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# EVALUATION OF *Beauveria bassiana* AND *Beauveria pseudobassiana* AGAINST *Tanymecus dilaticollis*

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## ABSTRACT

**The grey corn weevil, *Tanymecus (Episomecus) dilaticollis* Gyll., (Curculionidae: Entiminae) is the most destructive pest of maize and sun flower crops in Romania. In this article we report result of evaluation of native strains of entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. (Deuteromicotina: Hyphomycetes) and *B. pseudobassiana* Rehner et Humber against *Tanymecus dilaticollis* (Curculionidae: Entiminae) both in laboratory and field. In laboratory assay, two different strains of *B. bassiana* and one strain of *B. pseudobassiana* (BbLy) were applied on insects as $1\times10^8$ conidia/ml aqueous suspensions. The pure ATCC 74040 commercial strain of *B. bassiana* and the commercial mycoinsecticide based on this strain (Naturalis) were included in the laboratory assay for comparison. Adult mortalities were recorded daily, 14 days post-exposure. All the fungal strains have been shown to be pathogenic to *T. dilaticollis*. The *B. bassiana* strain (BbTd1) and the *B. pseudobassiana* strain (BbLy) were effective against adults of *T. dilaticollis* and comparable in percentage of mycosis and virulence to the *B. bassiana* strain ATCC 74040. The commercial product Naturalis was superior to the tested fungal strains killing the insects within a day. In the field, the strains BbTd1 and BbLy applied as conidia multiplied on barley grains ($1\times10^9$/g d.w) in the soil did not affect the *T. dilaticollis* density in maize crop.**

**Keywords:** *Beauveria bassiana*, *B. pseudobassiana*, *Tanymecus dilaticollis*, maize.

## INTRODUCTION

Maize or corn (*Zea mays*) is an important commercial crop in Romania, in the last year being the largest cultivated area and with the biggest production in the EU (Brodeală et al., 2022). Maize leaf weevil, *T. (Episomecus) dilaticollis* Gyll., (Curculionidae: Entiminae) is a major pest of maize in south, south-east and east of Romania, where 1 million ha of maize are attacked every year to varying degrees of intensity (Georgescu et al., 2018; Georgescu et al., 2021a, b). In Europe *T. dilaticollis* was also recorded in Turkey, Bulgaria, Greece, Serbia, Croatia, Hungary, Slovakia, Ukraine, Moldavia, Austria (Roşca and Istrate, 2009; Georgescu et al., 2014; Schuh et al., 2015; Yunakov et al., 2018) but the most severe economic damages are to maize and sunflower crops, in Romania, Bulgaria, and Serbia. Evidence from the literature shows that maize is attacked preferentially in all locations, followed by sunflower and wheat. At maize, the pest commonly feed on young plants at ground level, cutting of the stem and often causing the mass destruction of culture or consuming only the lateral parts of the leaf blade and delaying the vegetative growth and significantly decreasing the yield.

The management of the grey corn weevil, *T. dilaticollis* in Romania has become more and more difficult on the one hand because of the restrictions for pesticides and the lack of alternatives to the methods of chemical control and on the other hand because of the climatic conditions increasingly favorable for the multiplication of this pest. Further

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reasons include the increase in the areas of maize grown in a monoculture system due to the economically attractive market (Lup et al., 2013; Georgescu et al., 2014). In Romania, the most effective method of controlling this pest is by treating the seed with systemic insecticides from the class of neonicotinoids (Georgescu et al., 2014, 2018, 2021a, b; Trotuş et al., 2011, 2019). Starting with 2019, European directives have banned the use of treatments in seeds and vegetation with neonicotinoids. In Romania, they are still widely used through the derogations offered by the Member States. However, it is necessary to find alternative control materials and methods. Data from the literature about the natural enemies of the *T. dilaticollis* are very rare. Fungal pathogens causing mycosis to adults of the grey corn weevil were identified as *Beauveria bassiana* and *Metarhizium anisopliae* by Draganova et al. (2012). Also, Takov et al. (2013) reported rates of *B. bassiana* mycosis at epizootic level and also protozoan infection in *T. dilaticollis* populations.

