RESEARCH CONCERNING THE EFFECTIVENESS OF THE ENTOMOPATHOGENIC FUNGI FOR CONTROLLING THE MAIZE LEAF WEEVIL (*TANYMECUS DILATICOLLIS* GYLL) IN THE GREENHOUSE CONDITIONS

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Abstract
Maize leaf weevil (Tanymecus dilaticollis Gyll) is the most dangerous pest of the maize crop in the south and southeast of Romania. Each year there were attacked one million hectares cultivated with maize were. In case of high pest pressure, weevils can destroy maize plants. After the ban on neonicotinoids, no active ingredients are available in Romania for maize seed treatments to control this pest. This study there were evaluated the effectiveness of three entomopathogenic fungi, Beauveria bassiana (strains BbTd, BbTy), B. pseudobassiana (BpPA), and Metarhizium anisopliae (MaF), for controlling the T. dilaticollis weevils*, in the greenhouse conditions, using high pest pressure (4 weevils/plant). It has sowed maize in plastic pots. After the plant's emergence, it added insects collected from the field and it was pulverized solutions with entomopathogenic fungus at each variant. It has assessed phytotoxicity, attack incidence (%), attack intensity on a scale from 1 (plants not attacked) to 9 (plants destroyed), plant heights, and weevils mortality at eight days after infections with entomopathogenic fungi. The conditions of the high pest pressure from the greenhouse it has registered lower weevils mortality. Only in the case of M. anisopliae (MaF) and B. bassiana (BbTy) weevils was mortality higher than 10 %. At all experimental variants from the greenhouse, attack incidence was 100 %, while attack intensity ranged from 8.75 in the control variant to 7.10 in the case of M. anisopliae (MaF) fungus. In this study, there weren't significant statistical differences between control and treated variants concerning both weevils attack intensity and mortality ($p<0.05$).*

***Key words**: maize, weevil, entomopathogenic fungi, control*

INTRODUCTION

Maize leaf weevil (*Tanymecus dilaticollis* Gyll) is one of the main pests of the maize crops in Romania (Paulian *et* al., 1972; Paulian, 1978; Voinescu, 1985; Bărbulescu *et* al., 2001; Cristea et al., 2004; Popov *and* Bărbulescu, 2007; Trotuș et al., 2011, 2019; Georgescu *et* al., 2014, 2018; Badiu *et* al., 2019; Toader *et* al., 2020). If the attack occurs when maize is in early vegetation stages (BBCH 10-BBCH 14), the plants can be destroyed (Roșca *and* Istrate, 2009). The same authors mentioned that in later vegetation stages (BBCH 15-BBCH 16), the pest attack is less economically important, and maize plants survive. According to the data from the literature, maize leaf weevil is a polyphagous pest, with 34 host plants in Romania, exhibiting preferences for maize and sunflower crops, sometimes on soybean, lucerne, or cereals (Paulian, 1972; Paulian et al., 1979; Bărbulescu *and* Voinescu, 1998). The same authors mentioned pea (*Pisum sativum*) is a repellent crop for weevils. The most favorable areas of this pest are in the south and southeast of Romania. Every year, one million hectares cultivated with maize are attacked by this pest, the same as one-half million hectares with sunflower (Popov *et* al., 2004, 2005, 2007a). Recent data from the literature reveal damages made by the weevils in the area considered, until now, less favorable for this pest, such as South Transylvania (central part of Romania) or Botoșani county (North-East of Romania)(Antonie *et* al., 2012; Badiu et al., 2019). Possible explication for increasing the attack of maize leaf weevil in areas considered less favorable is climate changes and global warming (Diffenbaugh *et* al., 2008; Bozo, 2011; Choudhary *et* al., 2019).

