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Effect of bioinsecticides on the grey maize weevil *Tanymecus dilaticollis*

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Abstract: *Tanymecus dilaticollis* is an important pest of maize and sunflowers in eastern Europe. In the present study, we examined the effectiveness of two commercially available bioinsecticides against *T. dilaticollis* adults under laboratory conditions: Naturalis® based on the *Beauveria bassiana* fungus and an azadirachtin-based botanical product NeemAzal T/S®. Our results suggested that adults of the pests are more susceptible to *B. bassiana* than to azadirachtin. Naturalis®, tested at six concentrations (2.3×10^2 – 2.3×10^7 conidia/mL), showed a high lethal effect to *T. dilaticollis* adults, and 100% corrected mortality was observed in the treatments over 2.3×10^5 conidia/mL and 2.3×10^6 conidia/mL for females and males, respectively. NeemAzal T/S®, tested at four concentrations (0.5, 1, 2 and 4%) with two treatments, caused generally low to moderate mortality – 6–44% within 16 days. A field experiment consisted of two sprayings of maize experimental plots with Naturalis® at a rate of 200 mL/0.1 ha and then the recording the number of dead adults of *T. dilaticollis* in the treated and control plots was conducted in north-western Bulgaria. Twenty days after treatment, a significantly higher mean mortality of the pest caused by the mycoinsecticide than in the control variants was registered.

Keywords: maize pest; *Beauveria bassiana*; azadirachtin; mortality; lethal concentration; median lethal time

The grey maize weevil, *Tanymecus dilaticollis* Gyllenhal, 1834 (Coleoptera: Curculionidae), occurs in eastern Europe and south-western Asia (Alonso-Zarazaga et al. 2017). It is a polyphagous species, which is among the most important insect pests of maize and sunflowers in Bulgaria, Romania, Serbia and Hungary (Draganova et al. 2012; Georgescu et al. 2018a). To date, cultural practices and insecticide applications are the most common management strategies for the grey maize weevil control (Tomov & Yordanov 1984; Toader et al. 2016). Recently, as a consequence of the restrictions of the European Commission through Regulation (EU) No 485/2013 for the use of specific neonicotinoid insecticides (clothianidin, imidacloprid and thiamethoxam) and Regulation (EU) No 781/2013 for the use of the pyrazole fipronil, including their use for seed treatment to control early-season pests, the majority of the applied insecticides for *T. dilaticollis* control were withdrawn from the market. To compensate the loss of seed treatments with neonicotinoids and fipronil (CTIF), farmers relied mostly on increasing

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soil and foliar treatments with pyrethroids as the principal insecticide class. Several countries (including Bulgaria) have granted farmers derogations from the EU restrictions for certain CITF products and uses since 2014 (Kathage et al. 2018). The first way of reducing insecticide use in Europe in general, and neonicotinoids in particular, is the proper implementation of the Integrated Pest Management (IPM) strategies proposed by the European Directive 128/2009/EC on the Sustainable Use of Pesticides (Furlan & Kreutzweiser 2015). To date, effective control tools for IPM based strategies on pheromones have not been developed for *T. dilaticollis*, as no pheromone compounds have yet been reported for this pest. Two fungal pathogens, *Beauveria bassiana* (Balsamo) Vuillemin, 1912 and *Metarhizium anisopliae* (Metschnikoff, 1879) Sorokin, 1883 (Ascomycota: Hypocreales) (Draganova et al. 2012; Takov et al. 2013), are known as natural enemies of this species with perspectives of being used for the biological control of this pest.

During the last several years the need of alternatives to CITF in controlling the grey maize weevil and studies in this direction have been increasing (Georgescu et al. 2014a, b, 2015, 2016, 2018b; Ionel 2014; Toader et al. 2017, 2020). The effect of bioinsecticides on the *T. dilaticollis* control is not well known (Toader et al. 2017, 2020).

The aim of this study was to investigate the potential of the two bioinsecticides registered in the EU and their effectiveness against *T. dilaticollis* in laboratory and field bioassays and the possibilities for practical applications in pest control.

MATERIAL AND METHODS

Bioinsecticides

Two commercial bioinsecticides were used during the study: the mycoinsecticide Naturalis® [CBC (Europe) S.r.l., Italy] based on *B. bassiana* (ATCC 74040, containing 2.3×10^7 living spores per millilitre of the product; 0.0185%) and the botanical product NeemAzal T/S® (1% azadirachtin A) (Trifolio–M, GmbH, Germany).

