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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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1. INTRODUCTION AND OBJECTIVES

Maize is crucial crop next to rice and wheat, serving as staple food in many parts of the world (Shiferaw et al., 2011). In Hungary, maize is one of the major crops covering more than 0.8 million hectares every year (KHS 2022). Maize is used for human food, animal feed, and ethanol production as a fuel additive (Erenstein et al., 2022).

Western Corn Rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) is a new maize insect pest that has been a serious threat since the mid-1980s arrived in Central Europe starting to impact the way maize is grown (Bažok et al., 2021; Kiss et al., 2005). This pest undergoes complete metamorphosis, progressing through the stages of egg, larva, pupa, and adult. This univoltine species lays eggs that overwinter in the soil, with larvae hatching in the spring. The larvae go through three instar stages, feeding on maize roots, which can lead to plant lodging and significant yield loss. Adults emerge between mid-June and early August and can reduce yields through intensive silk feeding (Toepfer and Kuhlmann, 2006). In Hungary, the pest caused serious damage between the late 1990s and early 2000s (Bayar et al., 2003). Many growers learnt to manage the pest through crop rotation to their fields in order to interrupt its life cycle (Szalai et al., 2014). Others applied granular or fluid soil insecticides or used insecticide-coated maize seeds to target the root feeding larvae. Broad spectrum foliar insecticides are occasionally used against adults. Foliar insecticides are usually knock-down contact-insecticides with considerable non-target effects. Some soil insecticides and seed coatings are highly toxic and can impact non-target species, such as bees. This has led to public concern and the banning of neonicotinoid seed coatings in maize (Hladik et al., 2018) Positive controls, such as commonly used commercial insecticides, are

essential for various bioassays and experiments when testing new agents or active ingredients. This is also true for research focused on more sustainable and safer pest management solutions. However, the dose-response relationship is often published by the industry, leading to a methodological knowledge gap in noncommercial research. Specifically, information on LD_{50} and LD_{90} is often missing, which limits the use of positive controls and hinders the comparative evaluation of new agents.

Increasingly, microbial bio stimulants are entering the market as they are easier to register than plant protection products. These bio stimulants contain beneficial microbes that may also have biological properties. However, the effects of the ingredients or their combinations in these products is sometimes not entirely clear.

In my work, I first reviewed microbial bio stimulants from six countries to gather information on potential candidates (in terms of products and species) with plant protection properties against *Diabrotica virgifera virgifera* and other soil pests on maize, using the Web of Science and CABdirect search engines. Secondly, I tested commercial insecticides commonly used by growers in Hungary to establish a positive control for *Diabrotica virgifera virgifera*. This information is crucial for researchers and companies continuously searching for novel active ingredients and agents. To evaluate the insecticidal properties of microbial bio stimulants, I tested 10 different microbial bio stimulants against *Diabrotica virgifera virgifera* (egg, larvae, and adult) in both laboratory and greenhouse conditions. This was done to gain a better understanding of the effects of these bio stimulants on *Diabrotica virgifera virgifera* larvae and maize crops. This step is crucial for improving the understanding and proper use of microbial bio stimulants.

The specific objectives were as follows:

- To discover microbial bio stimulants (products and species) from six countries that have insecticidal properties against *Diabrotica virgifera virgifera* and soil insect pests using Web of Science and CABdirect tools.

- To establish the positive control of commercial insecticides that have a dose-efficacy response on *Diabrotica virgifera virgifera* (eggs, larvae, and adults) under standard laboratory bioassay conditions.

- To gain a better understanding of the breadth and diversity of insecticidal and crop-enhancing effects of microbial bio stimulants on *Diabrotica virgifera virgifera* eggs, larvae, and adults by conducting experiments under both laboratory and greenhouse conditions.

