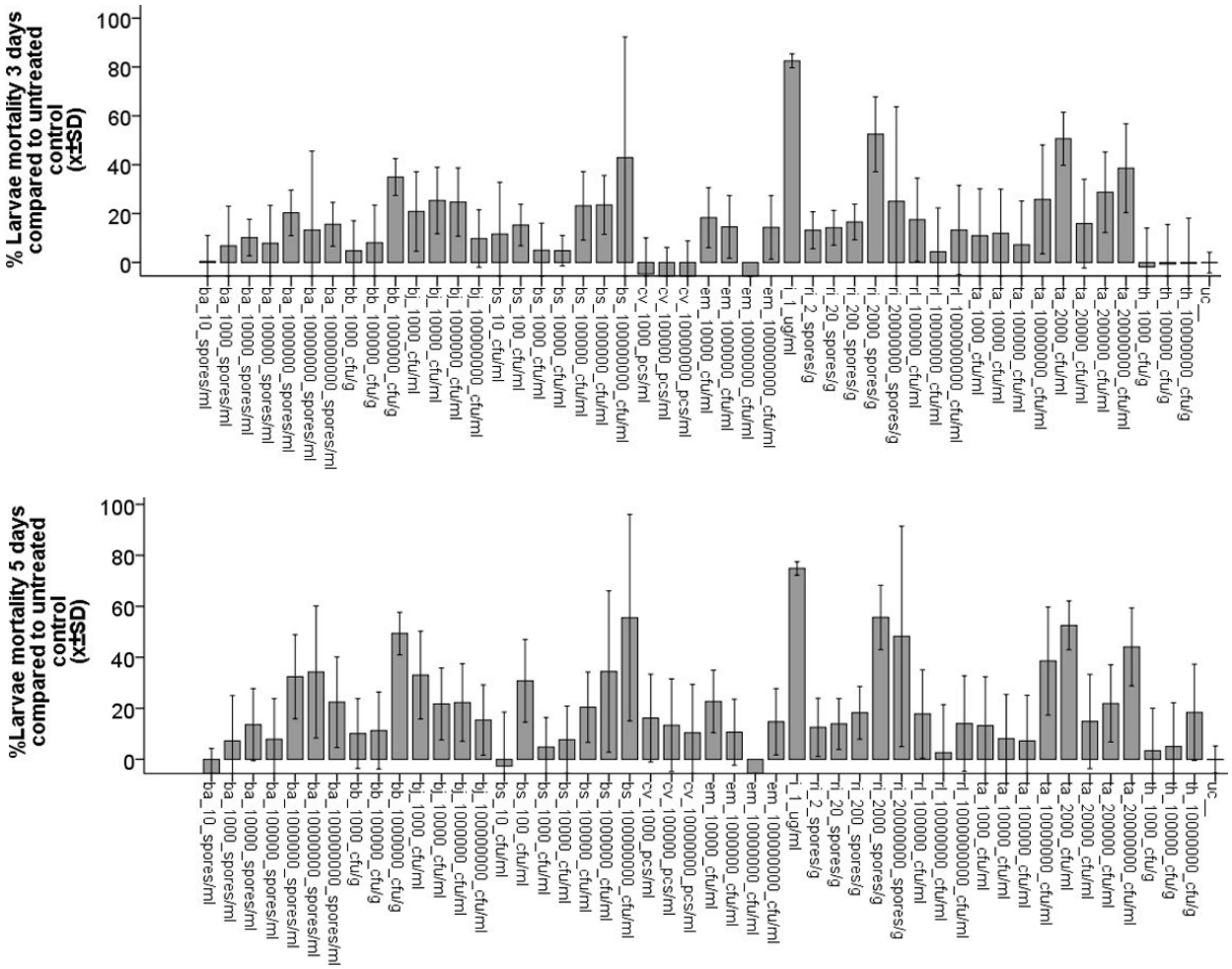


Figure 2. Effects of microbial biostimulant on egg hatching rate of *Diabrotica v. virgifera.* (treatment name on x-axis followed by the treatment code, referred to Table 2).



