Strain-Specific Virulence of *Beauveria bassiana* Vuill. (Hypocreales: Cordycipitaceae) on *Tettigonia viridissima* L. (Orthoptera: Tettigoniidae)

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Summary

Entomopathogenic fungi can play an important role as key biocontrol agent of agricultural pests' population. The present research aimed to test the effectiveness of *Beauveria bassiana* Vuill. (Hypocreales: Cordycipitaceae) strains against *Tettigonia viridissima* L. (Orthoptera: Tettigoniidae). In laboratory conditions study, the impact of different *B. bassiana* strains 214, 644, and 733 on green grasshoppers' larvae were determined. The enzyme activities (proteolytic, lipolytic, and chitinase) and the presence of the chitinase (*chit1*) gene in the studied strains of *B. bassiana* (214, 644, and 733) were also analyzed. As a result, it was found that all three tested strains exhibited proteolytic, lipolytic and chitin-degrading activity and amplified fragments with the expected length. It was also found that all the strains were effective on *T. viridissima*. The highest effect of the tested strains was found by strain 214 – 66.7%, followed by strains 644 – 55.6% and stains 733 – 44.4% on the 4th day after treatment. Effectiveness gradually increased and on the 7th day after treatment, mortality was found on 100% green grasshoppers' larvae. Although different effectiveness between strains 214, 644, and 733 was found on the 4th day after the test, the differences were not significant between the strains. The research shows the possibility of successful utilization of *B. bassiana* strains for controlling *T. viridissima*.

Key words

entomopathogenic fungi, biological control, hydrolytic enzymes, virulence

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Introduction

Grasshoppers are distributed worldwide and cause serious losses in agriculture. The green grasshopper *Tettigonia viridissima* L. (Orthoptera: Tettigoniidae) is a common species and damages crop plants (İlçin and Çelik, 2021). It is among the most widespread and dangerous pests of tobacco and the fight against it is chemical (Dimitrov et al., 2005). Continuously treatment by chemical pesticides leads to resistance of some pests (Borgi et al., 2016; Das et al., 2024). In recent years, as an alternative to chemical pesticides, more ecological ways of biological control through viruses, bacteria, fungi, etc. have been sought. Entomopathogenic fungi can be alternative because they can adhere to the cuticle, and their enzymes penetrate the pests. In this way, they can attack insect hosts (Dakhel et al., 2020).

The fungus *Empusa grylli* is mainly used against grasshoppers (Dimitrov et al., 2005), but it has fewer hosts compared to representatives of genus *Beauveria*. Entomopathogenic fungi of the genus *Beauveria* have an essential place in modern plant protection and organic agriculture (Petkova and Spasova-Apostolova, 2024). The potential of *Beauveria bassiana* Vuill. (Hypocreales: Cordycipitaceae) as an entomopathogen, antagonist to phytopathogenic fungi, endophyte and plant growth stimulator has been demonstrated (Deb and Dutta, 2021). Entomopathogenic fungi work as a selective pesticide, but have a more complex action than chemical pesticides (Sandhu et al., 2012) because they are living microorganisms and their entomopathogenicity depends on many factors (Gutiérrez et al., 2024) and can be inhibited by insect immune responses (Zhu et al., 2024). Since *B. bassiana* requires an optimal temperature, this allows grasshoppers to inactivate it, through their body temperature (Dakhel et al., 2020), but *B. bassiana* has good metabolic plasticity (Gutiérrez et al., 2024) allowing it to bypass inactivation. The hydrolytic enzymes of entomopathogenic fungi determine their virulence since the insect cuticle is composed of chitin, proteins and lipids (Golzan et al., 2023). The production of lipase, chitinase and protease is different for individual strains of *B. bassiana* and determines their virulence (Gebremariam et al., 2022; Petkova and Spasova-Apostolova, 2024).

The purpose of the present research was to study lipolytic, proteolytic and chitin-degrading activity of *B. bassiana* strains 214, 644, and 733 and to testing their effectiveness on *T. viridissima* L.

Materials and methods

Cultivation of the fungal strains

The microbiological studies were carried out in the Microbiology and Environmental Biotechnologies laboratory of the Agricultural University – Plovdiv, Bulgaria. The strains are native fungi from Bulgaria and were provided by Prof. Slavimira Draganova Agricultural Academy - Bulgaria, Institute of Soil Science, Agrotechnology and Plant Protection "Nikola Pushkarov". Spasova-Apostolova and Petkova (2024) molecularly identified fungi species by using ITS-specific primers. The three strains of *B. bassiana* used in the present experiment belonged to the family Moniliaceae, order Moniliales, class Deuteromycetes, and were isolated from various sources. Strain 214 was isolated from *Agrilus mokrzeckii* (Coleoptera: Buprestidae), and strain 644 was isolated from *Tanymecus dilaticollis* Gyll. (Coleoptera: Curculionidae), and strain 733 was isolated from *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Radev et al., 2024).