Insects for laboratory bioassays

Overwintered adults of *T. dilaticollis* were collected from the bare soil of an arable field belonging to the Maize Research Institute, Knezha (43°28'48.85"N; 24°3'22.03"E) (north-western Bulgaria) in April–May, 2019. The weevils were maintained in the laboratory at a natural light (L) to dark (D) photoperiod (approximately 14 : 10) and room temperature (23–25 °C). The adults were fed on a mixture of wild plant species from the Poaceae family [*Digitaria sanguinalis* (L.) Scop., *Hordeum murinum* Linnaeus and *Sorghum halepense* (L.) Pers.] collected in the field.

Laboratory bioassays

Bioassay with Naturalis®. The mycoinsecticide Naturalis® was tested at six concentrations, 2.3×10^7, 2.3×10^6, 2.3×10^5, 2.3×10^4, 2.3×10^3 and 2.3×10^2 conidia per mL, independently on male and female weevils to measure the sex differences. The bioassays were carried out by applying 1 mL from each concentration separately on a white filter paper disc covering the bottom of a glass Petri dish (120 × 20 mm) by a micropipette following the method described by Draganova et al. (2012). The filter paper discs in the control variants were treated with 1 mL of distilled water (i.e., no fungus). Due to the relatively high activity of the beetles, groups of eighteen to twenty-two males or females were transferred into each Petri dish. The experiment was replicated three times for each sex and treatment with the following exception – the lowest concentration, 2.3×10^2 conidia/mL, was tested on the female specimens only. A total of 771 adults (415 females and 356 males) were used in this bioassay. The laboratory test was conducted at room temperature (23–25 °C) with an approximate 14 : 10 h (L : D) photoperiod. The insect mortality was recorded at 24-h intervals during 10 days after treatment. The poaceous plants (described above) were available daily for feeding the weevils. Insects were considered dead when no leg or antennal movements were observed when prodded with forceps. The dead adults were removed each day to prevent horizontal contamination. They were placed on a moist filter paper in new Petri dishes (55 mm diameter) for a fungal pathogen exhibition and kept at 24 ± 1 °C.

Bioassay with NeemAzal T/S®. A separate bioassay was conducted in laboratory conditions (see above) in order to determine the potential insecticide effect of the commercial product NeemAzal T/S® against the adults of the grey maize weevil. The product was tested in four concentrations in distilled water – 0.5, 1, 2 and 4% against unsexed adults of the grey maize weevil. As azadirachtin has a higher toxicity by ingestion than by contact (Zanuncio et al. 2016), the amount of bioinsecticide solution (2 mL) was applied uniformly with a pipette on the

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food plants (the leaves of field-collected wild poaceous plant species) for the weevils placed on the filter paper disks in glass Petri dishes (150 × 25 mm). In the control treatments, the same volume of distilled water (2 mL) was applied on the leaves. After application, nineteen to twenty-two unsexed adults were introduced into the Petri dishes. The bioassay was conducted in three replicates and a total of 308 adults were used. Additional applications of NeemAzal T/S® concentrations and distilled water on fresh food plants was performed on the 8th day after the first treatment. Mortality was recorded at 24-h intervals for a total of 16 days. The plant leaves were renewed two days after the treatments, and during the next periods – each day.

Preliminary field bioassay

The field experiment was carried out during the period of 3–22 May 2020 in plots of a freshly ploughed field (1 ha) in Knezha, Bulgaria, where the soil is a typical chernozem (black-coloured soil) (Nankov & Glogova 2011). It started immediately after the maize seeds were planted by a hand planter in the bare soil in the field. The time of the application of the product and rate were according to the recommendations for its use against wireworms in Bulgaria, which are also very important pests of maize (BFSA 2013). Experimental plots of 49 m^2 (7 m length, 7 m width, 10 rows, 0.7 m distance between rows) were arranged in a complete block design. Naturalis® was sprayed with a plastic hand sprayer at a rate of 200 mL/0.1 ha on two different days – immediately after the maize seeds were planted in the field, and two weeks after the first spraying. The control plots were also sprayed twice at the same dates with tap water only. Each treatment was replicated three times. The distance between the plots and blocks was three meters.