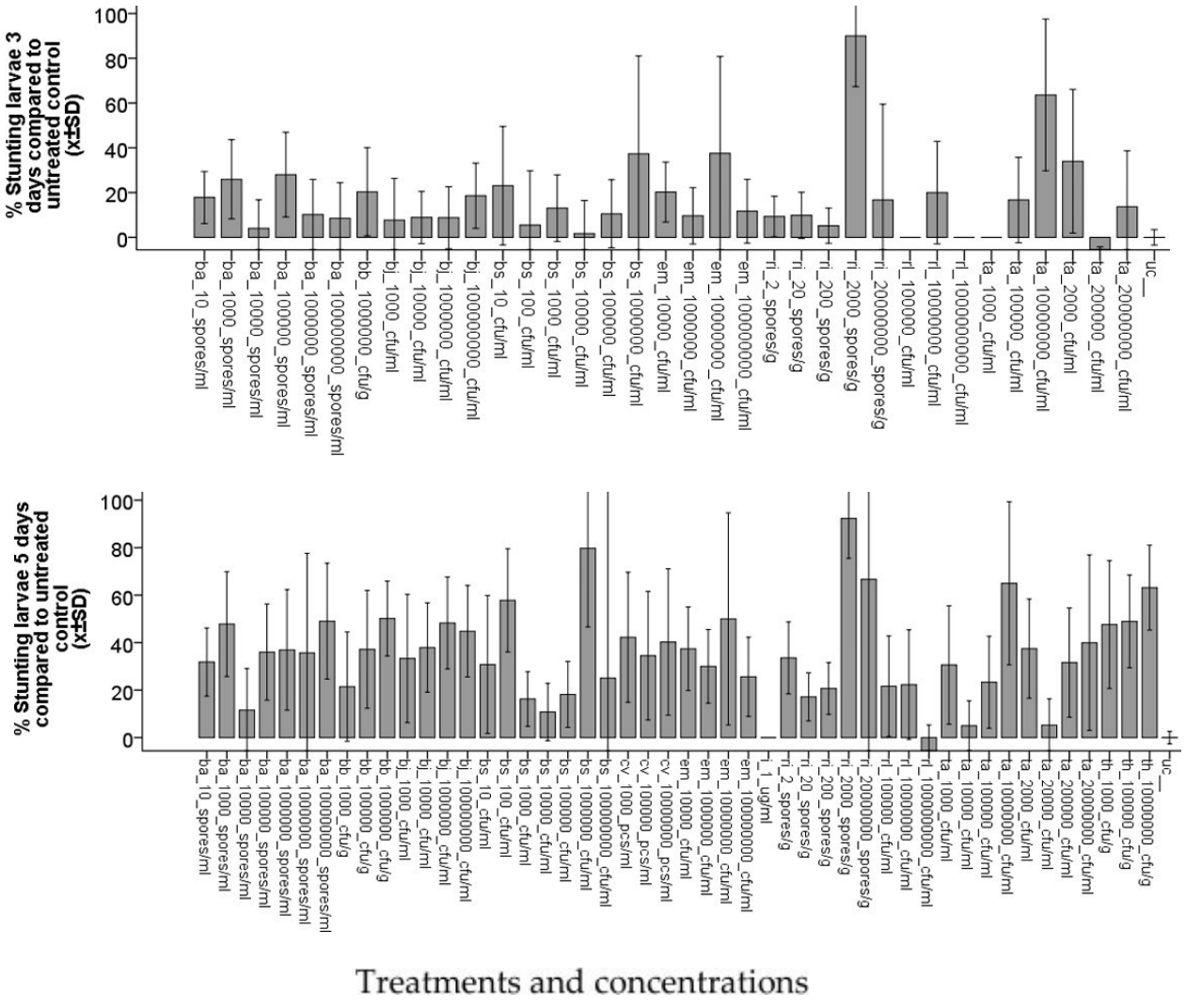
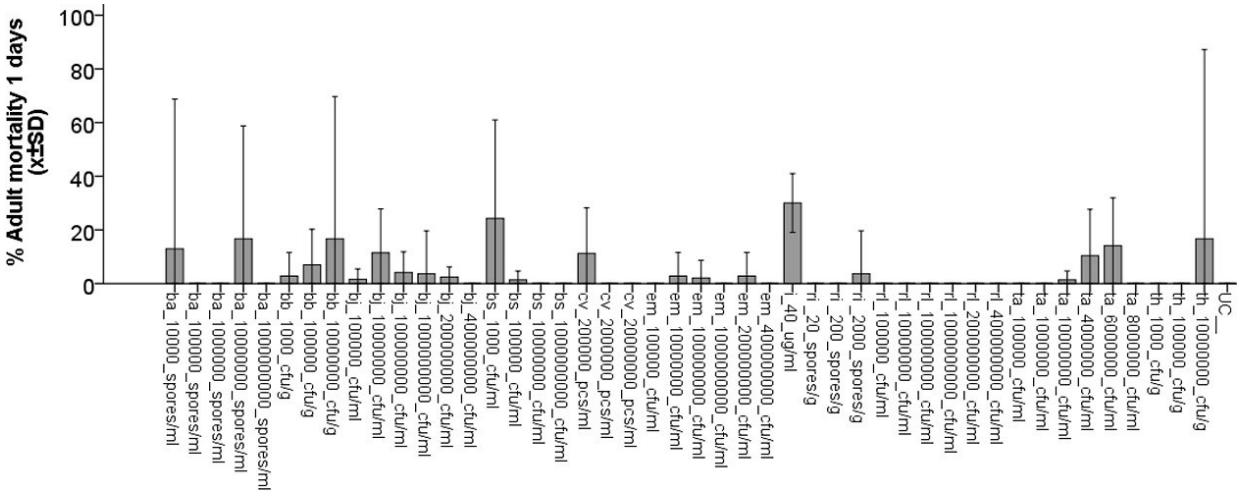


Figure 3. Effect of microbial bio stimulants on *Diabrotica v. virgifera* larvae (treatment name on x-axis followed by the treatment code, referred to Table 2).



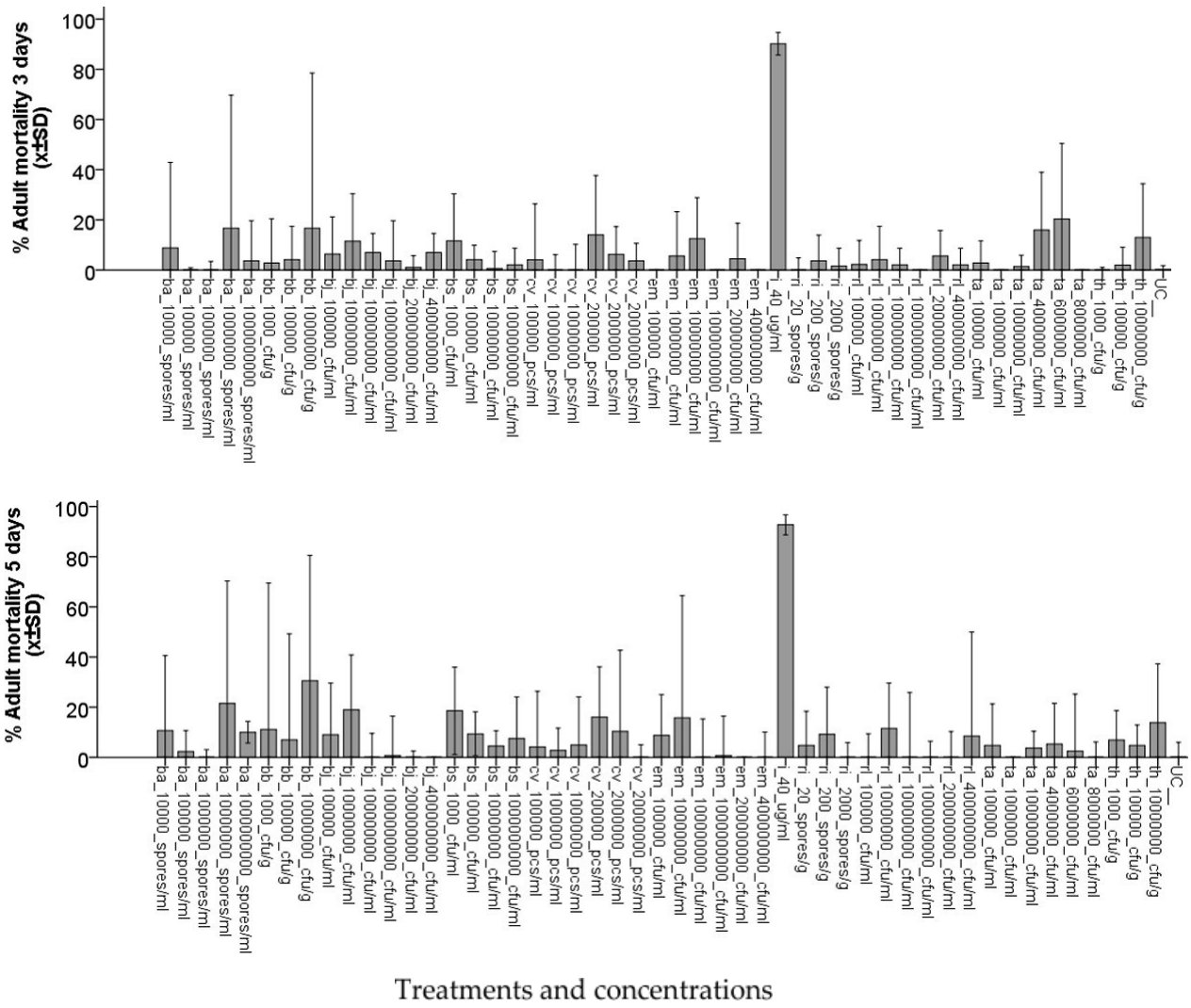
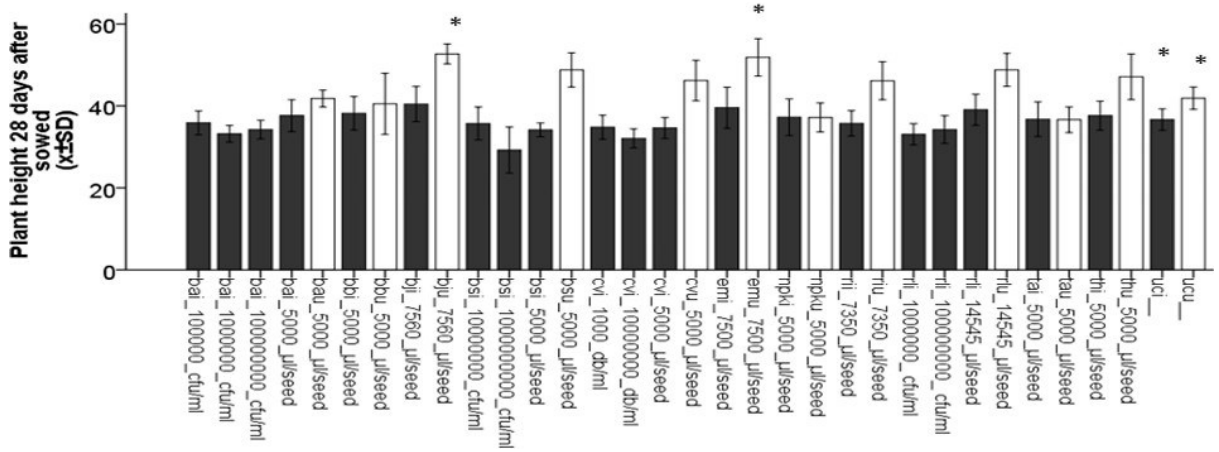
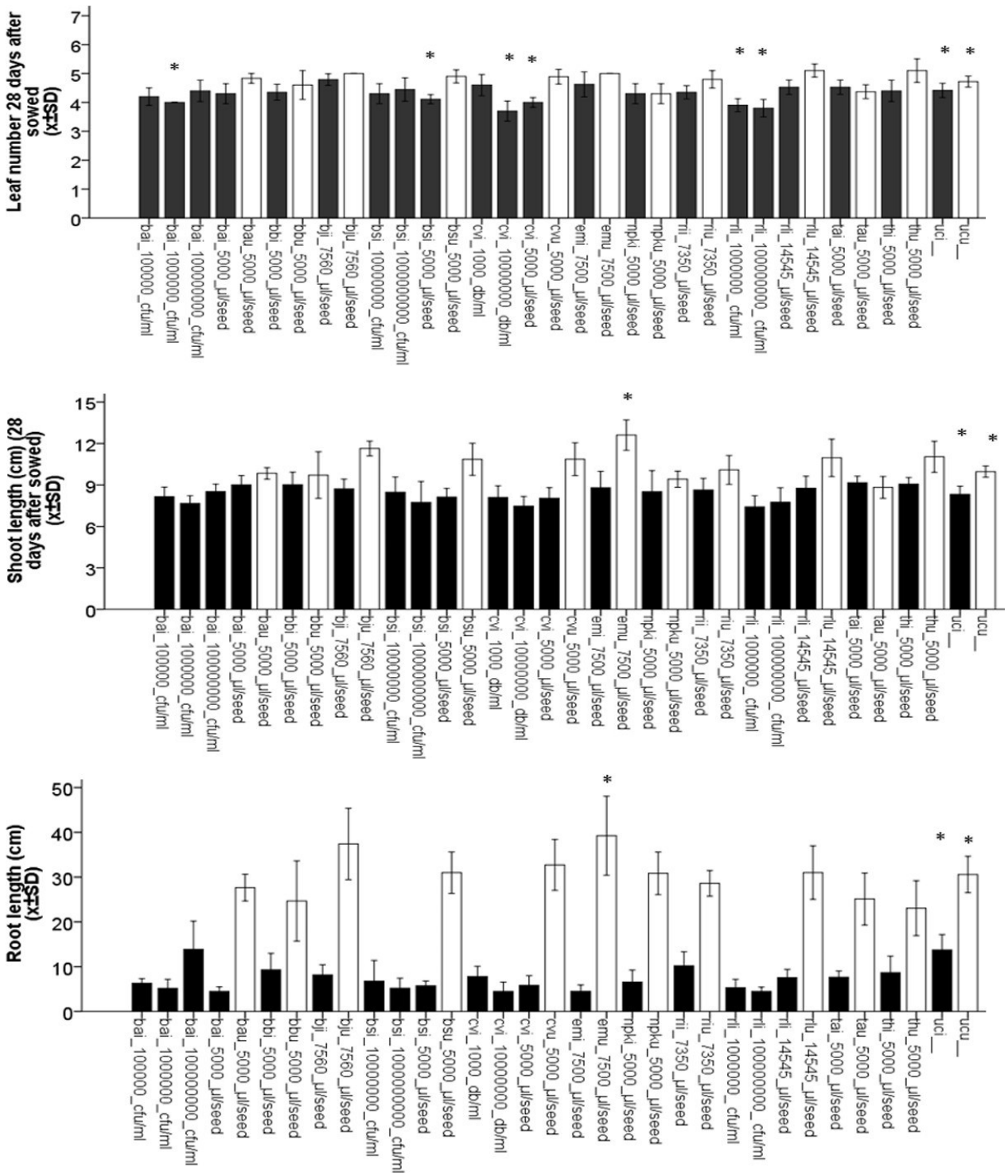


Figure 4. Effects of microbial bio stimulants on *Diabrotica v. virgifera* adult (treatment name on x-axis followed by the treatment code, referred to Table 2).