Researches made in Romania make in evidence that drought and high temperatures, registered in spring, when maize is in early vegetation stages (BBCH 10-BBCH 14), favored weevils' activity on the ground and feeding process (Paulian, 1972; Popov *et al.*, 2006; Roșca *and* Istrate, 2009). In the last ten years, weather conditions in this pest's favorable areas were atypical (Marin et al., 2014; Georgescu *et* al., 2015; Angearu *et* al., 2020). Research in Romania proves that maize cultivated in monoculture for several years increased the maize leaf weevils population from one year to another and the pest attack at this crop (Bărbulescu *et* Voinescu, 1998; Voinescu *et* Bărbulescu, 1998; Popov *et* Bărbulescu, 2007). The same authors mentioned that in case of short crop rotations or sunflower sowed after maize (both are host plants), maize leaf weevil populations can increase from one year to another. Researches from Romania make in evidence that seed treatment with systemic insecticides, with rapid translocation of the active ingredient in plants after their emergence, was the most effectiveness method for controlling maize pests that attack this crop in the first vegetation stages (BBCH 10-BBCH 14), including maize leaf weevils (Voinescu, 1985; Bărbulescu *and* Voinescu, 1998; Bărbulescu *et* al., 2001; Vasilescu *et* al., 2005; Popov *et* al., 2007b; Georgescu *et* al., 2014, Trotuș *et* al., 2019). Popov *and* Bărbulescu (2007) mentioned that seed treatment is a method with lower impact on environment, compared with foliar or granules application and higher effectiveness in controlling of several crop pest species that can destroyed plants in early vegetation stages. After the ban of the seed treatment and foliar application of the neonicotinoids active ingredients, as a result of the European Commission regulations 218/783, 218/784, and 218/785 (Official Journal of the European Union, 2018a,b,c), no alternative remain available in Romania for maize seed treatment to control maize leaf weevil attack. In the last years, several studies have been made, both in experimental fields and commercial farm conditions, to find alternatives to banned neonicotinoids active ingredients used in maize seed treatment (Georgescu *et* al., 2018, 2019, 2021, 2022; Trașcă F. *et* al., 2019; 2021; Toader *et* al., 2020; Amuza *et* al., 2021; Trașcă G. *et* al., 2021). All the alternatives studied in these researches have lower or zero effectiveness in controlling maize pests when plants are in early vegetation stages, including maize leaf weevil (*T. dilaticollis*). As a result, Romania obtains temporary authorizations for using the neonicotinoids active ingredients (imidacloprid, clothianidin, thiamethoxam) for maize seed treatment to control maize leaf weevil from 2014 to 2022 (MADR data, 2022). In the last decades, much research has been done concerning biological control of the agricultural, horticultural, forest, or stored product pests using entomopathogenic fungus (Storey *and* Gardner, 1988; Wraight *et* al., 2010; Dinu *et* al., 2013; Bradl *et* al., 2017; Fătu *et* al., 2020). Recent studies in laboratory conditions show the susceptibility of some pest species, such as *Plodia interpunctella* or *Calliteara pudibunda* larva, to *Beauveria bassiana* Romanian strains (Fătu *et* al., 2010; 2019). Several studies were made in our country concerning the endophytic potential of an autochthonous strain of *B*. *bassiana* isolated from *Lymantria dispar* pest or the effectiveness of granulated bio-product on the base of *B*. *bassiana* for protection of solanaceous crops (Dinu *et* al., 2016, 2018). In the literature is little information about the biological control of maize leaf weevil (*T. dilaticollis*), mainly from Bulgaria. Draganova *et* al. (2012) report that *B*. *bassiana* and *Metarhizium anisopliae* caused infection and death in weevils, while Takov et al. (2013) mentioned that gregarines (protozoan) and *B*. *bassiana* fungus caused diseases and mortality in *T. dilaticollis* populations. A recent study made in Bulgaria, in laboratory conditions, in Petri dishes, revealed that Naturalis mycoinsecticide, based on *B. bassiana* fungus, shows a high lethal effect (100% mortality) on *T. dilaticollis* males and females at a concentration of $2.3x10^5$ and $2.3x10^6$ conidia/mL. The same author concluded that in the field conditions, after 20 days from the two treatments with the Naturalis mycoinsecticide at a rate of 200 mL/0.1 ha, it registered a significant mean weevils mortality compared with the control (untreated) variant. In a very recent study published in Romanian Agricultural Research, Fătu *et* al. (2023, first online 2022) reveal that Romanian strains of *B. bassiana* (BbTd1) and *B. pseudobassiana* (BbLy) determined high mortality in *T. dilaticollis* population, in the laboratory conditions, in Petri dishes. The same authors mentioned that *B. bassiana* (BbTd1) and *B. pseudobassiana* (BbLy) applied in the field, as conidia multiplied on barley grains, incorporated in the soil, didn't affect the weevil's density in the maize field. This study aims to test the effectiveness of the entomopathogenic fungus (*B. bassiana*, *B. pseudobassiana,* and *M. anisopliae*) in controlling the maize leaf weevil (*T. dilaticollis*) at maize crop. It is the first study with biological control of the maize leaf weevil made in Romania in greenhouse conditions.