2. MATERIALS AND METHODS

1.1 Discovering microbial bio stimulants with insecticidal properties against *Diabrotica virgifera virgifera*: A review

In this study, we reviewed bio stimulants from all microbial plant bio stimulants registered in six countries: Hungary (NEBIH, 2020), Switzerland (Federal Office of Agriculture, 2020), Spain (Ministry of Agriculture, Fisheries and Food (MAPA), 2020), France (MFSC (matières fertilisantes et supports de culture): préparation fongique/préparation bactérienne, 2020), Indonesia (Directorate of Fertilizers and Pesticides, 2020), and Canada (Canadian Food Inspection Agency, 2020).

For each product, we recorded the microorganism species, orders, families, product trade names, and usage where available. After extracting all microbial plant bio stimulants, we reviewed each microorganism for its potential effects on insects. We utilized the literature databases of CAB Direct (1917 to 2020) and Web of Science (1973 to 2020). We searched abstracts of scientific publications for information on the effects of microorganisms using specific search terms.

1.2 Discovering microbial bio stimulants with potential effect to soil insect pest: A review

In this study, we listed 483 microbial plant bio stimulants registered in Hungary, Switzerland, Spain, France, Indonesia, and Canada (Tarigan et al., 2022). We reviewed each of the 245 identified microorganisms for their potential direct or indirect effects on soil insect pests, including rootworms, which are significant pests in the genus Diabrotica. Our review used the

literature databases of CAB Direct (CABI, 2022) and Web of Science (Clarivate, 2022).

1.3 Establishing the positive control of commercial insecticides that had dose-efficacy response on *Diabrotica virgifera virgifera* under standard laboratory

In this study, seven commercially available insecticides were tested in standard screening assays to determine their dose-responses on different life stages of *Diabrotica virgifera 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.

We examined the effects of seven insecticides at 5 or more concentrations on eggs, larvae, and adults. All insecticides were diluted in sterile tap water to the required doses. Doses in µg per ml and µg per experimental arena are presented in Table 1. 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 virgifera virgifera* under standardized laboratory conditions.

There are three experimental repetitions per treatment and dose for egg bioassay, there are two to five experimental repetitions per treatment and dose for larvae and adult bioassays.

Insecticide group	**Active ingredients**	**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	Reldan 22EC	225 mg/ml 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	Confidor 200SL	200 mg/ml 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	Mospilan 20SG	200 mg/g	granule	0.075--50	0.002-2	0.075-50
					0.0015-1	0.00004-0.04	0.003-20.0001-0.06
					0.00075-0.5	0.0001-0.1	
	Clothianidin	Poncho 600FS	600 mg/ml	liquid	1-10000	0.06-6000	7.5-2000
					0.02-200	0.0012-120	0.3-80
					0.01-100	0.003-300	0.07-3
Insect growth regulators	Novaluron	Rimon 10SC	100 mg/ml	liquid	0.1-10000	1-5000	7.5-75000
					0.002-200	0.02-100	0.3-3000
					0.001-100	0.05-250	0.01-100
Spinosyns	Spinosad	Laser Duplo	480 mg/ml	liquid	0.1-1000	0.02-20	7.5-75000
					0.002-20	0.0004-0.4	0.3-3000
					0.001-10	0.001-1	0.07-100
Pyrethroids	Cypermethrin	Supra 50EC	50 mg/ml	liquid	0.1-10000	0.08-8000	7.5-750
					0.002	0.0016	0.3-30

		-200 0.001 -100	-160 0.0008 -80	0.01-1
Sherpa 100EC	100 mg/ml liquid	0.1-1000 0 0.002 -200 0.001 -100	0.08-8000 0.0016 -160 0.0008 -80	7.5-750 0.3-30 0.01-1

For rearing *Diabrotica virgifera virgifera*, we obtained a non-diapause laboratory colony from USDA-ARS Laboratories (Brookings, SD, USA), reared for around 300 generations. These insects are expected to be susceptible to most pest management agents. The insects were maintained under standardized laboratory conditions at 23-25°C and 40-60% relative humidity. Eggs laid in soil-filled dishes were collected weekly, sieved, and washed with water containing <0.5% NaOCL. They were then stored in sterile river sand at 6-8°C for diapause in dark conditions. Eggs were periodically checked for contamination and kept moist with sterile tap water. Before bioassays, the eggs were incubated at 22-24° C for 7-10 days, then washed and sieved again, making them ready for use

For the egg bioassay, we assessed the effects and dose-responses of commonly used insecticides on eggs using standard screening methods under controlled semi-sterile conditions (Toepfer et al., 2021). Ready-to-hatch eggs were washed and placed on a 100 µm sieve. Treatments were prepared according to product label concentrations. Eggs were transferred using a sterilized stainless-steel spoon to treatment tubes, soaked for 1 hour, and then 20 µl of eggs were pipetted into Petri dishes for treatment.

