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Pathogenicity of a Native *Beauveria bassiana* Isolate Against the Stored-Grain Pests *Sitophilus granarius* and *Tribolium castaneum*

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ABSTRACT

Entomopathogenic fungi are among the most promising biological control agents for the sustainable management of insect pests. This study evaluated the pathogenicity of a native fungal isolate against the model host *Galleria mellonella* and the stored-product pests *Sitophilus granarius* and *Tribolium castaneum*. Amplification and sequencing of the ITS region identified the isolate as *Beauveria bassiana* (BbsDCj). Bioassays revealed a clear dose-dependent response, with mortality increasing with conidial concentration. Complete mortality (100%) of *G. mellonella* larvae was reached at 2×10^7 conidia mL^{-1}, while *S. granarius* adults reached 90% at the same concentration. *Tribolium castaneum* was less susceptible, with mortality of 16-32% after exposure to conidial suspensions, rising to 51-56% when spore powder was applied. Estimated LT50 values ranged from 6.54 to 6.75 days for *G. mellonella* and from 5.44 to 9.84 days for *S. granarius*. These results demonstrate the high pathogenic potential of the native *B. bassiana* isolate BbsDCj and support its further evaluation as a biological control agent within integrated pest management programs.

Keywords: entomopathogenic fungi, biological control, virulence, stored-grain pests.

List of Abbreviations: EPF - entomopathogenic fungi; PDA - Potato Dextrose Agar; PD - Potato Dextrose; DRBC - Dichloran Rose Bengal Chloramphenicol; ITS - internal transcribed spacer; LC50 - median lethal concentration; LT50 - median lethal time; ANOVA - analysis of variance; HSD - honestly significant difference.

INTRODUCTION

Growing concern over the environmental and health impacts of chemical pesticides has intensified interest in more sustainable pest management strategies. Owing to their high specificity, microbial biopesticides have limited impact on beneficial organisms, help preserve soil microbial balance, mitigate pesticide resistance, and reduce chemical residues in food and the environment, thereby lowering risks to consumer health (Kachhawa, 2017; Seiber et al., 2018; Thakur et al., 2020; Grădilă et al., 2022; Chaudhary et al., 2024; Sahoo et al., 2024; Wei et al., 2024; Wend et al., 2024). Within IPM programs, biological control is most effective when integrated with, rather than opposed to, chemical treatments, since such combinations can maintain efficacy at reduced pesticide rates. Biological and chemical control agents were shown to be compatible in combined seed treatments, providing effective and environmentally friendly protection against pests and diseases (Constantinescu et al., 2014)

Entomopathogenic fungi (EPF) occupy a distinctive position among microbial control agents because infection proceeds by direct penetration of the insect cuticle rather than through ingestion, allowing them to infect a broad range of arthropod pests, including soil-dwelling insects, aphids, mites and stored-product pests (Liu et al., 2023). Penetration is mediated by hydrolytic enzymes - proteases, chitinases and lipases - that degrade the cuticle and enable the fungus to invade the host (Dyczko et al., 2024).

Beauveria bassiana (Bals.-Criv.) Vuillemin is common in soil but also occurs on plant surfaces and as a latent endophyte within insects, reflecting its ability to establish both below- and above-ground interactions (Meyling et al., 2009). Because soils may also contain

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antimicrobial metabolites that reduce fungal infectivity (Vega et al., 2009), surveys of soil-associated fungal communities help characterize the diversity and distribution of entomopathogens and improve the chances of recovering novel isolates for biological control (Hallouti et al., 2020; Qayyum et al., 2021). Locally obtained isolates are often better adapted to the conditions of their region of origin, which makes them particularly attractive candidates for biological control (Zeina and Laing, 2022).

