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Fungal Pathogens of Grey Corn Weevil *Tanymecus dilaticollis* (Coleoptera: Curculionidae) and Bioassay with Some *Beauveria bassiana* Isolates

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Abstract: Fungal pathogens naturally occurred on adults of *Tanymecus dilaticollis* (Coleoptera: Curculionidae) were isolated in pure cultures and identified as *Beauveria bassiana* (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae). Morphological characteristics of some isolates of the entomopathogenic fungi were examined and presented. Results of conducted laboratory bioassays with three *B. bassiana* isolates against adults of the pest were evaluated and discussed. All isolates tested caused mycoses with rapid lethal effect higher than 97% cumulative daily mortality on the sixth day after treatment. Calculated values of the median lethal time varyed within narrow confidence intervals for all three isolates. This is the first report of pathogens isolated from *T. dilaticollis* adults and the first bioassays with *B. bassiana* isolates against adults of the pest.

Key words: entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, *Tanymecus dilaticollis*, virulence

Introduction

The Grey corn weevil *Tanymecus* (*Episomecus*) *dilaticollis* Gyllenhal (Coleoptera: Curculionidae) is an economically important pest of maize in Eastern and Central Europe, and Asia Minor (Meissle *et al.* 2010). It is a polyphagous species and beside maize it can cause serious yield loss also of sunflower and sugar beet (Gerginov 1989, Kacsó 1974, Kirkov 1967, Krusteva *et al.* 2006, Popov 1969, Sáringer, Takács 1994). The most damages are caused by overwintered adults. At high population level, the infestation of adults at early vegetation stages may even lead to crop devastation.

Various agro-technical measures (soil tillage, crop rotation, sowing terms, conditions favouring rapid seedling development and plant density) and chemical treatments are applied for *T. dilaticollis* control (Kirkov 1967, Krusteva *et al.* 2006). Inclusion of proper bioagents and in particular microbial insecticides in integrated pest management could reduce chemical treatments and problems connected with insecticide application.

Entomopathogenic fungi could be proper biological agents as some fungal species have wide hosts range which include many insect orders, infect different stages of their hosts, often cause natural epizootics, and there are minimal effects on non-target organisms (Shah, Pell 2003). At the best of our knowledge there are no literature data about identification of fungal pathogens in natural populations of the grey corn weevil or application of microbial insecticides for management of populations of the pest.

The aim of the current study was to identify fungal pathogens on *T. dilaticollis* and to estimate the virulence of three isolates of the entomopathogenic fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin to adults of Grey corn weevil.

Material and Methods

Samples with overwintered adults of *T. dilaticollis* were collected during a field survey in maize agrocoenoses in two regions of Northwestern Bulgaria – near the town of Knezha (43° 30′ N 24° 5′ E) in 2009 and 2010; and near the village of Selanovtsi (43° 41' N, 24° 1' E) in 2009. Weevils were maintained in the laboratory at a photoperiod of L14:D10 and temperature 25 ± 2 °C. Adults were fed on a mixture of 14-20 day-old maize plants (*Zea mays* L. hybrid Kn 509, Maize Research Institute, Bulgaria) grown in the laboratory and wild plant species mainly from family Poaceae collected in the field.

Dead adults of *T. dilaticollis* (26 collected from the first location and 3 from the second) after surface sterilization were placed in a moist chamber for sporulation of fungal pathogens. Conidia from sporulated fungi were transferred to SDAY (Sabouraud dextrose agar with yeast extract) in glass Petri dishes and cultured for 15 days at 25 ± 2 °C at a photoperiod of L12:D12. Obtained pure cultures of the isolates after repeated subculturing (not more than 3 times) on slopes of the same media in tubes were kept in a refrigerator at temperature 4 ± 2 °C. Conidia from pure cultures of isolates and small parts of cadavers were used to prepare smears stained by methylen-blue as well as slide preparations with lactophenol and aniline blue closed by nail vanish (Humber 1997).

Morphological characters of fungal pathogens were studied in order to determine their taxonomic status. Conidia and conidiogenous cells were examined in smears and slide preparations using a transmission interference microscope BX60 DIC Olympus equipped with digital camera and Cell B image capture software with calibrated Carl Zeiss micrometer. Sizes of conidia were measured only for isolates obtained in pure cultures. Minimum and maximum values of conidia measures were obtained from a minimum of 30 measurements. Fungal pathogens were identified according to Samson *et al.* (1988) and Humber (1997).