Eggs were placed in a moist filter paper of Petri dish (150 mm×25 mm) filter paper and then 100 µl of sterilized tap water was added. The pipette tip was replaced between treatments. The eggs that had been transferred were counted per filter paper and dish (1998 ± 325). The eggs were then incubated in the dishes at 24-25^0 C for 7 days, when the experiment was terminated. Egg hatching, larval mortality, and the day until hatching began were recorded at 1, 3, 5, and 7 days.

To evaluate the effects and dose-responses of insecticides on *Diabrotica virgifera virgifera* neonates, we conducted artificial diet-overlay bioassays under controlled semi-sterile conditions. Sterilized tap water was used as the untreated control. Each bioassay included 3 to 6 polystyrene plates, each with 96 wells. The wells had a volume of 330 µl, a diameter of 5 mm, a height of 10 mm, and a surface area of 0.34 cm². Each treatment was applied to 8 wells per plate.

The larval diet for the bioassay was prepared a day before treatment and infestation under semi-sterile conditions (Sutter et al., 1971; Toepfer et al., 2021). The diet included ground maize roots, 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, and chlortetracycline antibiotics. For 100 ml of diet, 13.8 g of ground maize roots were mixed with 88 ml of 60-70°C agar. After blending and cooling to 55-60° C, 0.75 g of ground lyophilized maize roots and 0.1 g of green food color were added. Then, 1.7-1.8 ml of 10% KOH was added to adjust the pH to 6.2-6.5. The mixture was blended again and stirred at 50-55° C. Next, 190 µl of the diet was pipetted into each 330 µl well, filling them about two-thirds full. The plates were dried in a laminar flow cabinet for 45 minutes and stored at 3-5°C overnight. The following day, 17 µl of treatment was applied to the 0.34 cm² diet surface,

ensuring good coverage. Treatments were rotated between plates to avoid edge effects. The plates were dried for 1-1.5 hours and then cooled for 1 hour in a 3-5° C fridge.

A single neonate larva was placed on the diet surface in each well using a fine artist brush. Healthy, fast-moving larvae were selected, lifted by the abdomen, and allowed to crawl off the brush onto the diet. Larvae were placed in a rectangular pattern to avoid systemic errors. After every 12 larvae, the brush was cleaned with 70% ethanol and sterile tap water. The plates were sealed with an optically clear adhesive qPCR seal sheet (#AB-1170, Thermo Scientific, USA or #BS3017000, Bioleader, USA) to allow data assessment without opening the plates. Four to five holes were made in each seal per well using 00-insect pins for aeration. The plates were incubated at 24±2°C and 50-70% relative humidity in the dark in a ventilated incubator for 5 days. Mortality and stunting of larvae were assessed at 3 and 5 days using a stereomicroscope (10×magnification, SMZ-B4, Optec, Chongqing, China) through the clear seals. Data were accepted only if the natural mortality in the untreated control did not exceed 37.5% (no more than 3 dead larvae out of 8 per column). This threshold is higher than the <10% natural background mortality commonly accepted in bioassays with other insects (Dulmage et al., 1990) due to the suboptimal nature of artificial diets for rootworm larvae (Huynh et al., 2018).

To evaluate the effects and dose-responses of commonly used insecticides on adult *Diabrotica virgifera virgifera*, artificial diet-overlay bioassays with varying dosages were conducted under controlled semi-sterile conditions. These standard screening methods, used by many researchers (Toepfer et al., 2005; Parimi et al., 2003), served sterilized tap water as the untreated control. Each bioassay included 6 polystyrene plates, each with 6 wells. Treatments were applied to 3 wells per plate. The adult diet was

prepared 1-7 days before treatment and infestation under semi-sterile conditions, following the methods of Brandson et al., (1971) and Pleau et al., (2002).