The species used in the bioassays were selected to cover complementary roles in the evaluation of a new isolate. Larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) are widely used as a reference host for entomopathogenic fungi because they are highly susceptible, easily reared under standardised conditions and yield reproducible dose- and time-response data, which makes them suitable for a first estimate of isolate virulence and for comparison with previously characterised isolates (Wojda, 2017; Champion et al., 2018; Cojanu et al., 2022). *Sitophilus granarius* and *Tribolium castaneum* were then included as economically relevant targets, being among the most damaging pests of stored cereals worldwide, where they cause both direct weight loss and deterioration of grain quality, while their management still relies largely on fumigants and residual insecticides to which resistance is now widespread - phosphine resistance has been detected in the majority of surveyed stored-product beetle populations, with resistance ratios averaging 32-fold in *T. castaneum* (Phillips and Throne, 2010; Machuca-Mesa et al., 2024). Testing the isolate against a primary, internally feeding weevil (*S. granarius*) and a secondary, externally feeding tenebrionid (*T. castaneum*), alongside a lepidopteran model host, further allowed us to assess whether an isolate recovered from *Leptinotarsa decemlineata* retains activity beyond its host of origin - a prerequisite for its use in stored-grain protection.

The objectives of this study were therefore to (1) isolate a native *B. bassiana* isolate from naturally infected adults of *Leptinotarsa decemlineata* collected in Romania (Buzău County), (2) characterize it by morphological and molecular methods, and (3) evaluate its pathogenicity against *Galleria mellonella*, *Sitophilus granarius* and *Tribolium castaneum* under laboratory conditions.

MATERIAL AND METHODS

Collected samples

Adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), showing symptoms consistent with fungal infection were collected from an organically managed potato crop in Buzău County, Romania (45.49036° N, 26.87257° E).

Isolation and purification of the fungal isolate

Insect cadavers were incubated in moist chambers at 23-25°C for 3-5 days to stimulate sporulation. Fungal propagules were aseptically transferred onto Potato Dextrose Agar (PDA) and Dichloran Rose Bengal Chloramphenicol (DRBC) agar and incubated at 25°C in darkness for seven days. Emerging colonies were subcultured and purified by serial dilution.

Morphological characterization

Macroscopic assessment covered colony colour, texture, shape, margin, aerial mycelium and reverse pigmentation, and was compared among subcultures to confirm phenotypic uniformity.

Conidiogenous structures and conidia were examined by light microscopy and compared with published descriptions of the genus *Beauveria* (de Hoog, 1972; Kepler et al., 2017).

Colonies with matching morphology were repeatedly subcultured on PDA at 22-23°C until pure cultures were obtained, which were then used for characterization, molecular identification and bioassays.

Molecular identification

For molecular identification, the isolate was grown in Potato Dextrose (PD) broth at 25°C for 5 days; actively growing mycelium

was then harvested and stored cold until extraction.

Genomic DNA was extracted with a commercial fungal kit (Zymo Research, USA) following the manufacturer's protocol with minor modifications: 50-100 mg of fresh mycelium was disrupted with ZR BashingBead tubes and the supplied lysis buffers, and DNA was purified on Zymo-Spin columns.

DNA concentration and purity were checked on a NanoDrop spectrophotometer, and the internal transcribed spacer (ITS) region of ribosomal DNA was amplified and sequenced to identify the isolate.

Pathogenicity bioassays

***Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae)**

Larvae of *Galleria mellonella* were used as a model host. The isolate was grown on PDA at 25°C in darkness for 14 days, and conidia were harvested and suspended in sterile distilled water with 0.001% Tween 80.

Five concentrations were tested - 1.1×10^5, 1.1×10^6, 2×10^6, 1×10^7 and 2×10^7 conidia mL^{-1} - using three to five replicates of five larvae each. Larvae were immersed in the suspension for 30 s and transferred to Petri dishes with filter paper and diet; controls were treated with distilled water containing Tween 80 (Cojanu et al., 2022).

Larvae were incubated at 25°C under a 14:10 h (L:D) photoperiod, and mortality was recorded daily for 10 days. Dead larvae were surface-sterilized in 70% ethanol for 1 min, rinsed in sterile distilled water, and held in moist chambers to confirm mycosis.