In this study we assessed the virulence of some native indigenous strains of *Beauveria* recovered from insects to adults of grey corn weevil, *T. dilaticollis* in laboratory and field bioassays.

## MATERIAL AND METHODS

### Fungal material

In laboratory, two native strains of *B. bassiana* (Bals.) Vuill. (Deuteromicotina: Hyphomycetes), one native strain of *B. pseudobassiana* and one commercial bioinsecticide Naturalis® [CBC (Europe) S.r.l., Italy] based on *B. bassiana* (ATCC strain 74040, containing $2.3\times10^7$ conidia/ ml, originating from United States) were tested in order to establish pathogenicity against *T. dilaticollis*. Fungal strains originated from dead adults of *T. dilaticollis* (BbTd1 and BbTd2) and a pupa of *Lymantria dispar* L. (BbLy). The isolates were obtained in pure cultures and maintained in RDIPP collection of entomopathogenic fungi on PDA medium. The fungus species was identified by the sequence of ITS region (internal transcribed spacer 1, 5.8 S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence). Fungal cultures subcultivated no more than 3 times were used in the tests. The material for testing consisted of conidial suspensions obtained by washing fungal cultures with a sterile aqueous solution and TWEEN 80® (0.01%). The suspension obtained was filtered through a sterile cotton filter and the concentration of aerial conidia was adjusted to $1\times10^8$ conidia/ml using a Burker hematocycometer. The commercial product Naturalis has not been diluted. Also from the commercial product the strain of *B. bassiana* ATCC 74040 was isolated on semi-selective culture medium containing antibiotics and antifungals (Dodine Supelco 0.1 g/l, Penicillin-G Sigma 0.4 g/l, Streptomycin sulfate Sigma Ald. 1 g/l, PDA Fluka medium 39 g/l, Cycloheximide Roth, 0.25 g/l) and introduced in the test. Once the sporulation took place, a conidial suspension was obtained by the same procedure as described for the other fungal isolates and was introduced into the laboratory test. The viability of all fungal cultures was checked before each test, this being >90%.

### Test insects

Adults of *T. dilaticollis* were collected from the proximity of arable land in Ialomiţa and Prahova counties between April and May, 2022. Insects were maintained in the laboratory, within RDIPP, in natural lighting conditions and at room temperature (23-25°C). Insects were fed with leaves of *Cirsium arvense* collected from the field.

### Laboratory testing

Bioassay in the laboratory was conducted using the method described by Draganova et al. (2012). More precisely, the contamination of the insects was achieved by placing them in Petri dishes (9 cm in diameter) lined with the filter paper rods on which one millilitre of conidial suspension was previously applied. The control consisted of insects treated with sterile aqueous solution and Tween 80 (0.01%). The paper discs were removed after 24 hours and the insects were fed with leaves by *Cirsium arvense*. The experiments were

conducted in the laboratory in natural lighting conditions, at room temperature (25±2°C), in three repetitions of 20 individuals each. Mortality of insects was recorded daily, for 14 days. Individuals were considered dead when they were no longer able to produce coordinated movements and no longer reacted to the touch. The dead individuals were removed to avoid cross-contamination and placed in damp chambers to encourage the development of mycosis at 24°C. Infection with *Beauveria* was confirmed by the appearance on the dead bodies of white mycelium with conidia.