For the adult diet, wheat germ and soy flour were used. For a 200 ml batch, 16.5 g of sucrose, 9 g of cellulose, 8 g of casein, 6 g of soy flour, 2.5 g of yeast, 0.6 g of Wesson salt mix, and 0.15 g of cholesterol were mixed with 165 ml of 60-70 °C agar. After blending and cooling to 55-60 °C, 6 g of ground wheat germ, 0.0064 g of chlortetracycline, and 0.0064 g of streptomycin sulphate were added. Then, 5.5 ml of glycerol was added to adjust the pH to 5 at 50-55 °C. The diet was poured into 5-6 sterile 11 mm Petri dishes, dried for up to 15 minutes under a laminar flow cabinet, and stored at 3-5 °C overnight.

The next day, diet cores were transferred using a screw iron and placed into all 6-well plates. Treatments (40 µl) were applied to the surface of each diet core (0.34 cm^3). Adults were then transferred from the rearing cage into the wells using a handheld tube aspirator, after being cooled in a fridge for 4 to 7 minutes to facilitate handling. Each well plate received 3 to 4 adults. The plates were incubated at 24-25 °C for 7 days. Adult mortality was recorded at 1, 3, and 5 days after treatment.

To compare experiments, data were standardized against the corresponding negative control, typically sterilized tap water, using the formula: standardized data = 100 × (data in negative control - data in treatment) / maximum (data in control or treatment). Data distributions were analysed using histograms and QQ normal and detrended normal probability plots. Skewness and kurtosis of residuals were checked for normality. Levene's test was used to assess equality of variances. Multiple comparisons were conducted using the Tukey HSD post hoc test for equal variances and the Games-Howell post hoc test for unequal variances. For each insecticide,

probit, linear, and logarithmic regression models were fitted to the dose-response data, with the best fit evaluated based on p, X^2, and R^2 values. Significant relationships were used to calculate doses causing 50% or 80% of relative effects (ED_{50}, ED_{80}). Statistical analysis was performed using IPM SPSS Statistical 22 software was used (Kinnear and Gray, 2000).

1.4 Assessing the effect of microbial bio stimulants on *Diabrotica virgifera virgifera* under both laboratory and greenhouse conditions

1.4.1 Tested commercial microbial bio stimulants under laboratory conditions

In this study, ten commercially microbial bio stimulants were evaluated using standard screening assays to assess their effects on different life stages (eggs, larvae, and adults) of *Diabrotica virgifera virgifera* under controlled laboratory conditions. The bio stimulants tested included bacterial strains (*Bacillus amyloquafaciens*, *Bradyrhizobium japonicum*, *Bacillus subtilis*, *Ensifer meliloti*, and *Rhizobium leguminosarum*), fungal strains (*Trichoderma asperellum*, *Beauveria bassiana*, *Trichoderma harzianum*, and *Rhizophagus irregularis*), and an algal strain (*Chlorella vulgaris*). Imidacloprid was used as the positive control, and sterilized tap water was used as the negative control. Each bio stimulant was tested at three to six different concentrations, with all solutions prepared by diluting the products in sterile tap water according to the dosage provided on their labels (Table 2). The effects of these bio stimulants on the eggs, larvae, and adults were systematically examined across the specified concentrations.

Table 2. Details of common microbial bio stimulants tested for their insecticidal effects on different life stages of *Diabrotica virgifera virgifera*

under standardized laboratory conditions. The egg bioassay treatments and doses were repeated six times, while the larvae and adult bioassays had three to five repetitions per treatment and dose.