The fungal strains were cultured in yeast extract agar with the composition yeast extract 3.0 $g \cdot L^{-1}$, peptone 5.0 $g \cdot L^{-1}$, agar 15.0 $g \cdot L^{-1}$, pH 7.2 ± 0.2. Petri dishes were maintained in an incubation chamber at 25 °C for 7 days.

Study of proteolytic, lipolytic and chitinolytic activity

The proteolytic activity of *B. bassiana* 214, 644, and 733 strains were tested by plating on milk agar (containing: casein 0.5%, yeast extract 0.25%, dextrose 0.1%, skimmed milk powder 2.5% and agar 1.5%) according to Vyas and Deshpande (1989). The strains were pre-activated in yeast extract agar for 7 days after a 9 mm disk from the mycelia was cut from the plates. The mycelial disks were placed on the skimmed milk agar and were incubated at 28 °C. The resulting diameter of the proteolytic zones was measured in mm and reported per day up to 7 days after inoculation.

Lipolytic activity was investigated by the agar diffusion method, like emulsified olive oil and resin-like substrate, according to the methodology of Pignede et al. (2000) in a 1:1 ratio. Results are reported as areas of colour change to red as a result of triglyceride absorption.

The chitinolytic activity was investigated by culturing the isolates on solid medium containing colloidal chitin as a carbon sink. Results are reported as and zone of clearance.

Isolation of DNA from tested *B. bassiana* strains

DNA isolation was performed using the HiPurA™ Fungal Genomic DNA Purification Kit (Canvax, Spain) following the manufacturer's protocol. Concentrations of isolated DNA samples were determined on an agarose gel compared with standard concentrations of lambda DNA (Thermo Scientific). Four concentrations (10, 25, 50, and 100 $ng \cdot \mu L^{-1}$) were prepared from a 300 $ng \cdot \mu L^{-1}$ stock solution of lambda DNA for comparison with a slight modification of the reverse primer *Bbchit1*-1 (5'- CCCTTCTACCCTTGACTTGTTC-3') and *Bbchit1*-2 (5'-ATCTACAAATATGTACCAAC-3') to amplify a 1225 bp fragment. The PCR reaction conditions were: 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C and 2 min at 72 °C with a final extension of 10 min at 72 °C. The PCR products were separated on a 1% agarose gel stained with SafeView (NBS Biologicals, UK) at 100 V for 50 min, as the VWR Mini Electrophoresis System is used for gel visualisation.

Molecular Detection of the *Bbchit1* Gene

Genomic DNA was extracted from mycelial biomass using a commercial fungal DNA isolation kit (e.g., GeneJET, Thermo Scientific) following the manufacturer's instructions. PCR was performed to detect the presence of the *Bbchit1* gene (ACC AY145440), which encodes a chitinase involved in cuticle degradation. The primer sequences used were based on Fang et al. (2005) with a modified reverse primer: Forward: 5'-ATCTACAAATATGTACCAAC-3' and Reverse: 5'-CCCTTCTACCCTTGACTTGTTC-3'. PCR amplification was carried out in a 25 µL reaction containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, 1 U Taq

polymerase, and 50 ng of template DNA. The thermal cycling conditions were: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 7 min. PCR products were visualized on a 1% agarose gel stained with SafeView (NBS Biologicals, UK) and run at 100 V for 50 min using a VWR Mini Electrophoresis System. A band of 1225 bp was considered indicative of the *Bbchit1* gene.

Insect Collection and Bioassay Design

Larvae of *Tettigonia viridissima* L. (Orthoptera: Tettigoniidae) were collected from wild populations in the vicinity of the Tobacco and Tobacco Products Institute (Markovo, Bulgaria). Healthy second to third instar larvae were selected and randomly assigned to treatment groups. Each fungal strain was tested on nine larvae placed in 100 × 20 mm Petri dishes previously inoculated with fungal mycelium and conidia. The assay was replicated three times per treatment, along with an untreated control. Petri dishes were maintained under ambient room conditions (22–25 °C, 60–70% RH, natural daylight). Larval mortality was monitored on days 1, 4, and 7 after exposure. Dead insects were identified based on lack of movement in response to mechanical stimulation.