**Preliminary field bioassay**

The field bioassay was conducted, on the Experimental Field of Plant Protection Department, National Agricultural Research and Development Institute Fundulea (NARDI), Călărași County, Romania, in 2022. This field had a history of maize leaf weevil with moderate to high infestation (more than 5 insects/$m^2$). The experimental field was located in a climatic region of the plain, with an average annual temperature of 11.3°C (Toader et al., 2020) and the soil type was cambic chernozem (Toader et al., 2016). The experiment was established in a complete block design. An individual plot size was 42 $m^2$ (10 m length × 4.2 m width 6 rows, 0.7 m distance between rows), with one-meter-wide pea strips between the plots (plant with repellent effect for maize grey weevil) and a buffer zone of 2 m between the replicates. Because the maize leaf weevils are mobile soil pest (Trotus and Buburuz, 2015) we decided to apply the fungus in a barley grain formulation to the soil. Two experimental treatments consisting of native *Beauveria* strains, BbTd1 and BbLy were applied as multiplicated conidia on barley grains ($1\times10^9$/g d.w). The control consisted of non-inoculated barley grains. On the 6 of April, before adults appear on the soil surface, the fungus on barley grains have been dispersed and incorporated in the soil, resulting a density of $5.0\times10^{11}$ conidia/$m^2$. Immediately after distribution, the incorporation into the soil of the barley grains inoculated with the fungus was executed with a rotary hoe. The first precipitation after application in the field of entomopathogenic preparations was recorded after 12 hours, and cumulated an amount of 7.6 mm. After another 24 hours, another 0.2 mm was recorded. Further, until the sowing of maize, 39.8 mm were recorded at the beginning of May. The maize was sowed 27 days after the treatment incorporation in soil. The seeds used for sowing were not treated, so that there was no interference with the entomopathogenic fungus tested. Surveys were carried out to establish the population density of *T. dilaticollis* after 7, 26, 34 and 47 days post emergence. On each row of maize (out of the four plants, at each experimental treatment) four determination points were established. Within each point, 5 plants were chosen, with a total of 20 plants per row (a total of 80 plants/plot). Both insects on corn plants and those at their base were counted. The surveys were conducted after 12:00 when the air temperature was higher than 20ºC and the sky was not cloudy. These conditions are known to favor the activity of grey maize weevil on the surface of the soil and insects can be noticed more easily.

**Statistical analysis**

The efficacy of fungal isolates was expressed as a cumulative percentage of mycosis. The effects of treatments with different strains of *Beauveria* on the survival degree of insects were analyzed using Kaplan Meier survival curves, a non-parametric statistical analysis, and the existence of differences between treatments was determined using the log rank test. The virulence of each fungus applied as an aqueous conidian suspension or commercial product has been estimated by values of the median survival time (MTS, the number of days until 50% of insects are dead). The differences in the degree of virulence were analyzed using ANOVA (one-way) followed by the Tukey post hoc test. The mycosis percentages were analyzed using variant analysis and the averages were compared using the Tukey test. The differences were

considered significant at a signification level of 5% (GraphPadPrism V.7 for Windows).

The Tukey's Honestly Significant Difference (HSD) test within ARM 8.5 program was performed to determine significant differences between insect density in the treated and control in field plots.

## RESULTS AND DISCUSSION

In the laboratory, the four strains of *Beauveria* used at a concentration of $1\times10^8$ conidia/ml were pathogenic for *T. dilaticollis* adults, as can be seen from the percentages of mycosis and the Kaplan Meier survival curves, which are significantly different from the control, except for the BbTd2 strain (Figure 1 and Figure 2). Although the mortality in the control variant was 38% (n=60) until the 14$^{th}$ day, the percentage of mycosis due to *Beauveria* infection was 16%. After seven days, treatments with *Beauveria* caused the death of more than 50% of *T. dilaticollis* adults with the exception of the strain BbTd2 for which the MTS value could not be calculated.