MATERIALS AND METHODS

The experience was carried out in the greenhouse at the National Agricultural Research and Development Institute (NARDI) Fundulea, Călărași County, Romania (latitude: 44°46' N; longitude: 26°32' E; alt.: 68 m a.s.l.) in the year 2021. Maize plants were sowed in plastic pots (12x12x10 cm). Each pot has placed five maize seeds at an equal distance (fig. 1). One seed was placed in the centre of the pot, and the other four were close to the corners. Before sowing, the pots were filled ¾ with soil obtained from the areas without chemical treatments. After the seeds were placed in the ground, plastic pots were filled with soil, and then the soil surface from each pot was slightly compressed and soaked with water to ensure the uniform emergence of the plants. Each experimental variant has used four pots, one pot representing a replication. This study has tested one strain of *B. pseudobassiana* (BpPa), two strains of *B. bassiana* (BbTd, BbTy), and one strain of *M. anisopliae* (MaF) (table 1).

Table 1. Experimental variants from the study in greenhouse conditions at NARDI Fundulea

Nr. crt.	Variant (strain)	Specie	Dose (UFC/ml)
1.	Control	—	—
2.	BpPa	*Beauveria pseubassiana*	$1x10^7$
3.	BbTd	*Beauveria bassiana*	$1x10^7$
4.	BbTy	*Beauveria bassiana*	$1x10^7$
5.	MaF	*Metarhizium anisopliae*	$1x10^7$

This experiment used the F423 maize hybrid (F470) created at NARDI Fundulea (Horhocea *et* al., 2020). After the emergence of the maize plants in the plastic pots, it has added 20 weevils in each pot (4 weevils/plant). This way, it has simulated conditions of high pest density from the field. The weevils were collected from the areas without any chemical treatment, located at the Centre for Organic Farming from NARDI Fundulea, and then were maintained inactive in the laboratory for a few days; at 15±2 ºC air temperature and 80-85 % air relative humidity. The weevils must be manipulated carefully.

Figure 1. Sowing maize in plastic pots. Green house assessment (NARDI Fundulea)

Immediately after the weevils were added, the pots were covered with isolators (fig. 2). After a few minutes, each variant it has spraying soil, emerged plants, and weevils with entomopathogenic fungus solution (fig. 3). Before the spraying, it was removed the upper side of the isolators. To avoid contamination, the pots from each variant were placed a few meters from each other. After spraying one variant (four pots), the technician changed the protection equipment. The solutions with entomopathogenic fungus were prepared at the Institute of Research-Development for Plant Protection, Bucharest.

Figure 2. Weevils added in the plastic pot. Green house assessment (NARDI Fundulea)

Assessments: **Phytotoxicity** was assessed for times, at 1, 2, 4, and 8 days after starting the experience, according to EPPO PP1/135 standard (EPPO standards, 2014). The **weevil's mortality** was recorded daily for eight days after starting the experiment in the greenhouse. The dead insects were removed from the pots and put in Petri dishes. At the same time, dead weevils were checked to see if they presented mycosis symptoms resulting from infection with entomopathogenic fungus.

The **attack incidence** was assessed for 1, 2, 4, and 8 days after the experience started.

The **attack intensity** was evaluated after eight days after the start of the experience, when maize plants from the pots arrived in the four leaves stage (BBCH 14), using a scale from 1 (plants not attacked) to 9 (plants destroyed), elaborated by Paulian (1972) and improved by Bărbulescu (1995). The attack intensity scale (fig. 4) is presented, in detail, below:

-note 1: plant not attacked;

-note 2: plant with 2-3 simple bites on the leaf edge;

-note 3: plants with bites or clips on all four leaves edge;

-note 4: plants with leaves chafed in the proportion of 25 %;

-note 5-plants with leaves chafed in the proportion of 50 %;

-note 6-plants with leaves chafed in the proportion of 75 %;

-note 7-plants with leaves chafed almost at the level of the stem;

-note 8-plants with leaves completely chafed and beginning of the stem destroyed;

-note 9-plants destroyed, with stem chafed close to the soil level.

Figure 3. Solution with entomopathogenic fungus, before spraying

Plants' height was measured eight days from the start of the experiment when maize plants arrived in the four leaves stage (BBCH 14). The air temperature was recorded during the greenhouse experiment with Klimalogg.pro data logger. These climatic data were recorded once time every 15 minutes.