Mortality data were corrected using Abbott's formula (Abbott, 1925):

$$E\ (\%) = (T - t)/T \times 100,$$

where T is the number of live insects in the control group, and t is the number of live insects in the treatment group.

Statistical analysis

Statistical analysis of the bioassay data was performed using one-way analysis of variance (ANOVA) in Microsoft Excel. Differences between means were considered statistically significant at $P \leq 0.05$. Graphs were generated using Microsoft Excel software with logarithmic regression for mortality over time.

Results

Under the selected experimental conditions, the three investigated strains of *B. bassiana* exhibited different levels of proteolytic, lipolytic and chitin-degrading activity (Table 1). *B. bassiana* 214 exhibited the highest proteolytic activity, but the highest lipolytic activity was observed in *B. bassiana* 644.

Table 1. Results of proteolytic, lipolytic and chitin-degrading activities of the three strains of *B. bassiana*

Beauveria strain / Activities	214	644	733
Proteolytic (mm)	> 15	< 10	< 10
Lipolytic (mm)	> 10	> 20	< 10
Chitinolytic (mm)	> 20	> 20	> 20

High chitinase activity was reported in all three studied strains. As a result of PCR analysis with primers designed by Fang et al. (2005) and located in the sequence of *Bbchit1* gene (Acc. AY145440), a fragment with the previously bioinformatically expected length of 1225 bp was found at all three studied genotypes (Fig. 1).

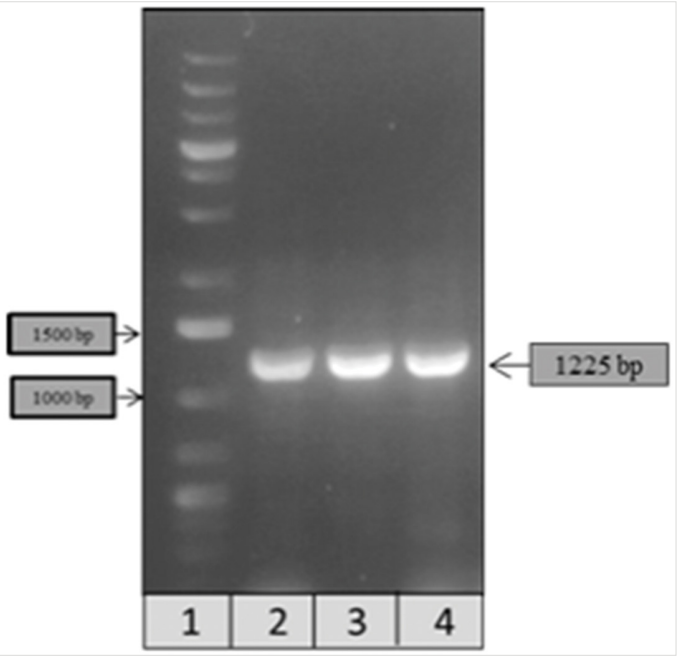

Figure 1. Agarose gel electrophoresis of amplified PCR products from *Bbchit1* gene of *B. bassiana* 214, 644 and 733 strains. Line 1: XY bp DNA ladder; Lane 2: *B. bassiana* strain 214; Lane 3: *B. bassiana* strain 644; Lane 4: *B. bassiana strain* 733

Therefore, since the fragment is single and intense, it can be assumed that at the DNA level a chitinase (*chit1*) gene is present in the genome of all three studied strains. They also exhibit chitin-degrading activity when cultured on a colloidal chitin medium.

The results of the test of effectiveness of *B. bassiana* strains on *T. viridissima* were presented (Fig. 2). The data showed that the *B. bassiana* strains 214, 644, and 733 showed effectiveness against larvae of green grasshoppers under laboratory conditions.

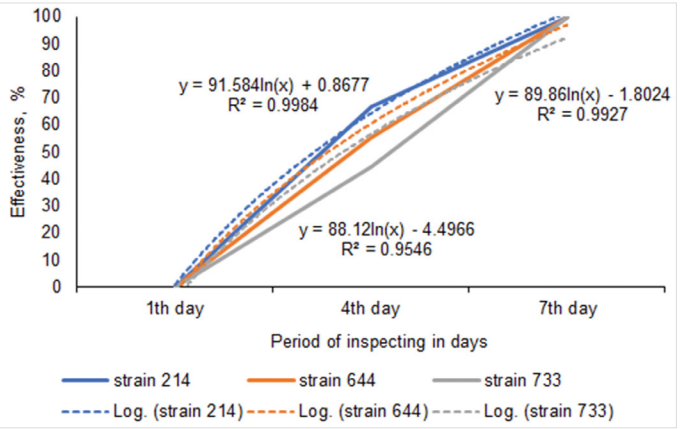

Figure 2. Effectiveness of *B. bassiana* strains 214, 644 and 733 against *T. viridissima*