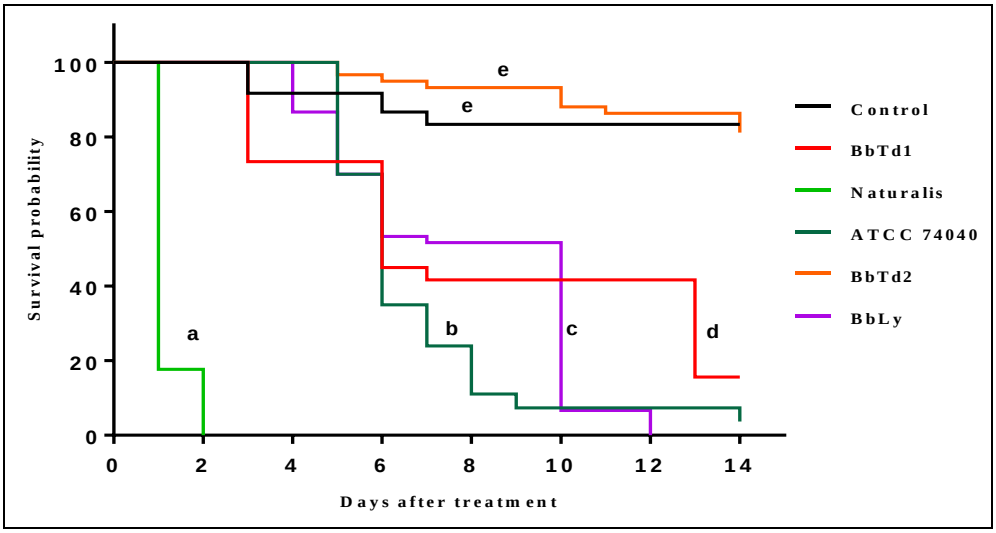


*Figure 1.* Kaplan Meier survival curves expressed as adult survival probabilities of *Tanymecus dilaticollis* 14 days after treatment with different *B. bassiana* strains (BbTd1, ATCC 74040, BbTd2), *B. pseudobassiana* (BbLy) and the commercial product Naturalis based on *B. bassiana* (strain ATCC 74040). The letters that accompany the Kaplan-Meier curves indicate a significant difference between the treatments with different fungal strains, applying the log-rank test, P<0.05.

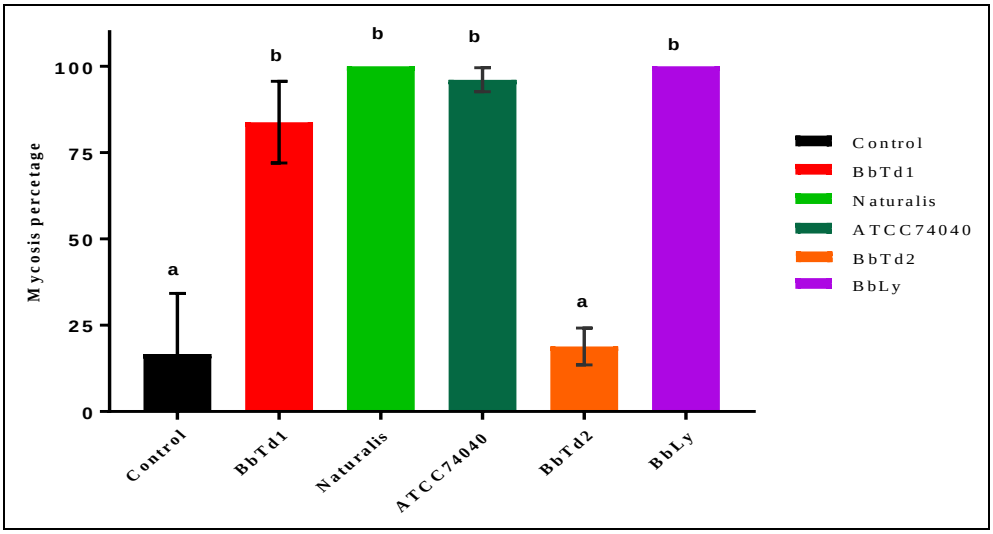


*Figure 2.* Mycosis percentage of *Tanymecus dilaticollis* adults at 14 days after treatment with different *Beauveria bassiana* strains (BbTd1, ATCC 74040, BbTd2), *B. pseudobassiana* (BbLy) and the commercial product Naturalis based on *B. bassiana* (strain ATCC 74040)

The lowest probability of survival and the highest virulence were recorded in the treatment with the commercial product Naturalis, these being significantly different from those recorded in the case of the other treatments (Figure 1 and Table 1).

*Table 1*. The virulence of different *B. bassiana* strains (BbTd1, ATCC 74040, BbTd2), *B. pseudobassiana* (BbLy) and *B. bassiana* (strain ATCC 74040) formulated as Naturalis

| Fungal treatment | Virulence (MTS)± SD[1] | Confidence interval (CI) | ANOVA[3] |
|---|---|---|---|
| BbTd1 | 6±0.2 | 5.4-6.8 | a |
| BbTd2 | Undefined[2] | - | - |
| Naturalis | 1±0 | 1-1 | b |
| ATCC74040 | 6±0 | 6-6 | a |
| BbLy | 8.6±2.3 | 2.9-14.4 | a |

[1]MST - Median survival time, SD - standard deviation;
[2]Undefined - the value was not calculated because the percentage of survival exceeded 50% during the test period;
[3]Different letter indicates significant difference between treatments (Tukey post-hoc test).