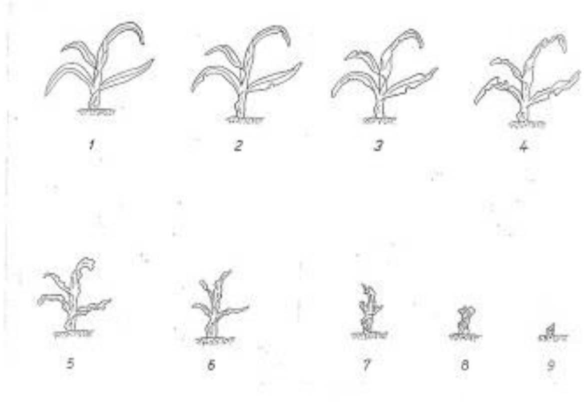

Figure 4. Attack intensity scale (1-9), NARDI Fundulea

The data from greenhouse assessments were **statistically analyzed** with ARM 20xx software using the Student-Newman-Keuls test (Student, 1927; Neuman, 1939; Keuls, 1952). Correlations graphs are made with Microsoft Excel 2003 software.

RESULTS AND DISCUSSIONS

The experiment in the greenhouse conditions at NARDI Fundulea started on 25 May and ended eight days later, on 2 June. Analyzing the temperature during the study, it can be concluded that the maximum temperature was higher than 25 °C, even higher than 29 °C (fig 5).

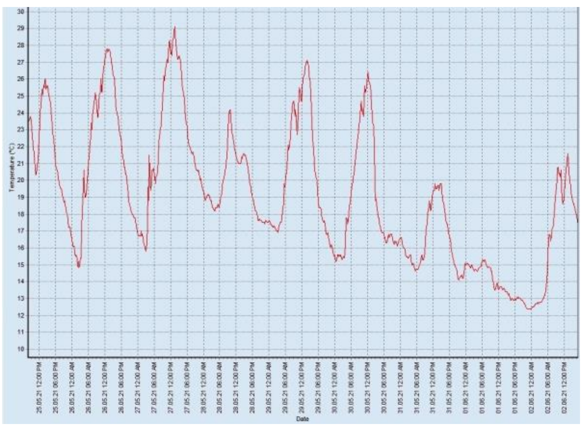

Figure 5. Air temperature during greenhouse experience, at NARDI Fundulea

Generally, the minimum temperature was over 15 °C (except on 26 May). According to Roșca *and* Istrate (2009), weevil activity is favored at temperatures higher than 20 °C during the daytime. Analyzing meteorological data from the greenhouse experiment, these conditions were registered between 25 and 30 May. However, between 31 May and 2 June, air temperatures in the greenhouse decreased below 20 °C. As a result, weevils' activity on the ground and feeding processes were reduced. The most favorable conditions for weevil activity were registered in the first five days from the start of the experiment.

Table 2. Phytotoxicity (%)
Greenhouse experiment at NARDI Fundulea

Variant (strain)	Phytotoxicity (%)			
	1 day*	2 days	4 days	8 days
Control	0a	0a	0	0a
BpPa	0a	0a	0a	0a
BbTd	0a	0a	0a	0a
BbTy	0a	0a	0a	0a
MaF	0a	0a	0a	0a
LSD (P=0.05)	0	0	0	0
Standard deviation (SD)	0	0	0	0
Variation coefficient (CV)	0	0	0	0

*days after starting of the greenhouse experiment
Means followed by the same letter do not significantly differ
(P=0.05, Student-Newman-Keuls test)

During this experiment, in the greenhouse conditions, treatments with one strain of *B. pseudobassiana* (BpPa), two strains of *B. bassiana* (BbTd, BbTy), and one strain of *M. anisopliae* (MaF) are safe for maize plants, in first vegetation stages (BBCH 10-BBCH 14). No phytotoxic effect occurred (tab. 2). In this experience, it registered lower daily weevils mortality (fig. 6). At *B. pseudobassiana* (BpPa) strain, first mortalities were recorded at four and five days after the starting of the experience (3.75 %). At the *B. bassiana* (BbTd) strain, first weevils mortality was recorded five days after the start of the experience and then had an increasing tendency. However, the maximum daily mortality in this variant was below 4 %. In the case of the *B. bassiana* (BbTy) strain and *M. anisopliae* (MaF) strain, it has recorded maximum daily weevils mortality at three days from the start of the greenhouse experiment (6.25 %).