Bioassays with three isolates of *B. bassiana* 579Bb, 581Bb and 586Bb obtained from adults of Grey corn weevil were carried out on *T. dilaticollis* at the same laboratory conditions as described above and at 60 ± 1 % RH.

Fungal isolates were cultured for 15 days on slopes of SDAY in tubes at 25 ± 2 °C at a photoperiod of L12:D12 and obtained conidia were washed down with sterilized water containing 0.05% Tween 80. The concentrations of conidia were determined by enumeration of conidia in Bürker chamber after serial dilution of aqueous suspensions. Suspensions applied in bioassays were prepared at concentration of $3x10^8$ conidia/mL.

Adults of *T. dilaticollis* used in bioassays were collected from natural populations of the pest from maize crops near the town of Knezha in 2010. Insects were treated for 24 h by a surface contact with 1 mL of conidial suspensions placed on filter paper discs (90 mm in diameter) in glass Petri dishes (Draganova, Staneva 1990). Adults in control variants were treated with 1 mL of water containing 0.05% Tween 80 instead of conidial suspension. The filter paper disks were removed 24 h after the treatments and the insects were fed on maize leaves. Experiments were conducted in the laboratory at a photoperiod of L14:D10 and temperature 25 ± 2 °C in three replicates with 21-32 unsexed adults of mixed age per a replicate.

Insect mortality was checked daily for 8 days after contamination. Dead insects in all treatments were removed daily and were placed on moist filter paper in new Petri dishes for a fungal pathogen exhibition. The presence of the white mycelium and conidia of *B. bassiana* over the cuticle of cadavers was evidence of death by mycosis. Results of the bioassays were evaluated as percentages of cumulative daily mortality due to mycosis corrected with mortality in the control treatment following Abbott (1925). Differences among lethal effects of mycoses to adults of *T. dilaticollis* in treatments with conidial suspensions of *B. bassiana* isolates were estimated by t-test for independent samples applying Descriptive statistics. Values of $p<0.05$ were considered significant. Statistical analyses were performed using the software STATISTICA^R version 6.0 of Stat Soft Inc. Virulence of each fungal isolate was estimated by values of the median lethal time (LT_{50}), calculated by log probit analysis (Finney 1971).

Results and Discussion

Microbiological analyses of the collected samples showed that mycoses observed on *T. dilaticollis* cadavers were caused mainly by following fungal species – *Beauveria bassiana* (Cordycipitaceae) and *Metarhizium anisopliae* (Metschnikoff)

Sorokin (Clavicipitaceae), anamorphs Ascomycota (Hypocreales).

Mycoses caused by *B. bassiana* were established in 19 adults of Grey corn weevil collected from maize crops in the region of Knezha and in one – from the region of Selanovtsi. Nine of the isolates were isolated in pure cultures (Table 1). When dead adults of *T. dilaticollis* were placed into a moist chamber the fungus started growing out through the inter-segmental membranes of the host covering it by dense white to cream mycelium. *B. bassiana* isolates formed on SDAY round raised colonies with powdery surface, with pigmentation from white to cream, reverse of the colonies – with pale cream pigmentation. Conidiogenous cells were densely clustered in whorls, hyaline, smooth and short. In smears and slide preparations these cells were with a globose base terminated in a narrow extended denticulate apex with a distinctly zig-zag threadlike appearance (a denticulate rachis) with one conidium per denticle. Conidia were single-celled, hyaline, thin-walled, hydrophobic, subglobose, mainly broadly elliosoidal in shape, similar in size for different isolates. Isolate 579Bb was with the smallest conidia measured while isolates 581Bb and 585Bb were among isolates with the biggest conidia (Table 1). Isolate 579Bb was with the smallest conidia measured as (1.15-2.60) 1.55 ± 0.07 x 0.99 ± 0.04 (0.64-1.56) μm. Isolates 581Bb and 585Bb were among isolates with the biggest conidia (1.30-2.53) 2.10 ± 0.05 x 1.52 ± 0.04 (1.23-2.17) μm and (1.37- 3.22) 2.02 ± 0.04 x 1.53 ± 0.03 (0.86-2.14) μm, respectively. These measures were very similar to size of conidia 2-3 x 2-2.5 μm described by Brady (1979a), Evlakhova (1974), Humber (1997), Rehner, Buckley (2005), Samson *et al.* (1988) and Zimmermann (2007).