***Sitophilus granarius* (Linnaeus) (Coleoptera: Curculionidae)**

Adults of *Sitophilus granarius* were maintained at 25 ± 2°C and 60 ± 5% relative humidity under a 12:12 h (L:D) photoperiod. Inoculum was prepared as above, and adults were immersed for 30 s in suspensions of 2×10^5, 2×10^6 or 2×10^7 conidia mL^{-1} (three replicates of ten adults each), then transferred to ventilated containers with 10 g of wheat grain.

Mortality was scored daily for 14 days, and dead insects were processed as described for *Galleria mellonella*.

***Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)**

Adults of *Tribolium castaneum* were maintained at 27 ± 2°C and 70 ± 5% relative humidity under a 12:12 h (L:D) photoperiod and fed wheat flour and oat flakes supplemented with skimmed milk powder.

Two application methods were compared: immersion for 30 s in suspensions of 2×10^6 or 2×10^7 conidia mL^{-1}, and spore powder incorporated into wheat grain at 10 or 20 mg per 10 g grain.

Each treatment comprised five replicates of 20 adults with 10 g grain; insects were kept at 25°C and mortality was recorded daily for 14 days. Dead insects were processed as described above.

Statistical analysis

Mortality was expressed as the percentage of dead insects per replicate. Because control mortality was zero in all assays, no Abbott's correction was applied. Percentage data were arcsine square-root transformed and compared among treatments by one-way analysis of variance (ANOVA) followed by Tukey's HSD test at $P < 0.05$; treatments sharing the same letter did not differ significantly. Median lethal concentrations (LC50), with 95% confidence intervals, were estimated by logistic (probit) regression of mortality on log10 conidial concentration. Median lethal times (LT50) were derived from cumulative daily mortality. Analyses were performed in Python 3 (SciPy).

RESULTS AND DISCUSSION

Several fungal colonies were recovered from naturally infected *L. decemlineata* adults collected in Buzău County, Romania (Figure 1).

Figure 1. Leptinotarsa decemlineata adult naturally infected: (a) Mycosis of the cadaver showing white mycelial growth; (b) Cadaver exhibiting fungal sporulation following incubation in moist chamber.

Serial dilution and cultivation on PDA and DRBC media yielded multiple colonies with similar morphology (Figure 2a, b). Repeated subculturing produced pure cultures. Colonies were uniform - white, circular and with regular margins - initially velvety and becoming powdery with abundant conidiation; surfaces were dense and slightly raised, with little or no reverse pigmentation (Figure 2c).