Active ingredients	Trade name	Treatment code	Active ingredient concentration in product	Formulation	Tested dosage range			
					eggs	larvae	adults	unit
Bacillus amyloliquefaciens	CAP ITO BIO	ba	$5x10^9$ spore/ml	liquid	10^2-10^8	10^1-10^8	10^4-10^9	spores/ml
Bradyrhizobium japonicum	Phylazonit NG	bj	$2x10^9$ cfu/ml	liquid	10^2-10^8	10^3-10^8	10^5-10^9	cfu/ml
Bacillus subtilis	AmazoN	bs	$5x10^9$ cfu/g	granule	$2x10^3$-$2x10^7$	10^1-10^8	10^3-10^9	cfu/ml
Ensifer meliloti	RhizoFix ® RF-50	em	$1x10^9$ cfu/ml	liquid	10^2 - 10^8	10^4-10^8	10^5-10^9	cfu/ml
Rhizobium leguminosarum	RhizoFix ® RF-40	rl	$1x10^9$ cfu/ml	liquid	$2x10^2$ - $2x10^6$	10^5-10^9	10^5-$2x10^8$	cfu/ml
Trichoderma asperellum	Hi-SPore	ta	$3.5x10^7$ cfu/g	liquid	10^2 - 10^7	$1x10^3$-$2x10^7$	10^5-10^7	cfu/ml
Beauveria bassiana	Bora R	bb	5 m/m %	powder	10^3 - 10^7	10^3-10^6	10^3-10^7	cfu/g
Trichoderma harzianum	Tricho immun	th	$2x10^8$ cfu/g	powder	10^2 - 10^8	10^3-10^7	10^3-10^7	cfu/g
Rhizophagus irregularis	Lalrise® Max	ri	2000 spore/g	powder	$4x10^2$ - $2x10^3$	$2x10^1$-$2x10^7$	$2x10^1$-$2x10^3$	spore/g
Chlorella vulgaris	Bioplasm algatragy	cv	$2x10^7$ cell/ml	liquid	10^2 - 10^7	10^3-10^7	10^5-2	cell/ml

	a						x10^7	
Imidacloprid	Confidor 200SL	i	200 mg/ml	liquid	0.1-10000	0.02-20	7.5-7500	µg/ml
Untreated control (sterelized tap water)		uc						

For the egg bioassay, we assessed the effect of microbial bio stimulants on eggs using standard screening methods under controlled semi-sterile conditions, as described in section 2.2. For the larvae bioassay, we evaluated the effect of microbial bio stimulants on neonates of *Diabrotica virgifera virgifera* using artificial diet-overlay bioassays under controlled semi-sterile conditions, as detailed in section 2.2. For the adult bioassay, we tested the effect of microbial bio stimulants on adult *Diabrotica virgifera virgifera* by conducting artificial diet-overlay bioassays with varying dosages under controlled semi-sterile conditions, as outlined in section 2.2.

1.4.2 Experimental set up and testing of microbial bio stimulants under greenhouse conditions

To evaluate the insecticidal effects of bio stimulants on *Diabrotica virgifera virgifera* larvae, a systematic controlled trial (SCT) was carried out using plastic cups and plants under semi-natural conditions within a glass greenhouse. The experiment spanned approximately 41 days. 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 3).

Each treatment consisted of 20 plastic cups: 10 cups inoculated and infested with *Diabrotica virgifera virgifera* larvae, and 10 cups inoculated but uninfested. This resulted in a total of 20 data points per treatment

(sample size). The experiment was replicated once (1 true replicate), with a total of 2 replicates conducted. Maize was sown individually in 0.5 liters of soil within 1-liter plastic cups (8 cm inner diameter, 14 cm height). Initially, each plastic cup was filled with 0.5 liters of soil. One maize seed was placed in each cup, followed by the application of 20-40 ml of water. Treatments were administered using a liquid pipette directly onto the soil surface around the maize seed. Subsequently, an additional 0.5 liters of soil was added, burying the treatment and seeds 3 cm deep, resulting in a soil surface diameter of 9 cm within the plastic cup.