Using the method of Abbott (1925), the logarithmic trendline results for: strain 214 y = 91.584In(x) + 0.8677 R^2 = 0.9984; strain 644 y = 89.86In(x) - 1.8024 R^2 = 0.9927 and for strain 733 y = 88.12In(x) - 4.4966 R^2 = 0.9546 showed 100% effectiveness against larvae of green grasshopper on the 7th day. The strain 214 showed the highest mortality of 66.7%, and strain 733 showed the lowest efficacy of 44.4%, while the strain 644 efficacy is in the middle with 55.6% effectiveness against larvae of *T. viridissima* on the 4th day after treatment. No effectiveness against green grasshopper larvae was found on 1st day after treatment (Fig. 2). No mortality of green grasshopper larvae in the control group was found. Although, they were found to have different effectiveness between strains 214, 644, and 733 on the 4th day after the test, the differences were not significant between the strains (F ≤ F crit, P = 0.9).

Discussion

The present research gives essential knowledge about the biocontrol of *T. viridissima* larvae under laboratory conditions by using *B. bassiana* strains. The insect *T. viridissima* is one of the pests that first appear in crops. The purpose of the present scientific work was to find out the activity of entomopathogenic fungus *B. bassiana* against this pest. Entomopathogenic fungi cause insect death through mechanical damage as a result of tissue invasion, depletion of food resources, toxicosis and toxin production in the insect (Sandhu et al., 2012).

The specific arrangement of the insect cuticle suggests easier degradation by the most virulent strains of entomopathogenic fungi. *B. bassiana* can synthesize various extracellular enzymes that enhance the potential of the entomopathogenic fungi as a biocontrol agent against pests (Nguyen et al., 2024). These strains had previously shown biocontrol activity against T. viridisima in tobacco plants (Radev et al., 2024). In the present study, all three tested strains caused death to *T. viridissima*, but a difference in their virulence was found, correlating with the difference in the production of proteolytic, lipolytic and chitin-degrading enzymes. Various authors have established the relationship between the virulence of genus *Beauveria* and the production of hydrolytic enzymes. A study of *B. bassiana* strain Bv065 showed only chitinolytic and proteolytic activity, suggesting that its virulence depends on higher proteolytic activity (Gutiérrez et al., 2024). In our study, strain 214 had the highest proteolytic activity and the highest insecticidal activity on day 4th, and 100% mortality of *T. viridissima* was achieved on day 7th in all three *B. bassiana* strains tested. Other studies showed that the high virulence is related to the high proteolytic and lipolytic activity of the entomopathogenic fungi (Abd-ElAzeem et al., 2024), and no relationship between the isolates virulence and the activity of exochitinases (Golzan et al., 2023). In terms of chitinolytic activity, light zones were found in the 3 studied strains, being clearer in *B. bassiana* 214 and 644 strains.

Two chitinase genes *Bbchit1* and *Bbchit2* have been cloned and characterized from *B. bassiana* genome (Fan et al., 2007). In 2005, the sequence of the *Bbchit1* gene (Acc. AY145440) was deposited in the NCBI database (Fang et al., 2005). The results obtained in this study are consistent with the study of Golzan et al. (2023) in which entomopathogenic fungi cause larval death, but there are significant differences in their virulence. In the present study, the two strains 214 and 644 showing the highest total activity also showed higher insecticidal activity on day 4th than strain 733. Regardless of the results in the present study, as well as in others, a dependence between virulence and enzyme activities was reported, it should be taken into account that many factors influence entomopathogenic processes (Golzan et al., 2023).

Overall, our findings suggest that strain-specific enzymatic profiling could be a valuable selection criterion for developing more effective and faster-acting *B. bassiana* biopesticides. Strains 214 and 644, in particular, exhibited high hydrolytic activity and early virulence, making them promising candidates for further field evaluation and formulation development.

CREDit Autorship Declaration

Zheko Radev: Conceptualization, methodology, field and laboratory experiments, writing – original draft preparation, supervision, data analysis, funding acquisition. **Velichka Spasova-Apostolova:** Conceptualization, methodology, analysis, manuscript draft preparation. **Mariana Petkova:** Conceptualization, methodology, microbiological analysis, laboratory experiments, investigation, data analysis, original draft preparation.

Declaration of Competing Interest

The authors declare that they have no known competing f inancial interests or personal relationships that could have appeared to influence the work reported in this paper.

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