The mycosis established on a *T. dilaticollis* adult from Knezha was due to *M. anisopliae*. *M. anisopliae* isolate (619Ma) was obtained in a pure culture as well. After it was placed in a moist chamber, *T. dilaticollis* cadaver was covered by dark-green dense powdery mat. The isolate 619Ma formed round colonies with dark-green powdery surface on SDAY, while the reverse of colonies was colourless. Conidiophores were in compact patches. Conidia were single-celled, cylindrical, produced in dry chains. Colour of the spore mass was dark-green. Size of *M. anisopliae* conidia isolate 619Ma obtained from adults of *T. dilaticollis* was (5.12-7.61) 6.22 ± 0.11 x 2.64 ± 0.04 (2.17-3.13) μm (Table 1) which was similar to 5.0-7.0 x 2.0- 3.5 μm – size of conidia of *M. anisopliae* strain ARSEF 7487 (Bischoff *et al.* 2006), 5-8 x 1.5- 3.5 μm (Brady 1979b) or 6.4-8 x 1.6-2.8 μm – size of conidia of *M. anisopliae* strain IMI 333138 (Bridge *et al.* 1993). Evlakhova (1974) discribed *M. anisopliae* with smaller conidia measured as 4.8 x 1.6 μm.

Reasons for death of the other 8 insects collected from both locations were not established. Fungi belonging to genus *Fusarium* Link (established in 2 insects) and to species *Aspergillus flavus* Link (in 5 insects) and *A. ochraceus* Wilhelm (in one insect) were found in smears, slide preparations and cultures on SDAY. Isolates of them were not obtained in pure cultures. In our opinion these fungi are secondary pathogens or contaminants grown at insect cadav-

Table 1. Size of the conidia of *B. bassiana* (Bb) and *M. anisopliae* (Ma) isolates obtained from dead adults of *T. dilaticollis.*

Species (isolate)	Size of conidia: L-Length; W-Width (μm)		
	L (min – max) ± SE	W (min – max) ± SE	Average size (L x W)
Bb (579)	(1.15 – 2.60) ± 0.07	(0.64 – 1.56) ± 0.04	1.55 ± 0.07 x 0.99 ± 0.04
Bb (580)	(1.48 – 2.42) ± 0.05	(1.20 – 2.03) ± 0.04	1.87 ± 0.05 x 1.60 ± 0.04
Bb (581)	(1.30 – 2.53) ± 0.05	(1.23 – 2.17) ± 0.04	2.10 ± 0.05 x 1.52 ± 0.04
Bb (582)	(1.50 – 2.82) ± 0.05	(1.25 – 2.25) ± 0.04	2.06 ± 0.05 x 1.66 ± 0.04
Bb (583)	(1.51 – 2.95) ± 0.05	(0.90 – 2.44) ± 0.05	1.99 ± 0.05 x 1.74 ± 0.05
Bb (584)	(1.36 – 2.59) ± 0.06	(0.86 – 1.77) ± 0.05	1.97 ± 0.06 x 1.47 ± 0.05
Bb (585)	(1.37 – 3.22) ± 0.04	(0.86 – 2.14) ± 0.03	2.02 ± 0.04 x 1.53 ± 0.03
Bb (586)	(1.36 – 2.95) ± 0.05	(0.86 – 2.17) ± 0.04	1.97 ± 0.05 x 1.43 ± 0.04
Bb (587)	(1.37 – 3.22) ± 0.07	(0.86 – 2.14) ± 0.06	2.00 ± 0.07 x 1.52 ± 0.06
Ma (619)	(5.12 – 7.61) ± 0.11	(2.17 – 3.13) ± 0.04	6.22 ± 0.11 x 2.64 ± 0.04

ers after death of the host. Found species from genus *Aspergillus* are weak entomopathogens and they could not play significant role in pest mortality.