The inspections were performed the following day after the spraying; and then the following three and two inspections were performed at 5–7-day-intervals after the first and second treatments, respectively. These observations were performed between 8 and 10 a.m. Two people searched thoroughly for dead *T. dilaticollis* adults in each plot and collected them separately in labelled Petri dishes.

The daily meteorological records on the minimum and maximum air temperature (°C), relative humidity (RH) and precipitation (mm) were obtained from the meteorological station located near the experimental field in Knezha.

Statistical analyses

The mortality data were corrected using Schneider-Orelli's formula. The data were transformed to log (x 1) to normalise the variances and were subjected to an ANOVA. The significant differences among the treatment means were separated using the least significant difference (LSD) test. The data about treatments with Naturalis® were subjected to a factorial ANOVA for the concentration, sex, and interaction effects. The untransformed data are presented in the tables. A probit analysis was used to estimate the concentration lethal to 50% (LC_{50}) and 90% (LC_{90}) of the test individuals, and the median lethal time LT_{50} (time necessary to kill 50% of the test individuals).

The *t*-test was used to detect the mortality differences between the weevils in the treated and control field plots.

A probability $P \leq 0.05$ was accepted as statistically significant and all data analyses were performed using Statistica (version 7.0.).

RESULTS

Laboratory bioassays

Bioassay with Naturalis®. The adults of *T. dilaticollis* were highly susceptible to Naturalis®, and the lethal effect increased with an increasing conidial concentration. Both the concentration ($F_{4, 19} = 11.83$, $P < 0.001$) and sex ($F_{1, 19} = 6.08$, $P = 0.02$) affected the mortality. There was also significant interaction between the concentration and sex ($F_{4, 19} = 3.32$, $P = 0.03$). The corrected cumulative mortalities of female and male specimens in the treatments with different conidial concentrations are shown in figure 1. For all the concentrations, with the exception of the lowest ones (2.3×10^2 conidia/mL and 2.3×10^3 conidia/mL), the mortality was observed from the first day. The mycoinsecticide caused 100% corrected mortality of the exposed weevils when tested at concentrations over 2.3×10^5 conidia/mL and 2.3×10^6 conidia/mL for the females and males, respectively. For both sexes together, there was no significant difference in the mortality between the treatments with concentrations above 2.3×10^4 conidia/mL (Table 1).

The lethal concentration values, LC_{50} and LC_{90}, with their associated 95% confidence intervals are presented in Table 2.

The median lethal time (LT_{50}) for the females and males was concentration dependent with an increased

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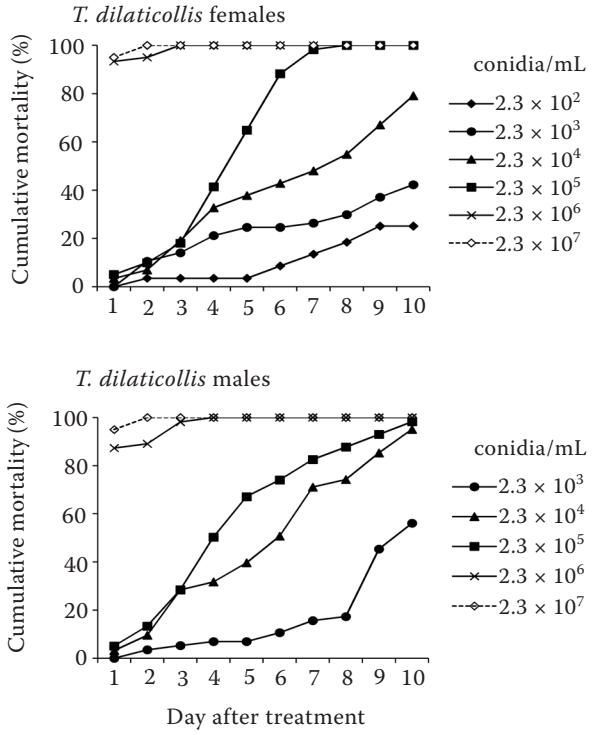

Figure 1. Cumulative mortality (%) of *Tanymecus dilaticollis* adults caused by the different conidial concentrations of Naturalis®

mortality at the exposure time (Figure 2). For the water-diluted treatments of the mycoinsecticide, the LT_{50} values ranged from several hours (males: three hours; females: eight hours) at a concentration of 2.3×10^6 conidia/mL to 16–19 days after exposure at a concentration of 2.3×10^3 conidia/mL. At the highest concentration (2.3×10^7 conidia/mL), Naturalis® had the shortest LT_{50} – a few minutes.