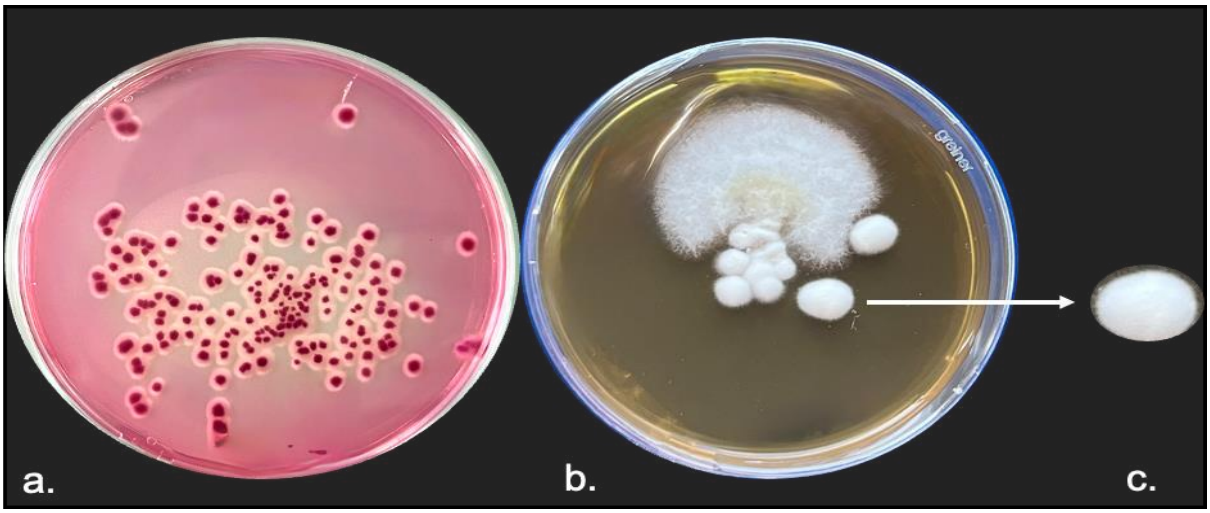

Figure 2. Beauveria bassiana BbsDCj on culture media: (a) colonies recovered on DRBC medium; (b) pure colony on PDA showing characteristic morphology; (c) close-up of the powdery white conidial mass.

Microscopy revealed no substantial differences among colonies, indicating a single species, and the macro- and micromorphology were consistent with the genus *Beauveria*.

Molecular identification by amplification and sequencing of the ITS region with primers ITS1/ITS4 produced a single fragment of ~600 bp, and the extracted DNA was of high quality (69 ng μL^{-1}) (Figure 3).

Comparison with NCBI records confirmed the isolate as *Beauveria bassiana*. It was designated BbsDCj and used in all subsequent experiments.

Pathogenicity against *Galleria mellonella*

Treatment of *G. mellonella* larvae with BbsDCj produced concentration-dependent mortality. After 10 days, mortality rose from 8.0% at 1.1×10^5 conidia mL^{-1} to 32.0% at 1.1×10^6, 73.3% at 2×10^6, 88.0% at 1×10^7 and 100% at 2×10^7 (Table 1).

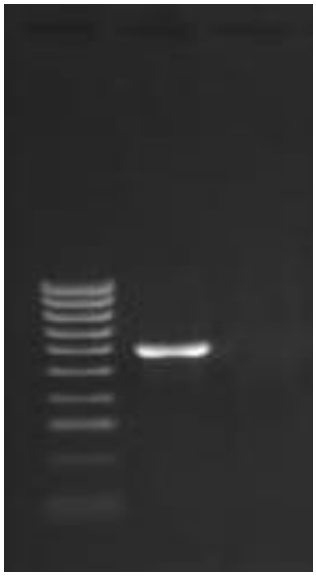

Figure 3. The amplicon resulting from the amplification of the 5.8S-ITS region using the ITS1 / ITS4 primers. Sample order: 1 = 100 bp marker, 2 = BbsDCj, 3 = Negative Control.

Table 1. Mortality of *Galleria mellonella* larvae 10 days after inoculation with different concentrations of *Beauveria bassiana* isolate BbsDCj

Concentration (conidia mL^{-1})	Replicates	Initial larvae (n)	Dead larvae (n)	Mortality (%)	SD	SE	LT50 (days)
1.1×10^5	5	25	2	8.0 a	10.95	4.90	Not reached
1.1×10^6	5	25	8	32.0 a	10.95	4.90	Not reached
2×10^6	3	15	11	73.3 b	23.09	13.33	6.75
1×10^7	5	25	22	88.0 b	10.95	4.90	6.72
2×10^7	3	15	15	100.0 b	0.00	0.00	6.54

Note: Control mortality was 0%, so no Abbott's correction was applied. Means with the same letter do not differ significantly (Tukey HSD, $P < 0.05$).

Dead larvae showed typical signs of fungal infection - melanization, reduced mobility, mummification and fungal outgrowth – and, after incubation in moist chambers, the white mycelium and sporulation characteristic of *B. bassiana* (Figure 4).