Original sources of *B. bassiana* and *M. anisopliae* isolated from *T. dilaticollis* adults are unknown. Entomopathogenic fungi are usually identified as such based on the fungal growth observed on insect cadavers (Vega *et al.* 2009). In our investigations the adults of *T. dilaticollis* could have been infected either before their collection from the field or have acquired the pathogens during feeding with field collected plants.

Obtained results show that *T. dilaticollis* is an insect species from the host range of both fungal species *B. bassiana* and *M. anisopliae*.

These entomopathogens are world-wide, very adaptive to different climate conditions. Both fungal species are frequently isolated as causal agent of mycoses which can develop into epizootics dramatically reducing population density of arthropod pests (Butt *et al.* 2001). Fungi *B. bassiana* and *M. anisopliae* have a large infectious range. *Beauveria* is known to infect more than 700 arthropod species (Goettel *et al.* 1990) and according to Veen (1968) the infectious range of *M. anisopliae* includes over 200 insect species. They are species which strains are usually applied for developing microbial biopesticides (Zimmermann 2007).

According to Pilz *et al*. (2007) species *B. bassiana* and *M. anisopliae* were among naturally occurring pathogens on another serious pest of maize – *Diabrotica virgifera virgifera* Le Conte (Coleoptera: Chrysomelidae) invasive species recently introduced into Europe. Experiments showed that obtained fungal isolates were virulent to adults and larvae of the pest.

Conducted bioassays revealed that adults of *T. dilaticollis* were susceptible to the studied fungal isolates of *B. bassiana*.

Mortality of adults of *T. dilaticollis* in variants treated with conidial suspensions of the isolates 579Bb, 581Bb and 586Bb increased from 7.14%, 19.62% and 5.85% on the third day to 90.54% ± 15.28, 97.10 ± 15.31 and 90.08% ± 16.29, on the fifth day respectively. For a period of 6 days 581Bb caused mycosis with the highest lethal effect – 100% ± 15.31, following by 586Bb – 98.71% ± 16.29 and 579Bb – 97.33% ± 15.28. Only 8.00% ± 1.05 of adults died in the control variants within the same period (Fig. 1). No mycosis was found on adults of control treatments. As it could be seen SE-values were rather high. This could be explained with different age of insects as they were collected from natural population.

Rapid increase of cumulative mortality established in dynamics of fungal infections 3 days after the treatment could be explained as due to toxic com-

Table 2. Differences between mean lethal effects of mycoses to adults of *T. dilaticollis* in treatments with conidial suspensions ($3x10^8$ conidia/mL) of *B. bassiana* isolates for eight-days period of study.

Variants compared	Mean lethal effect Group 1	Mean lethal effect Group 2	t	df	p	t separ. var.est.	df	p 2-sided	Valid N Group 1	Valid N Group 2	Std. Dv. Group 1	Std. Dv. Group 2	F-ratio variances	p variances
579Bb vs. CTR	0,5698	0,065	3,1497	14	0,0071	3,1497	7,0606	0,0162	8	8	0,4523	0,0298	230,9614	0
581Bb vs. CTR	0,5763	0,065	3,2008	14	0,0064	3,2008	7,061	0,0150	8	8	0,4509	0,0298	229,5046	0
586Bb vs. CTR	0,5471	0,065	2,8779	14	0,0122	2,8779	7,0554	0,0237	8	8	0,4729	0,0298	252,5002	0
579Bb vs. 586Bb	0,5698	0,54718	0,0977	14	0,9235	0,0977	13,9723	0,9235	8	8	0,4523	0,4729	1,0933	0,9094
581Bb vs. 579Bb	0,5763	0,5698	0,0291	14	0,9772	0,0291	13,9999	0,9772	8	8	0,4509	0,4523	1,0063	0,9936
581Bb vs. 586Bb	0,5763	0,5471	0,1263	14	0,9012	0,1263	13,9682	0,9012	8	8	0,4509	0,4729	1,1002	0,903