Bioassay with NeemAzal T/S®. The *T. dilaticollis* adults exposed to NeemAzal T/S® in the laboratory conditions showed a delayed initial mortality effect (Table 3). The mortality slightly increased with the concentration after the second treatment. A weak insecticide activity was registered at the lowest concentrations of 0.5 and 1% throughout the 16-d bioassay.

The Neem product had a LC_{50} of 5.7% (confidence intervals of the LC_{50} at 95% probability: 3.9–9.2%). The LT_{50} was 16.22 days for the highest concentration (4%) (confidence intervals: 13.18–20.89 days).

Preliminary field bioassay

Twenty days after the beginning of the test, a significantly higher mean number of dead *T. dilaticollis* adults was recorded in the experimental plots treated with Naturalis® (200 mL/0.1 ha) (0.87 ± 0.22 adults per plot/inspection) in comparison to the control

Table 1. Percent mortality (mean ± SE) of *Tanymecus dilaticollis* adults caused by different suspensions of Naturalis® ten days after treatment under laboratory conditions

Concentration conidia/mL	Percent corrected mortality (mean ± SE)		
	females	males	females and males
2.3×10^2	16.7 ± 8.3^c	–	16.7 ± 8.3^b
2.3×10^3	36.6 ± 13.2^b	43.3 ± 26.9^b	40.0 ± 13.5^b
2.3×10^4	77.4 ± 10.9^a	94.2 ± 3.7^a	85.8 ± 6.4^a
2.3×10^5	100.0 ± 0.0^a	97.5 ± 2.5^a	98.8 ± 1.2^a
2.3×10^6	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a
2.3×10^7	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a

The means within the columns followed by the same letters are not significantly different at $P < 0.05$, ANOVA followed by LSD test for pair-wise comparisons

Table 2. LC_{50} and LC_{90} of different concentrations of Naturalis® against *Tanymecus dilaticollis* adults under laboratory conditions

Sex	LC_{50} conidia/mL	Confidence intervals		LC_{90} conidia/mL	Confidence intervals		Slope ± SE
		lower bound	upper bound		lower bound	upper bound	
Females	2.0×10^3	6.2×10^2	1.1×10^4	4.6×10^4	8.9×10^3	4.9×10^5	0.9 ± 0.2
Males	0.9×10^3	0.3×10^3	5.1×10^3	3.6×10^4	6.3×10^3	4.8×10^5	0.8 ± 0.2
Females and males	1.9×10^3	8.7×10^2	5.8×10^3	5.2×10^4	1.6×10^4	5.8×10^5	0.9 ± 0.1

LC – lethal concentration

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Table 3. Percent mortality (mean ± SE) of *Tanymecus dilaticollis* adults in NeemAzal T/S® treated and control Petri dishes 8 days and 16 days after treatments

Treatment concentration (%)	Corrected mortality (%) (mean ± SE)	
	days after treatment	
	8	16
0.5	12.0 ±1.5^{ab}	17.0 ± 6.5^{b}
1	13.5 ± 4.7^{ab}	23.4 ± 1.8^{b}
2	4.8 ± 4.8^{b}	30.2 ± 4.2^{ab}
4	22.7 ± 9.5^{a}	48.5 ± 9.9^{a}

The means followed by same letter within a column are significantly different ($P < 0.05$, ANOVA followed by LSD test)

plots (0.27 ± 0.12 adults/plot/inspection) (*t*-test, $P = 0.018$).

The average values (± SE) of the minimum air temperature, maximum air temperature, mean air temperature, relative humidity and amount of precipitation during the period of the field test were 10 (± 1) °C, 23 (± 1) °C, 17 (± 1) °C, 69 (± 2) % RH and 4 (±1) mm, respectively.