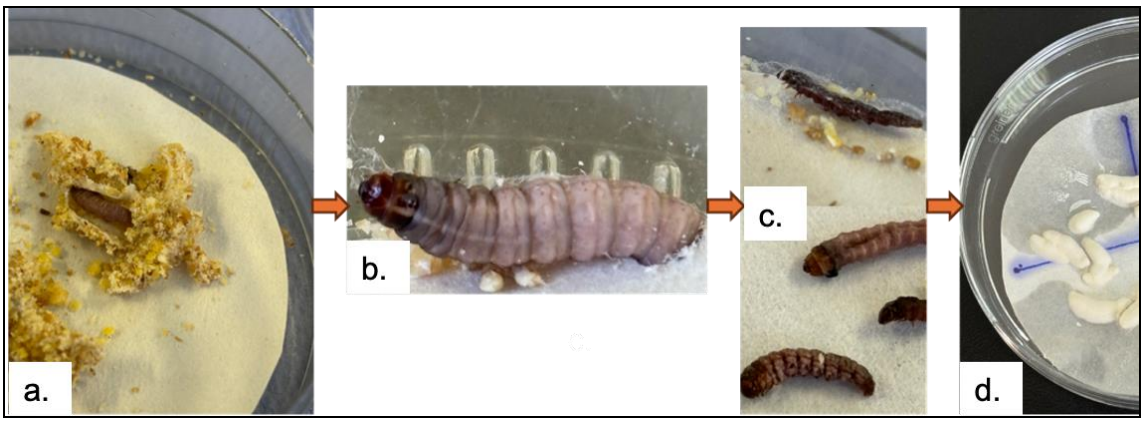

Figure 4. Progression of fungal infection caused by *B. bassiana* BbsDCj: (a) larvae exhibit reduced activity; (b) discoloration appears, with the cuticle turning purplish; (c) progressive dehydration of the larvae; (d) complete fungal sporulation on the cuticle surface.

Differences among treatments were significant [F (4,16) = 23.72, P < 0.001], with concentrations ≥2 × 10⁶ conidia mL⁻¹ differing significantly from the two lowest ones (Tukey HSD). Logistic regression estimated an LC50 of 1.40 × 10⁶ conidia mL⁻¹ (95% CI 9.34 × 10⁵-2.11 × 10⁶) at 10 days (Figure 5a). LT50 was reached only at 2 × 10⁶, 1 × 10⁷ and 2 × 10⁷ conidia mL⁻¹ (6.75, 6.72 and 6.54 days, respectively). Larval death was concentrated around day 7 at all lethal concentrations (Figure 5b), so concentration affected the number of larvae killed, not the timing.

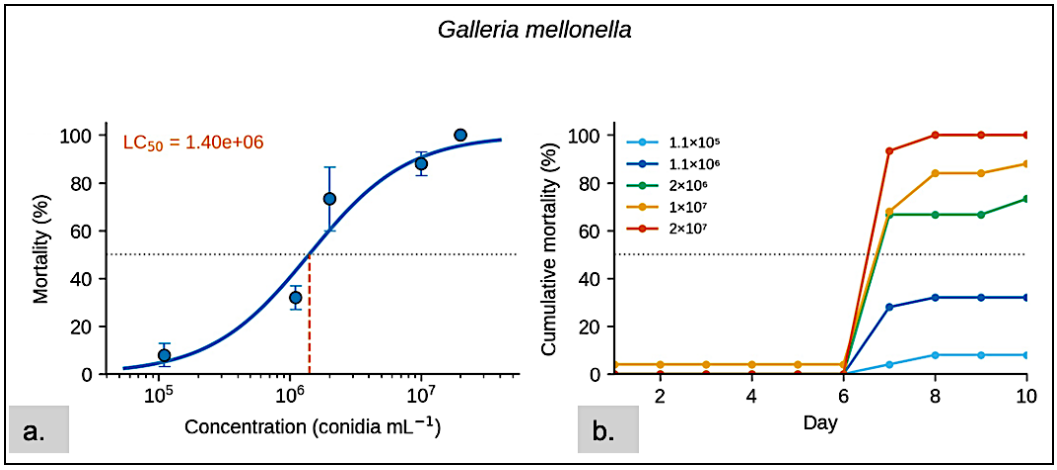

Figure 5. Pathogenicity of *Beauveria bassiana* BbsDCj against *Galleria mellonella*: (a) dose-response with LC50 (mean ± SE); (b) cumulative mortality over time.