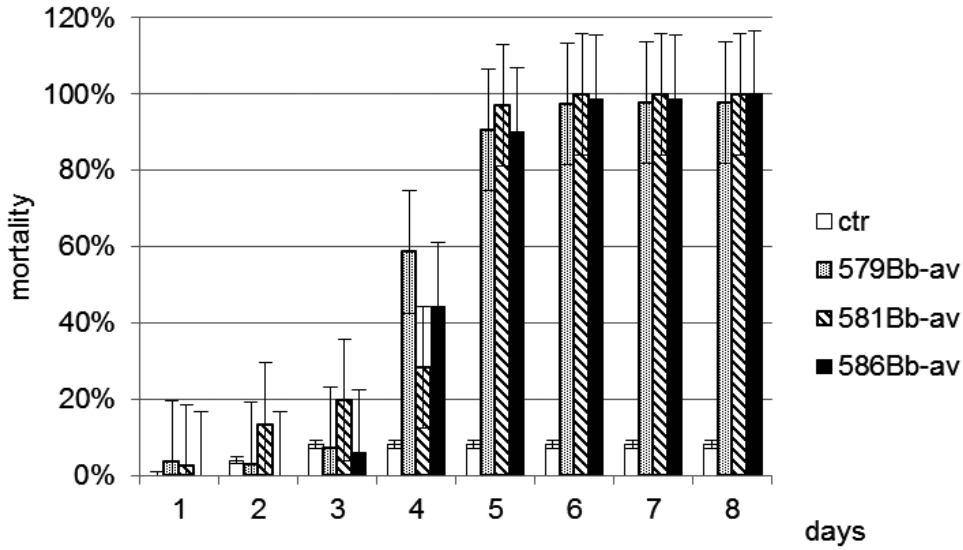

Fig. 1. Lethal effect (% cumulative daily mortality) of mycoses caused by the isolates 579Bb, 581Bb and 586Bb of *B. bassiana* to *T. dilaticollis* adults using conidia suspensions ($3x10^8$ conidia/mL).

Table 3. Virulence of isolates of the entomopathogenic fungus *B. bassiana* to adults of *T. dilaticollis* using conidia suspensions ($3x10^8$ conidia/mL).

Isolate of *B. bassiana*	**Median lethal time LT_{50} (days)**			**Regression coefficient (b ± SE)**	**t-test* (t_{Table}=1.96)**
	Average values	Confidence intervals*			
		from	to		
579Bb	3.703	3.568	3.845	7.811 ± 0.240	2.776
581Bb	3.199	3.026	3.383	7.251 ± 0.304	3.182
586Bb	4.016	3.873	4.165	10.383 ± 0.321	2.776

*p-level < 0.05

pounds released by the pathogen during it's development in insect body which is typical of this fungal species (Roberts 1981, Strasser *et al.* 2000).

The results of the laboratory bioassay showed that isolates 579Bb, 581Bb and 586Bb of *B. bassiana* caused significantly higher mortality when compared to control treatments ($p<0.05$) (Table 2). Small differences were found between lethal effects in treatments with conidial suspensions of *B. bassiana* isolates for eight-days period of the study but they could not be proved as significant as values of t-test were calculated at p-level > 0.05.

Calculated values of the median lethal time varied within narrow confidence intervals for all three isolates – from 3.568 to 3.845 days, from 3.026 to 3.383 days, and from 3.873 to 4.165 days, respectively (Table 3). Significant differences between confidence intervals were not proved at p-level < 0.05. Regression coefficients for the variants treated with conidia of *B. bassiana* isolates were with high values what was an expression of the sharp lethal effect of mycoses.

Literature data about the natural enemies of *T. dilaticollis* are scarce (Cămprag, Sekulic 2002). Sáringer, Takács (1994) mentioned that in humid and cold soils, entomopathogenic fungi can evoke death of adults of the grey corn weevil but no genus or species names were reported.

The present research is the first report about finding and isolation in pure cultures of fungal pathogens from natural populations of the grey corn weevil. This is also the first report on the results of bioassays with some of them against adults of the pest.

Conducted bioassays are only the first step to future studies but they are a hopeful sign as well. They show that search for a proper bioinsecticide with potential for practical utilization in agriculture for *T. dilaticollis* control could be directed to development of new mycopesticide or to selection of

proper one among commercially available formulations with *B. bassiana* isolate as an active substance with coleopteran pests in its host range. Both directions are attended with a hard investigation work in the laboratory and under field conditions.

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