DISCUSSION

The results from the current study showed that the bioproducts, Naturalis® and NeemAzal T/S®, had a different mortality effect against the *T. dilaticollis* adults of this species. Naturalis® was very effective on both sexes of *T. dilaticollis* in the laboratory conditions - it achieved a high mortality rate with a rapid lethal effect. The highest concentrations of the mycoinsecticide tested (from 2.3×10^5 conidia/mL) resulted in a 97–100% mortality, which is comparable to the high mortality of the grey maize weevil caused by *B. bassiana* isolates from this host applied at 3×10^8 conidia/mL (Draganova et al. 2012). Reddy et al. (2016) evaluated the effect of six commercially available biorational insecticides against *Hypera postica* (Gyllenhal, 1813) (Curculionidae) larvae under laboratory conditions including *B. bassiana* and azadirachtin based products, and showed that

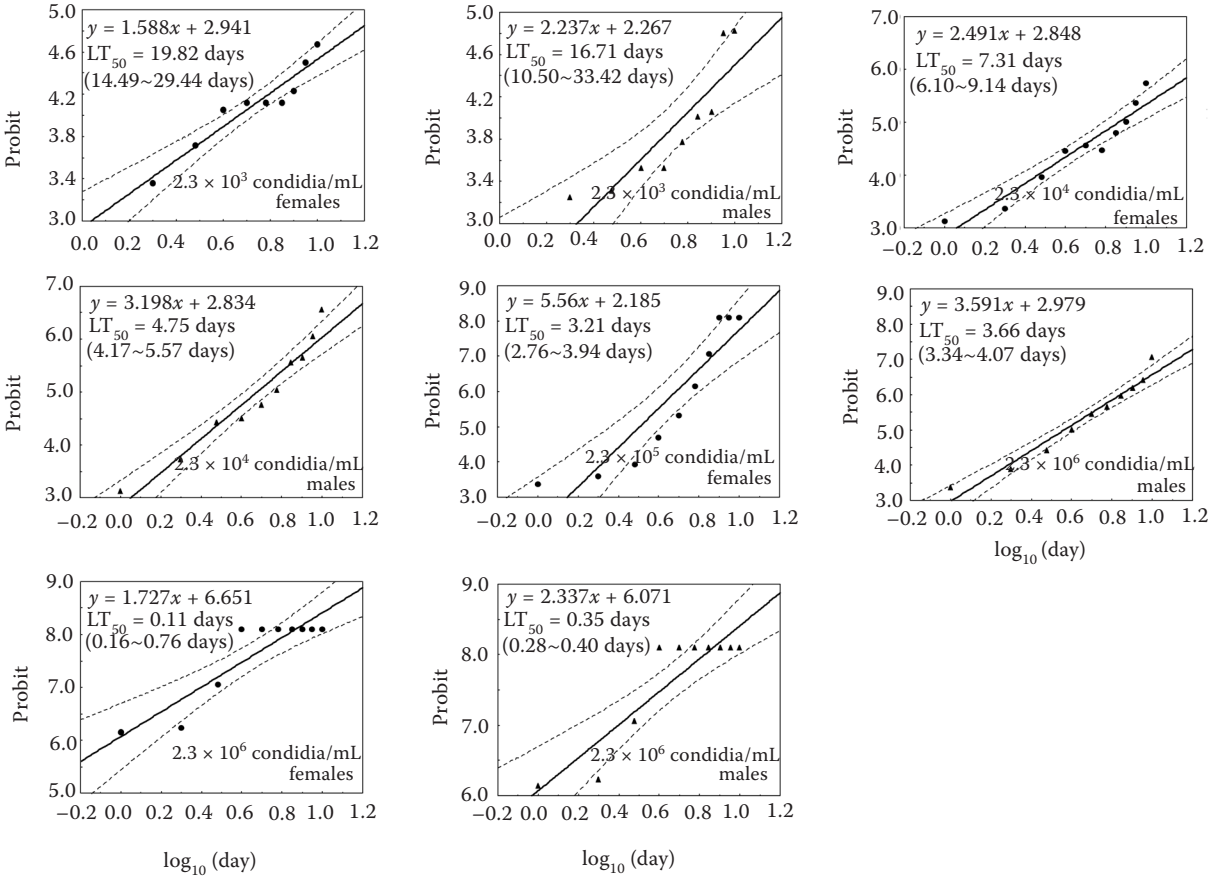

Figure 2. Probit analysis on LT_{50} of Naturalis® treated *Tanymecus dilaticollis* adults at different concentrations
LT_{50}– the median lethal time

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Entrust WP® (spinosad 80%) was the most effective, causing 100% mortality within three days after treatment. However, other products tested also killed 100% of the *H. postica* larvae, but with delayed effect – 5–9 days after the treatments.