Leaf number 28 days after sowed (x±SD)
Shoot length (cm) (28 days after sowed) (x±SD)
Root length (cm) (x±SD)
bai_100000_cfu/ml
bai_1000000_cfu/ml
bai_10000000_cfu/ml
bai_5000_µl/seed
bau_5000_µl/seed
bbi_5000_µl/seed
bbu_5000_µl/seed
bji_7560_µl/seed
bju_7560_µl/seed
bsi_10000000_cfu/ml
bsi_100000000_cfu/ml
bsi_5000_µl/seed
bsu_5000_µl/seed
cvi_1000_db/ml
cvi_10000000_db/ml
cvi_5000_µl/seed
cvu_5000_µl/seed
emi_7500_µl/seed
emu_7500_µl/seed
mpki_5000_µl/seed
mpku_5000_µl/seed
mii_7350_µl/seed
miu_7350_µl/seed
mli_1000000_cfu/ml
mli_100000000_cfu/ml
mli_14545_µl/seed
mlu_14545_µl/seed
tai_5000_µl/seed
tau_5000_µl/seed
thi_5000_µl/seed
thu_5000_µl/seed
uci__
ucu__

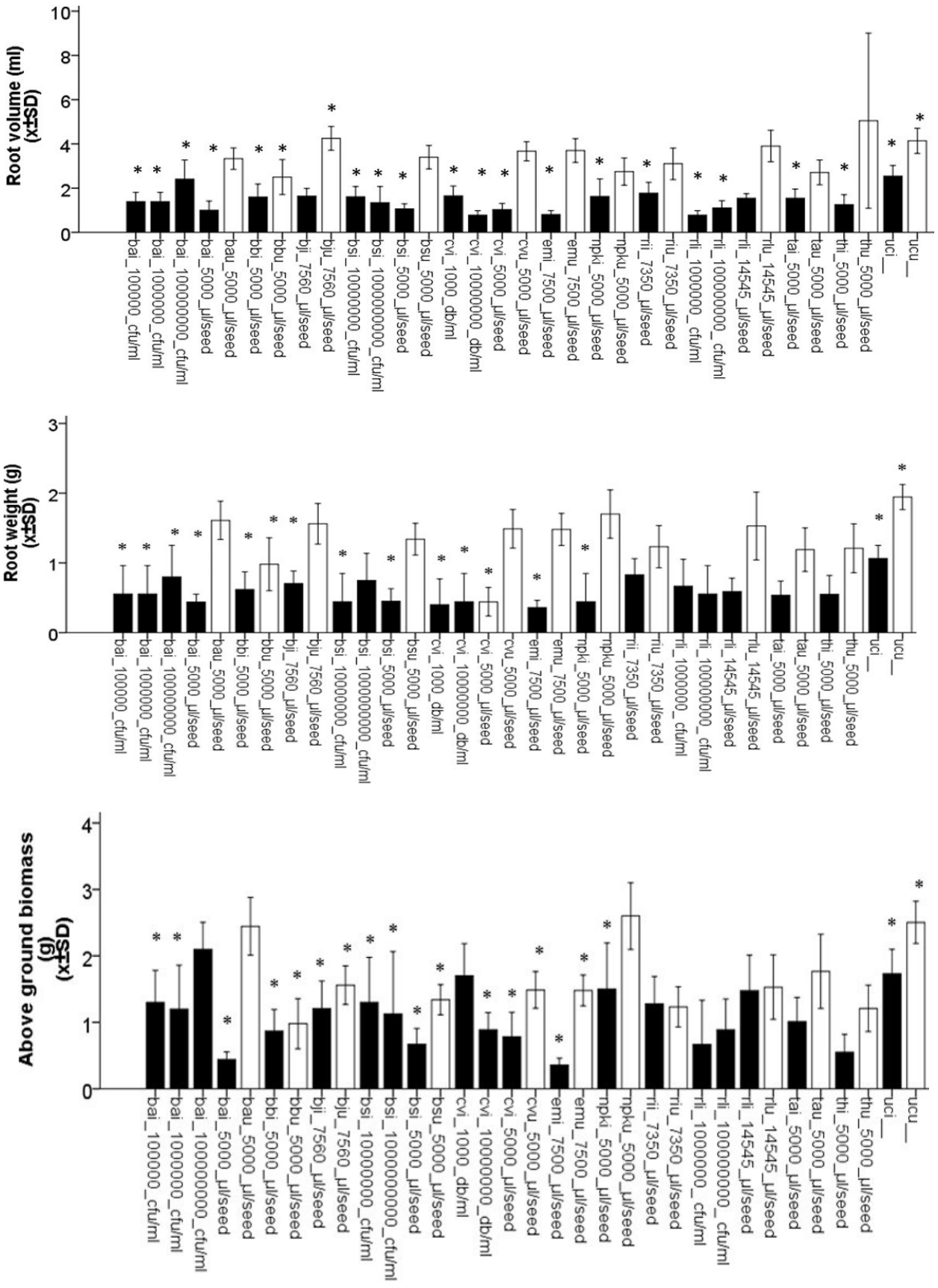

Root volume (ml) (x±SD)
Root weight (g) (x±SD)
Above ground biomass (g) (x±SD)
bai_100000_cfu/ml
bai_1000000_cfu/ml
bai_100000000_cfu/ml
bai_5000_µl/seed
bau_5000_µl/seed
bbi_5000_µl/seed
bbu_5000_µl/seed
bji_7560_µl/seed
bju_7560_µl/seed
bsi_10000000_cfu/ml
bsi_1000000000_cfu/ml
bsi_5000_µl/seed
bsu_5000_µl/seed
cvi_1000_db/ml
cvi_10000000_db/ml
cvi_5000_µl/seed
cvu_5000_µl/seed
emi_7500_µl/seed
emu_7500_µl/seed
npki_5000_µl/seed
npku_5000_µl/seed
rii_7350_µl/seed
riu_7350_µl/seed
rli_1000000_ cfu/ml
rli_100000000_ cfu/ml
rli_14545_µl/seed
rlu_14545_µl/seed
tai_5000_µl/seed
tau_5000_µl/seed
thi_5000_µl/seed
thu_5000_µl/seed
uci__
ucu__
Treatments and concentrations

Figure 5. Effect of microbial bio stimulants on the maize crop. (The name of the treatments on x-axis has been followed by the treatment code referred to in Table 2; the bar chart with white colour represents maize crops treated with bio stimulants and un infested with *Diabrotica virgifera-virgifera* larvae, while the bar chart with black colour represents maize crops treated with bio stimulants and infested with *Diabrotica virgiferavirgifera* larvae).