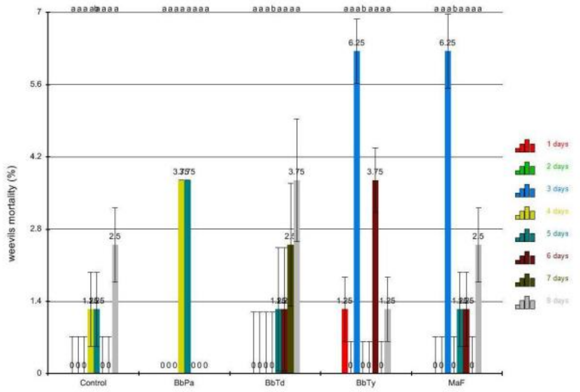

Figure 6. Daily weevils mortality (%) in the green house experiment, at NARDI Fundulea

At the end of the greenhouse experiment, eight days after the introduction of the weevils in the plastic pots with maize plants and spraying with entomopathogenic biopreparates, weevil mortality was low. Only in the case of *B. bassiana* (BbTy) and M. *anisopliae* (MaF) strains the weevils mortality was higher than 10 % (table 4).

Table 3. Weevils attack incidence in the greenhouse experiment at NARDI Fundulea

Variant (strain)	Attack incidence (%)			
	1 day*	2 days	4 days	8 days
Control	100a	100a	100a	100a
BpPa	100a	100a	100a	100a
BbTd	100a	100a	100a	100a
BbTy	100a	100a	100a	100a
MaF	100a	100a	100a	100a
LSD (P=0.05)	0	0	0	0
Standard deviation (SD)	0	0	0	0
Variation coefficient (CV)	0	0	0	0

*days after starting of the greenhouse experiment
Means followed by the same letter do not significantly differ
(P=0.05, Student-Newman-Keuls test)

According to Student-Newman-Keuls (SNK) test, there weren't significant statistical differences concerning weevils mortality at the control (untreated) variant and treated variants ($p<0.05$). A possible explanation is that the fungus period that can affect weevils is too low. In the experiences made in a laboratory, using Petri dishes, direct exposure of the weevils to *B. bassiana* fungus causes high mortality in the weevils in a short period (Toshova *et* al., 2021). The Romanian research team arrived at the same conclusions (Fătu *et* al., 2023, first online, November 2022). However, in this experiment, made in the greenhouse, the suspension with conidia applied on the plants, weevils and soil immediately after the weevils were added to the pots wasn't effective in controlling this pest. Another possible explication is weevil behaviour. Not all the insects stay at the soil surface, on the pots, after their introduction. Some were hidden in the soil cracks. This is the maize leaf weevil's natural behaviour (Paulian *et* al., 1972). Another possible explication is that higher temperatures registered in the first five days from the start of the experience didn't favour entomopathogenic fungus spores germination.

Table 4. Weevils attack incidence in the greenhouse experiment at NARDI Fundulea

Variant (strain)	Attack intensity (1-9)	Weevils mortality* (%)
Control	8.75a	5.00
BpPa	7.60a	7.50
BbTd	8.65a	8.75
BbTy	7.45a	12.50
MaF	7.10a	11.25
LSD (P=0.05)	1.471	1.312
Standard deviation (SD)	0.955	0.851
Variation coefficient (CV)	12.070	47.300

*weevils mortality at the end of the experience
Means followed by the same letter do not significantly differ (P=0.05, Student-Newman-Keuls test)

Data from the literature reveal that *for B. bassiana* fungus development, the optimum temperature is between 23 and 28 °C, the minimum temperature is between 5 and 10 °C, and the maximum temperature is between 30 and 38 °C (Fătu *et* al., 2020).

The same authors mentioned that optimal temperatures for conidia germination of *M. anisopliae* fungus are very different and varied with regions where the different strains originated. As a result, the optimal temperature for *M. anisopliae* conidia germination has a wide variation, from 2.5 to 37 °C. Analyzing the temperatures during the greenhouse experiment can ascertain a variation of this parameter between 15 and 27 °C. Another critical factor for entomopathogenic fungus evolution is soil humidity. According to Luz *and* Fargues (1997), cited by Fătu *et* al. (2020), conidia of *B. bassiana* germinate at 90 % humidity. However, these conditions are not registered in the most favorable area of *T. dilaticollis*, the south, and southeast of Romania (Popov *et* al., 2006).