The insects treated with this commercial product started to dies in the first hours after inoculation, while the treatment with the *Beauveria* strain (ATCC 74040), which is the basis of this product, did not show a significantly different virulence and no percentage of mycosis compared to the native strains of *B. bassiana* (BbTd1) and *B. pseudobassiana* (BbLy) (Table 2 and Figure 2). The mycosis percentages of *T. dilaticollis* adults due to the treatments with fungal spores varied from 18.8% to 100%, the lowest percentage being determined by *B. bassiana* (BbTd2).

In the field the average number of *T. dilaticollis* per $m^2$ counted on each plot did not vary significantly from control (Table 2). In each period of observations, the density of *T. dilaticollis* in different treated plots varied within tight limits, between 7.21 and 7.89 insects/$m^2$. Insects were generally evenly distributed within experimental plots and active at the soil surface or on plants. No dead insects showing symptoms of infection with entomopathogenic fungi were found.

*Table 2.* The average number of *Tanymecus dilaticollis* per $m^2$ measured in different treatments and on different sampling dates after maize emergence, in 2022

| Fungal treatment | Days after maize emergence | | | |
|---|---|---|---|---|
| | 7 days | 26 days | 34 days | 47 days |
| *Beauveria pseudobassiana* (BbLy) | 7.20±1.03 a | 5.91±1.38 a | 3.41±1.35 a | 0.31±0.10 a |
| *Beauveria bassiana* (BbTd1) | 7.89±1.32 a | 5.94±0.89 a | 3.54±0.59 a | 0.34±0.38 a |
| Control | 7.46±1.16 a | 5.96±0.87 a | 3.60±0.45 a | 0.35±0.19 a |

The same letter indicates no significant difference between treatments (Tukey HSD test).

This is the first report of signaling the natural infection with *B. bassiana* to *T. dilaticollis* and also the first reporting of the susceptibility of adults of *T. dilaticollis* to *B. pseudobassiana*, in Romania. Only a few studies on host diversity and virulence of *B. pseudobassiana* are available maybe because of the many morphological similarities to *B. bassiana*. In previous works it was probably identified as *B. bassiana*. Recently *B. pseudobassiana* has been shown to have great potential in the biocontrol of numerous insect pests (Kocaçevik et al., 2016; Wang et al., 2020).

In this study, the laboratory results showed that the entomopathogen fungi *B. bassiana* (BbTd1) and *B. pseudobassiana* (BbLy) were effective against adults of *T. dilaticollis* and comparable in percentage of mycosis and virulence to the commercial strain ATCC 74040. In laboratory tests, high mortality

rates (>83%) were obtained by applying a concentration of $1\times10^8$ conidia/ml of *B. bassiana* and *B. pseudobassiana* after 14 days. In a study to identify pathogenic fungi against *T. dilaticollis* and to estimate the virulence of three fungal isolates of *B. bassiana*, after the exposure to a concentration of $3\times10^8$ conidia/ml, after 6 days, all isolates of *B. bassiana* resulted in a mortality rate of over 97% with no differences in their virulence (Draganova et al., 2012). The results reported here showed that there are significant differences in pathogenicity between the two strains of *B. bassiana* (BbTd1 and BbTd2). Although it was isolated from the same host insect species as BbTd1, the BbTd2 strain was not virulent for adults of *T. dilaticollis* so it did not show specificity for this host.