Pathogenicity against *Sitophilus granarius*

Exposure of *S. granarius* adults to BbsDCj also caused a clear concentration-dependent increase in mortality, reaching 46.7%, 60.0% and 90.0% at 2 × 10⁵, 2 × 10⁶ and 2 × 10⁷ conidia mL⁻¹, respectively, after 14 days (Table 2).

Table 2. Mortality of *Sitophilus granarius* adults 14 days after exposure to different concentrations of *Beauveria bassiana* BbsDCj isolate

Concentration (conidia mL⁻¹)	Replicates	Initial adults (n)	Dead adults (n)	Mortality (%)	SD	SE	LT50 (days)
2×10⁵	3	30	14	46.7 a	11.55	6.67	Not reached
2×10⁶	3	30	18	60.0 a	20.00	11.55	9.84
2×10⁷	3	30	27	90.0 b	10.00	5.77	5.44

Note: Control mortality was 0%. Means with the same letter do not differ significantly (Tukey HSD, P < 0.05).

Mortality first appeared on day 5 and increased until the end of the assay. The highest concentration gave the fastest progression (LT50 = 5.44 days), compared with 9.84 days at 2 × 10⁶ conidia mL⁻¹ (Figure 6b).

Final mortality differed significantly among treatments [F (2,6) = 6.59, P = 0.031]; only the highest concentration (90%) differed significantly from the lower ones (Tukey HSD). The estimated LC50 was 3.83 × 10⁵ conidia mL⁻¹ (95% CI 1.15 × 10⁵-1.27 × 10⁶) at 14 days (Figure 6a), confirming a dose-dependent response and the pathogenicity of BbsDCj against *S. granarius*.

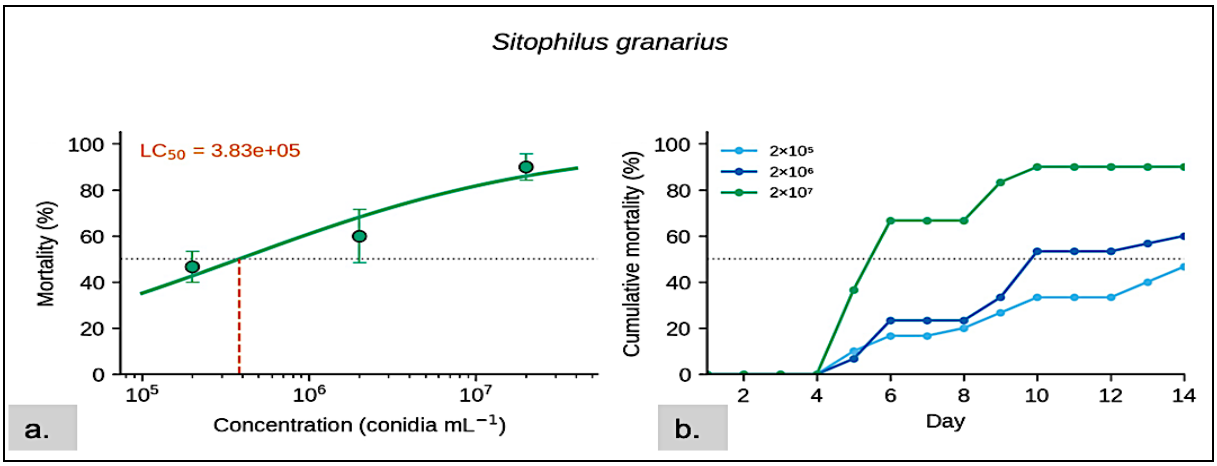

Figure 6. Pathogenicity of *Beauveria bassiana* BbsDCj against *Sitophilus granarius*: (a) dose-response with LC50 (mean ± SE); (b) cumulative mortality over time.