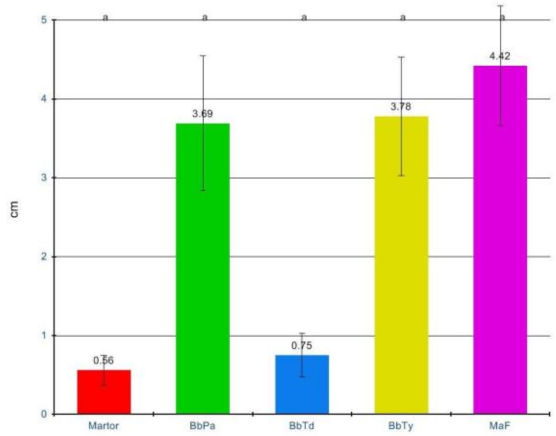

Figure 7. Plants height at 8 days from start of the green house experiment, at NARDI Fundulea

Regarding **attack incidence**, data from table 3 show that all maize plants from plastic pots were attacked by the weevils (attack incidence 100 %). The insect started feeding with maize immediately after they were added to the pots. The activity of the weevils was favored by high temperatures, especially in the first five days after the start of the experiment.

Data from table 4 reveal that weevils' attack intensity, registered when maize was in the first vegetation stages, on a scale from 1 (plants not attacked) to 9 (plants destroyed) was almost maximum at the control variant.

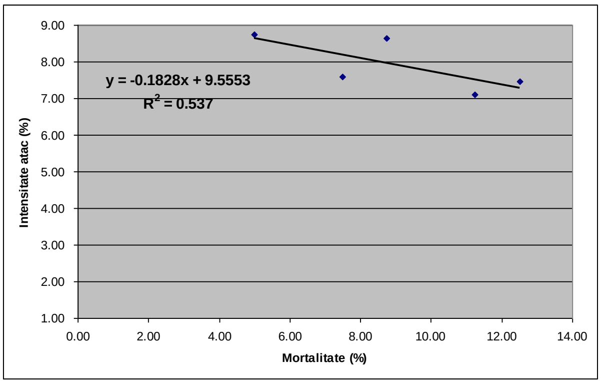

Figure 8. Relation between weevils mortality and attack intensity at greenhouse experiment

The lowest values of the attack were registered in the case of variants BpPa, BbTy, and MaF, where attack intensity ranged from 7.10 to 7.60, plants were almost destroyed in these cases too, and only a few could recover after the attack.

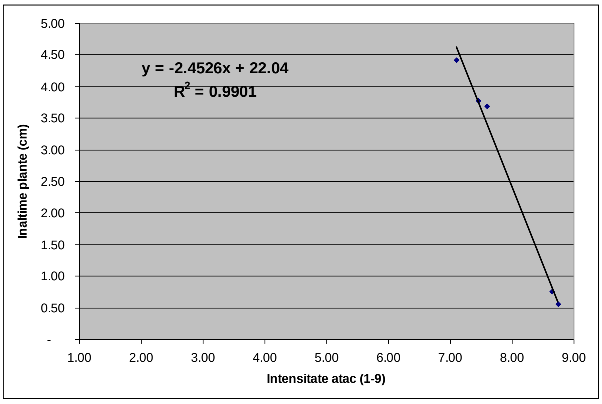

Figure 9. Relation between weevils attack intensity and plants height at greenhouse experiment

According to the SNK test, it hasn't registered significant statistical differences between weevils' attack intensity at maize plants from the control variant and weevils' attack intensity at the treated variants ($p<0.05$). Plants' height at eight days from the start of the greenhouse experiment, when maize was in the four leaves stage (BBCH 14), have low values due to high weevils attack intensity. According to the SNK test, it hasn't registered significant statistical differences between plants' height in the untreated variant and plants' height in the treated variant (fig. 7). In the greenhouse experiment, it has registered a negative correlation between weevil's mortality and weevils attack intensity and negative correlation between weevils attack intensity and plants height (fig. 8 and 9). In conditions of high pest pressure, from the greenhouse, at NARDI Fundulea, in the spring of 2021, only two weevils from a total number of 400 used in this experience present symptoms of infection with entomopathogenic fungus. Further researches are necessary, both in the greenhouse and field conditions, to find the most effective formulation of bioinsecticides based on the entomopathogenic fungus for controlling maize leaf weevils and protecting maize plants in the first vegetation stages in harsh conditions from the favorable pest area in Romania (drought and high temperatures).

CONCLUSIONS

In the greenhouse experiment, in the spring of 2021, at NARDI Fundulea, in the conditions of high pest pressure (4 weevils/plant) spraying with a solution on the base of entomopathogenic fungus *B. pseudobassiana* (strain BpPa), *B. bassiana* (strains BbTd and BbTy) or *M. anisopliae* (strain MaF) wasn't effective in the protection of the maize plants, in early vegetation stages (BBCH 10-BBCH 14), against maize leaf weevil attack.

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