The soil used in this study was black clay loam field soil, without any added garden soil (pure soil only). Temperature and relative humidity were monitored using a standard temperature tool, maintaining conditions at 21-25 °C and 55% relative humidity in the greenhouse. Plants were watered with 90-100 ml of water per week, averaging 20-30 ml per week, totalling 0.3 liters over the experimental period. Approximately 100 eggs, ready to hatch, were transferred to the plants at the 3 or 4 leaf stage, three weeks after sowing. The eggs were placed in 5-10 cm holes made in the soil. To prepare the eggs, 0.2 g of agar powder was dissolved in 1 liter of water to create a dilution. A 100 µl aliquot of this egg dilution was pipetted onto filter paper in a Petri dish, and the eggs were counted under a stereomicroscope. The filter paper was then folded and placed over the hole next to the maize seed, and water was slowly dripped onto the filter paper until all the eggs had entered the hole. The hole was then gently covered until no eggs were visible on the soil surface.

Seed germination was recorded 3 days after sowing, while plant height, leaf number, and shoot length were measured 3 weeks post-planting. At 5 weeks (28 days), root length, fresh root weight, root damage, fresh root volume, above-ground biomass, and the number of living larvae were

assessed. The effects of microbial bio stimulants were evaluated at the second and early third instar stages (5-7 leaf stage). Each maize plant was carefully removed from the soil, shaken to remove loose soil, and cut 1 cm above the roots to measure fresh weight, leaf number, and plant height. Soil from each cup was dried on a plastic screen to allow larvae to emerge onto wet tissue paper, following the Berlese method. Larvae were counted after 1 and 3 days. The untreated control aimed for at least 20% infestation with second or third instar larvae, and larvae were recovered from 100% of the infested pots in the untreated control.

Table 3. Specifications of commercial microbial bio stimulants tested for their insecticidal effects on *Diabrotica virgifera 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	$5x10^9$; 10^7;10^9 cfu/g
Ensifer meliloti	RhizoFix® RF-50	em	$1x10^9$ cfu/ml	liquid	10^9 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	10^5 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	2ml/L

3. RESULTS

3.1 Microbial bio stimulants with insecticidal properties against *Diabrotica virgifera virgifera*: A review

Our review identified 483 different products and 245 microorganisms registered as microbial plant bio stimulants in Hungary, Switzerland, Spain, France, Indonesia, and Canada. On average, each country had 181±157 products and 64±27 species

Among the products, 82% contained bacteria (133±106 products), 63% contained fungi (77±59 products), and 14% contained protists, including algae (2324 products). Approximately one-third of the products were mixtures of bacteria, fungi, and/or protists, and 48% contained more than one microorganism. Around 53% of the products (137±121) included microorganisms known for their insecticidal properties, covering 36% of the species (23±9), although the underlying mechanisms are often unknown. Additionally, about 67% of the products (149±133) contained microorganisms reported to protect plants from insects, representing 54% of the species (35±10).

We found that the most common bio stimulant 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*.

3.1 Microbial bio stimulants with potential effect for soil insect pest control: A review

Our reviewed revealed that many microorganisms found in commercial plant bio stimulants may also affect soil insect pests, according to scientific

literature. Approximately 44% of microorganisms (19±6 species) registered as microbial plant bio stimulants were reported to affect soil insect pests, which corresponds to about 30% of commercial products (103±86 products). Bacterial bio stimulants that affect soil insect pests include strains of *Bacillus thuringiensis* and *Pseudomonas fluorescens*. Among the most frequently used fungi with reported effects on 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*.

Regarding rootworms, 9% of microorganisms (6±2 species) registered as bio stimulants have been reported in the literature to affect rootworms, mostly through indirect effects. This corresponds to about 20% of commercial products potentially impacting rootworms (41±46 products). Most of these microorganisms are bacteria (3±1 species, or 6%; 16±13 products, or 11%), followed by fungi (3±2 species, or 1%; 29±35 products, or 33%). 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*.

3.2 Positive control of commercial insecticides tested against *Diabrotica virgifera virgifera* under standard laboratory bioassay

In general, we observed a dose-response relationship for all insecticides tested against *Diabrotica virgifera virgifera*, including eggs, neonate larvae, and adults. Dipping assays with ready-to-hatch eggs showed that several ingredients caused mortality; but imidacloprid and clothianidin are the 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.