Pathogenicity against *Tribolium castaneum*

Tribolium castaneum adults showed moderate mortality that differed by application method. After 14 days, liquid suspensions caused 16.0% mortality at 2 × 10^6 and 32.0% at 2 × 10^7 conidia mL^{-1}, whereas spore powder gave higher mortality - 51.0% at 10 mg and 56.0% at 20 mg (Table 3; Figure 7a).

Table 2. Mortality of *Tribolium castaneum* adults 14 days after exposure to different application treatments of *Beauveria bassiana* BbsDCj isolate.

Concentration (conidia mL^{-1})	Replicates	Initial adults (n)	Dead adults (n)	Mortality (%)	SD	SE	LT50 (days)
2×10⁶	5	100	16	16.0 a	8.94	4.00	Not reached
2×10⁷	5	100	32	32.0 a	2.74	1.22	Not reached
10 mg spore powder	5	100	51	51.0 b	17.10	7.65	13.92
20 mg spore powder	5	100	56	56.0 b	6.52	2.92	13.54

Note: Control mortality was 0%. Means with the same letter do not differ significantly (Tukey HSD, $P < 0.05$). LC50 was not calculated because the treatments combined conidial suspensions and spore powder, which are on different dose scales.

Cumulative mortality increased gradually, with most dead adults after the first week (Figure 7b). LT50 was not reached with the liquid suspensions but was ~13.92 and 13.54 days for the 10 and 20 mg spore-powder treatments, respectively. Differences among treatments were significant (one-way ANOVA on arcsine-transformed data [$F(3,16) = 16.38$, $P < 0.001$], with spore powder causing significantly higher mortality than the conidial suspensions (Tukey HSD), indicating that *T. castaneum* was less susceptible than the other hosts but that the powder formulation improved efficacy.

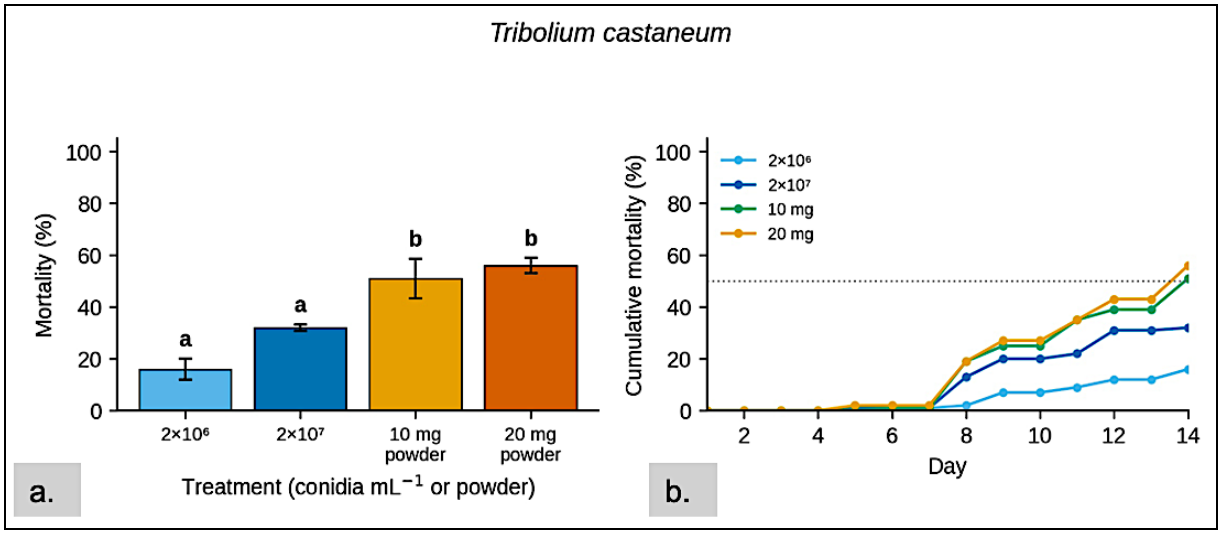

Figure 7. Pathogenicity of *Beauveria bassiana* BbsDCj against *Tribolium castaneum*: (a) final mortality per treatment (mean ± SE; same letter = no significant difference, Tukey HSD); (b) cumulative mortality over time

The native *Beauveria bassiana* isolate BbsDCj showed different levels of pathogenicity depending on the host and the application method.