Similar to our results, sex differences in the susceptibility to an infection by *B. bassiana* have been previously reported for the Eucalyptus snout-beetle *Gonipterus scutellatus* Gyllenhal, 1833 (Curculionidae) (Echeverri-Molina & Santolamazza-Carbone 2010) while the mortality rates for males and females of *Prostephanus truncatus* (Horn, 1878) (Bostrichidae) treated with the entomopathogenic fungus did not differ (Bourassa et al. 2001).

Gargani et al. (2016) applied Naturalis® in two laboratory experiments with a soil treatment (label dose) and a direct application on adults of the fig tree weevil, *Aclees* sp.cf. *foveatus* Voss, with 1 µL suspension of the product, and recorded 100% and 90% mortality, respectively. The LT_{50} for the first assay was 8.5 days and 7 days for the second one. However, in our study, the LT_{50} of 1 µL suspension of the product showed a shorter lethal time in the range of a few minutes. The LT_{50} values obtained demonstrated that Naturalis® has a high lethal effect to *T. dilaticollis* adults at concentrations above 2.3×10^4 conidia/mL.

In our bioassay with the botanical bioproduct, there was a low to moderate effect of NeemAzal T/S® on the mortality of the *T. dilaticollis* adults up to 8 days (after the first treatment) and 16 days (after the second treatment), respectively. Laboratory studies on the effects of NeemAzal T/S® on the hazelnut weevil, *Curculio nucum* Linnaeus, 1758, showed no mortality of adult individuals in 72 hours (Tuncer et al. 2007). Later research on the efficiency of azadirachtin on the hazelnut weevil by these authors, carried out in semi-field cage experiments, showed that azadirachtin caused 75–86% mortality at different application doses over a 10-day-period. Athanassiou et al. (2005) found that this botanical product was not equally effective against the adults of the stored-grain beetle species *Sitophilus oryzae* (Linnaeus, 1763), *Tribolium confusum* Jacquelin du Val, 1868 and *Rhyzopertha dominica* (Fabricius, 1792) and showed that *T. confusum* was less susceptible after 14 days of exposure at the highest rate (200 ppm of azadirachtin A). Kowalska (2008) showed that NeemAzal-T/S® (concentration 0.5%) applied to the surface of the soil in pots with the larvae of the black vine weevil, *Otiorhynchus sulcatus* (Fabricius, 1775) completely stopped the physiological development of the larvae.

The results of our preliminary field test showed that the aqueous formulation of Naturalis® induced a relatively low, but significantly higher mortality of *T. dilaticollis* adults when compared with the untreated control plots in natural field conditions. Similarly, Paparatti and Speranza (2005) reported a higher effectiveness of a Naturalis® product (99.5%) in comparison with the control (63.5%) against the hazelnut pest *C. nucum* during trials carried out in cages in a hazelnut orchard in Italy. Recently, Toader et al. (2020) reported that seed treatment with a Neem oil (Neem oil, at a dose of 2.5 mL/250 g of seed) has provided relatively good protection for maize plants against *T. dilaticollis*, with an average of 81% saved plants (four-leaf stage) during 2016–2018 in southeastern Romania, while in plots treated with Spinosad (Laser 240 SC, at a dose of 2.5 mL/250 g of seed), 54% *Bacillus thuringiensis* (Bactospeine DF, at a dose of 0.01%) and control (untreated) plots, the values were 79, 75 and 63%, respectively.

Our study with Naturalis® and NeemAzal T/S® was the first one reporting results about the efficacy of commercial bioproducts against the grey maize weevil in Bulgaria. Naturalis® was very effective on both sexes of the pest under laboratory conditions. It achieved a high mortality rate with a rapid lethal effect; however, more promising field bioassays are needed to be conducted to prove its insecticide effect against this pest in field conditions. Further investigations are necessary to test the hypothesis that the infected adults can horizontally transmit the fungal pathogen to uninfected partners during mating (Kreutz et al. 2004). More research is needed to determine the influence of Naturalis® on other insect pests and non-target arthropods in the maize agrocenoses.

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