3.3 Effect of microbial bio stimulants on *Diabrotica virgifera virgifera* life stages under laboratory

Our results revealed that among the ten bio stimulants tested, 10% had a positive effect on *Diabrotica virgifera virgifera* eggs with 40% of bio stimulants having insecticidal effects on *Diabrotica virgifera virgifera* larvae including *Beauveria bassiana, Rhizophagus irregularis, Trichoderma asperellum* (all fungi) and *B. japonicum* (bacterium). None of bio stimulants affected *Diabrotica virgifera virgifera* adults.

3.4 Effects of microbial bio stimulants on *Diabrotica virgifera virgifera* second instar larvae under greenhouse conditions

Our results revealed that among the ten bio stimulants tested, 20% of bio stimulants tested promoted maize growth without *Diabrotica virgifera virgifera* larvae infestation, particularly *B. japonicum and Ensifer meliloti* and 30% of bio stimulants (*Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Ensifer meliloti*) positively enhanced plant defense against *Diabrotica virgifera virgifera* larvae assessed by IOWA root damage scale.

4. CONCLUSIONS AND OUTLOOK

In conclusion, our reviewed work revealed that all commercial bio stimulants registered from 6 countries had multiple effects. About 53% of products (137±121) contained microorganisms 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 bio stimulant 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*.

Additionally, 44% of microorganisms (19±6 species) were reported to affect soil insect pests these include bacterial strains such as *Bacillus thuringiensis* and *Pseudomonas fluorescens* and fungal strains like *Rhizophagus irregularis* (syn. *Glomus intraradices*, *Rhizophagus intraradices*, *G. irregulare, Rhizoglomus irregulare, G. irregular*), *Glomus mosseae* (syn. *Funneliformis mosseae*), and *Beauveria bassiana*. Growers should be aware of the multiple effects of microorganisms in bio stimulants. It is important to note that these effects often depend on the specific strain of the microorganism, which is frequently not mentioned on the product label or in the scientific literature.

For positive control in insecticide use, scientists or researchers can use imidacloprid or clothianidin for egg bioassays of *Diabrotica virgifera virgifera*, as they show a strong dose-response in reducing egg hatching and causing mortality in hatching neonates. Imidacloprid is also suitable for larvae bioassays of *Diabrotica virgifera virgifera* due to its effective dose-

mortality response and sublethal effects. Acetamiprid is recommended for adult bioassays of *Diabrotica virgifera virgifera* because of its optimal dose-mortality response. The provided ED_{50} and ED_{80} values, along with dose-response equations, offer valuable insights for researchers. These metrics help in selecting suitable positive controls for screening new crop protection agents or assessing resistance levels across different life stages of this pest.

In the lab, 10% of bio stimulants tested positively affected *Diabrotica virgifera virgifera* eggs, while 40% had insecticidal effects on larvae, including fungi like *Beauveria bassiana*, *Rhizophagus irregularis*, *Trichoderma asperellum*, and *the bacterium B. japonicum*. No bio stimulants affected adults. In the greenhouse, 20% of bio stimulants (50% of bacterial ones) promoted maize growth without larvae infestation, particularly *B. japonicum* and *Ensifer meliloti*. Additionally, 30% enhanced plant defence against larvae, including *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *E. meliloti*. These results suggest that these bio stimulants could boost maize resilience against *Diabrotica virgifera virgifera*, supporting sustainable pest management. Further research and field trials are needed to optimize their use.

5. NEW SCIENTIFIC RESULTS

- I found that approximately 53% of bio stimulant products (137±121) and 36% of species (23±9) contained microorganisms reported to have insecticidal properties. Additionally, 67% of products (149±133) and 54% of species (35±10) contained microorganisms were reported to enhance plant defences against insects.

- I found that around 44% of microorganism-based bio stimulants (19±6 species) were reported to have an impact on soil insect pests.

- I identified bio stimulants with reported insecticidal effects, including strains of *Rhizophagus irregularis*, *Bradyrhizobium japonicum*, *Rhizobium leguminosarum, Bacillus megaterium*, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *Penicillium bilaiae*, *B. pumilus*, and *Ascophyllum nodosum*.