Against *G. mellonella* larvae, BbsDCj achieved complete mortality (100%) at the highest concentration, confirming the well-known susceptibility of this host and the strong virulence of the isolate. Oreste et al. (2012) similarly reported 80-100% mortality after 17 days with 2×10^6 conidia mL^{-1}, with the fastest isolates killing larvae within about 2.2-2.3 days. Kryukov et al. (2018) obtained 63% mortality after 12 days at 10^8 conidia mL^{-1}, attributing part of the increase to horizontal transmission among grouped larvae. BbsDCj matched or exceeded these results at lower concentrations, underscoring its high virulence.

BbsDCj was also highly pathogenic to *S. granarius* adults, reaching 90% mortality at 2×10^7 conidia mL^{-1}, which compares favourably with previous reports. Batta (2018) obtained at most 55% mortality even at 2×10^8 conidia mL^{-1} when conidia were mixed with wheat flour for ingestion, whereas the immersion method used here produced higher mortality at lower doses, suggesting that direct cuticular contact enhances infection.

Environmental conditions also influence performance. Athanassiou and Steenberg (2007) found that *S. granarius* mortality varied with temperature and humidity, peaking at 25-30°C and 55% relative humidity, and reported only about 52% mortality with dry spores despite much higher doses than used here.

The application method is likewise important. Ak (2019) reported 93.66% mortality after spraying a suspension of 1×10^8 conidia mL^{-1}. The high efficacy observed here supports the view that isolate-specific virulence and inoculation method jointly determine treatment success against *S. granarius*.

Tribolium castaneum was the least susceptible host: mortality was low with conidial suspensions but higher with spore powder, indicating that both host susceptibility and formulation shape the outcome.

This agrees with previous work. De Souza et al. (2024) obtained only 22% mortality at 10^8 conidia mL^{-1} despite a 10-min exposure, whereas BbsDCj reached up to 32% as a suspension and over 50% as spore powder. The low susceptibility of *T. castaneum* to *B. bassiana* - also noted by Zamani et al. (2013) - probably reflects physiological and structural traits of the host, confirming it is much less susceptible than *G. mellonella* or *S. granarius*.

CONCLUSIONS

A native *Beauveria bassiana* isolate (BbsDCj), recovered from naturally infected

Leptinotarsa decemlineata adults in Romania, was characterized using morphological and ITS-based molecular approaches. Under laboratory conditions, the isolate exhibited clear host- and dose-dependent pathogenicity, with the highest susceptibility observed in *Galleria mellonella* and *Sitophilus granarius*. Mortality of *G. mellonella* reached 100% at 2×10^7 conidia mL^{-1}, while *S. granarius* mortality reached 90% at the same concentration. In contrast, *Tribolium castaneum* was substantially less susceptible to liquid conidial suspensions, although application as spore powder resulted in higher mortality.

These findings demonstrate that BbsDCj has significant entomopathogenic activity, particularly against *S. granarius*, and that its efficacy is influenced by both host species and application method. The comparatively low mortality of *T. castaneum* also indicates that susceptibility should be considered when assessing the isolate's potential for stored-product pest management.

Overall, BbsDCj represents a promising laboratory candidate for further investigation as a microbial control agent against selected stored-grain pests. Further studies should evaluate its efficacy under realistic storage conditions, optimize formulation and application methods, determine its persistence and environmental tolerance, and assess its compatibility with other components of integrated pest management before practical application can be recommended.

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