In recent studies, Toshova et al. (2021) evaluated the possibilities for practical applications of two commercial bioinsecticide against adults of *T. dilaticollis*, Naturalis and NeemAzal T/S (based on azadirahtin). Mycoinsecticide applied to adults of *T. dilaticollis* at a concentration of $2.3\times10^6$ conidia/mL caused a high mortality percentage in a short time (100% within 3 days), the insects dying within a few minutes at the concentration of $2.3\times10^7$ conidia/ml, but the effect of NeemAzal T/S treatment was reduced to moderate (6-44% within 16 days). Our study showed that the commercial micoinsecticide Naturalis at $2.3\times10^7$ killed all the adults of *T. dilaticollis* in one day and the ATCC strain 74040 that underlies this product, has not been shown to be more virulent than the other two native strains of *Beauveria*, BbTd1 and BbLy. Our results are in line with those obtained by Oreste et al. (2012) who tested the pathogenicity of 23 isolates of *Beauveria* and 4 isolates of *M. anisopliae* (Metsch.) Sorokin against the larvae of *Galleria mellonella* L. and *Tenebrio molitor* L. They noticed that Naturalis, at a concentration of $2\times10^6$ conidia/ml, causes the death of test insects within a day after inoculation. Also, the *B. bassiana* strain ATCC74040 contained in the tested product did not cause different results than the most effective isolates tested. Naturalis, showed also its efficacy against *Tetranychus urticae* Koch on tomato in glasshouse (Chlander et al., 2005) and on pepper in field (Marcic et al., 2013). Duso et al. (2008) investigated the six commercially used insecticides, one of which was Naturalis-L, on Mediterranean populations of *T. urticae* and their predator *Phytoseiulus persimilis* Evans in the laboratory, found that *B. bassiana* was the most effective pesticide in reducing the hatching of *T. urticae* treated eggs.

In our field experiment, in the first decade of May, after the emergence of maize plants (3-rd of May), the density of *T. dilaticollis* adults was higher than the economic threshold (5 adults/$m^2$). The high air temperatures recorded in the first decade of May, as well as the low rainfall (30 mm) favored the activity of this insect. After 34 days (in the first decade of June) there was a significant decrease in the density of *T. dilaticollis* in the experimental plots, under the economical threshold (Table 2). This is because on the one hand, the maximum temperatures, higher than 30°C, caused the pests to hide and circulate less at the soil surface, on the other hand, in June there is a decrease in the activity and number of insects (Roşca and Istrate, 2009). However, in the past there were situations when insects were active in the first half of June. This can happen after a cold and rainy spring.

The results of our preliminary field test showed no efficacy of entomopathogenic fungus applied as colonized barley grains in soil. Soil texture and climatic factors as sunlight, moisture or temperature can affect the success or failure of the entomopathogenic fungus (Jaronski, 2010; Scheepmaker and Butt, 2010). Infection and mortality of third instar sugarbeet maggot larvae by *M. anisopliae* F52 were significantly affected by soil type and moisture in five soils and three moisture levels (10, 15, 30 % field saturation for each soil type) (Jaronski et al., 2005). In our trials although the precipitation was recorded two days after the fungus application, the moisture content of the soil probably was not in favor of the fungus efficacy considering that two months before the application and severe drought was recorded (5.4 and 12.3 mm

amount of average precipitation in February and March, respectively).

Up to now, we have founded only two reports about the field trials of entomopathogenic microorganisms against *T. dilaticollis*. Toader et al. (2020) reported that *Bacillus thuringiensis* (Bactospeine DF, at a dose of 0.01%) applied on maize seeds or in vegetation gave similar values as control plots in terms of *T. dilaticollis* adult density per $m^2$ (9.6, 9.05 and 10.9, respectively). Two sprayings of maize with *B. bassiana* (Naturalis, 200 mL/0.1 ha) induced a relatively low, but significantly higher mortality of *T. dilaticollis* adults when compared with the untreated control plots in natural field conditions (Thoshova et al., 2021).

## CONCLUSIONS

This is the first report about a bioassay against *T. dilaticollis* using an autochthonous strain of *B. pseudobassiana*. The results from the laboratory test demonstrated that *T. dilaticollis* adults are susceptible to *B. bassiana* and *B. pseudobassiana* strains when treated with $1\times10^8$ conidia/ml. The *B. bassiana* strain originated from naturally infected *T. dilaticollis* adult has a similar virulency as the ATCC74040 strain, contained by the commercial product Naturalis. The inconsistent results on *T. dilaticollis* adult density in field one month after the fungus application in soil could be attributed to climatic conditions.

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