- I identified bacterial bio stimulants reported to affect soil insect pests, including strains of *Bacillus thuringiensis* and *Pseudomonas fluorescens*. Additionally, fungal bio stimulants included strains of *Rhizophagus irregularis* (syn. *Glomus intraradices*, *Rhizophagus intraradices*, *G. irregulare, Rhizoglomus irregulare, G. irregular*), *Glomus mosseae* (syn. *Funneliformis mosseae*), and *Beauveria bassiana*.

- I have assessed that imidacloprid and clothianidin are suitable positive controls for egg bioassays, imidacloprid

is suitable for larvae bioassays, and acetamiprid is suitable for adult bioassays.

- I found that 10% of the bio stimulants tested positively affected *Diabrotica virgifera virgifera* eggs. Additionally, 40% of the bio stimulants tested exhibited insecticidal effects on *Diabrotica virgifera virgifera* larvae, including the fungi: *Beauveria bassiana*, *Rhizophagus irregularis*, *Trichoderma asperellum*, and the bacterium: *Bradyrhizobium japonicum*.

- I discovered that 20% of the bio stimulants tested (including 50% of the bacterial ones) enhanced maize growth in the absence of *Diabrotica virgifera virgifera* larvae infestation, with *Bradyrhizobium japonicum* and *Ensifer meliloti* showing particularly notable effects.

- I discovered that 30% of the bio stimulants (all bacteria: *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Ensifer meliloti*) positively enhanced plant defence against *Diabrotica virgifera virgifera* larvae, as assessed by the IOWA root damage scale.

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7. PUBLICATIONS AND SCIENTIFIC ACTIVITIES

6.1 Peer-reviewed papers (In English)

TARIGAN, SI., TOTH, S., MARK, S., JOSZEF, K., TUROCZI, G., STEFAN, T. (2022). Biological control properties of microbial plant bio stimulants. *Biocontrol Science and Technology* 32:12, 1351-1371. (DOI: 10.1080/09583157.2022.2129589) (IF 0.46) **(Scopus Q2 & WOS).**

TARIGAN, SI., JOZSEF, K., TURÓCZI, G., NHU PYD., STEFAN, T**.** (2024). Effects of microbial bio stimulants on maize and its pest, the western corn rootworm, *Diabrotica virgifera virgifera*. *Agronomy* (Under revision) (IF 3.3) **(Scopus Q1 & WOS).**

MOHAMMAD, H., RACHMI, M., SUHARTININGSIH, DN., ANKARDIANSYAH, PP., FARIZ, KA., DWI TS., FARCHAN, MAR., RISMAYANI, **TARIGAN, SI. (2024).** The Potential of Deleterious rhizobacteria to Control Primary Weed *Echinochloa* sp. on Rice. *Biosaintifika* (DOI:https://doi.org/10.15294/biosaintifika.v16i2.3422) (Accepted, In press) (IF 0.2) **(Scopus Q4 & WOS).**

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5.4 International conference talks

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International and national posters

TARIGAN, S.I., SZALAI, M., TOTH, S., TOEPFER, S. (2021) Biological control properties of biofertilizers. 2nd International Congress of Biological Control. Davos, Switzerland, 26 to 30 April 2021. (Online) (Poster).

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5.5 Awards

2-4 May 2023. IOBC Global Travel Award-IWGO Conference at Nairobi-Kenya under link:(https://iobc-wprs.org/wp-content/uploads/2023/08/IOBC-Global_Newsletter_113_2023.pdf).

September 2020-September 2024. Awarded Stipendium Hungaricum Scholarship-PhD study at Hungarian University of Agriculture and Life Sciences under registration number: SHE-02988-004/2020/

October 2024-April 2025 Awarded Dissertation Scholarship-PhD study at Hungarian University of Agriculture and Life Sciences under educational identification number: 73612737064.

September 2021-September 2024. Awarded LPDP Scholarship-Financial Support for Indonesian PhD students under reference number: RPL/019/BLU/LPDP.