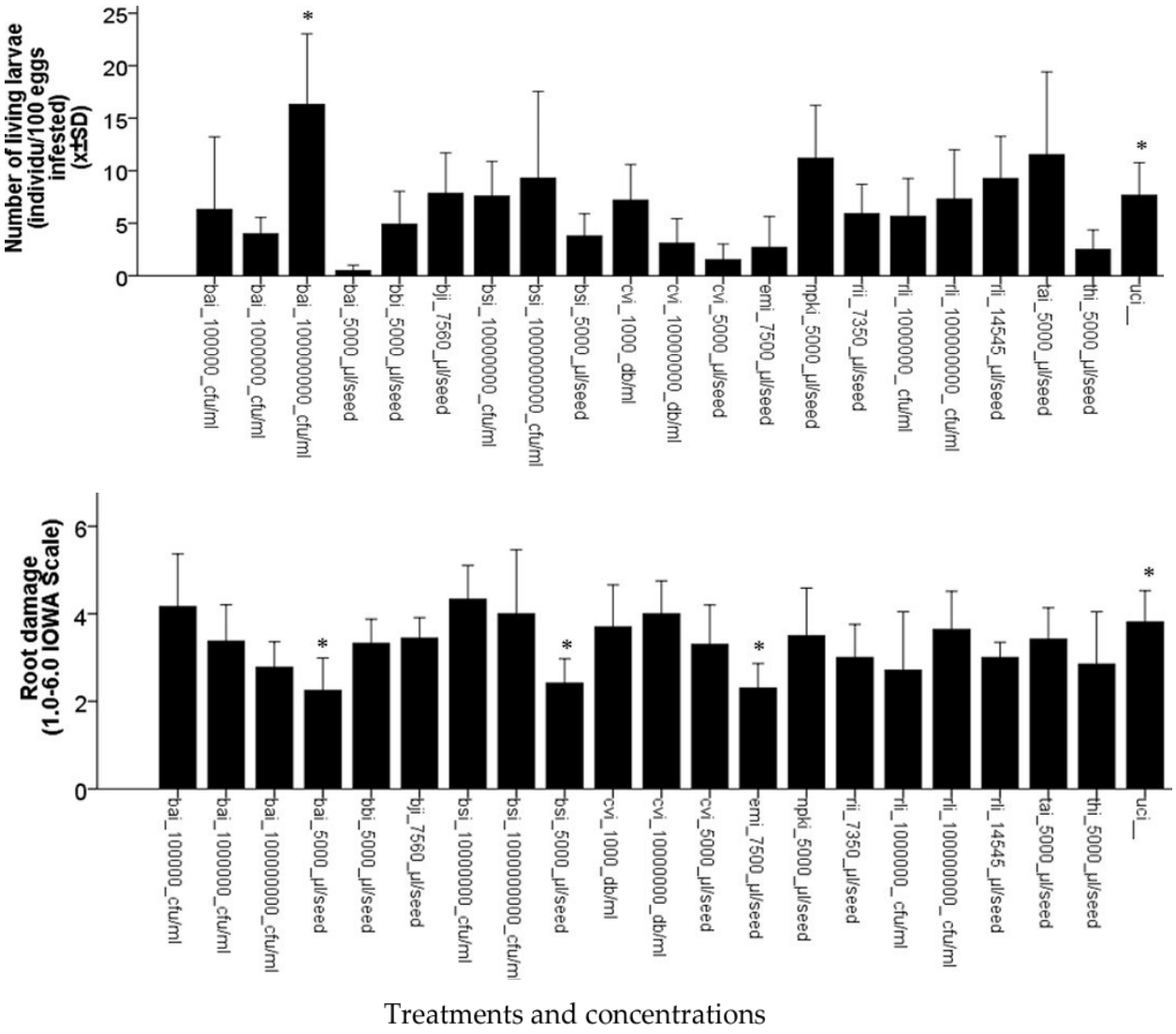


Figure 6. Effect of microbial bio stimulants on *Diabrotica v. virgifera* larvae. (The name of the treatments on x-axis has been followed by the treatment code referred to in Table 2; the bar chart with black colour represents maize crops treated with bio stimulants and infested with *Diabrotica v. virgifera* larvae).

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