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TEMPERATURE EFFECTS ON THE ENTOMOPATHOGENIC FUNGI *BEAUVERIA BASSIANA* STRAIN CNMN-FE-01: VEGETATIVE GROWTH, SPORULATION, GERMINATION RATE

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Abstract
As part of an approach for selecting potential mycoinsecticides for biocontrol control of weevils, the physiological properties of the strains are to be considered. The paper aimed to investigate the temperature effects on the vegetative growth, conidia germination, and viability of the fungal strains Beauveria bassiana CNMN-FE-01. As a result of the provided analyses, it was shown that the radial growth of the investigated strain follows a linear model. Based on the rate of average radial growth data, the optimum temperature growth at 25°C was determined, while at 35°C, the growth stops. Furthermore, it was found that the maximum rate of sporulation and germination of B. bassiana strain CNMN-FE-01 are at 25°C. At this temperature, the strain maintains the viability of the spores in a proportion of 86% for 90 days. However, temperatures above 25°C significantly reduce the viability of spores. The inhibition in growth of the investigated strain Beauveria bassiana CNMN-FE-01 at 30°C and higher and considerable reduction of its spore viability after extended exposure to 30°C testify to the need for repeated application when used as a biological control agent.

Keywords: Beauveria bassiana, germination, growth, sporulation, temperature

1. INTRODUCTION

Beauveria bassiana (Bals.-Criv.) Vuill. (1912) (Hypocreales: Cordycipitaceae) is an entomopathogenic fungus widely used as a biological control agent (Charnley, Collins, 2007; Wraight, Inglis, Goettel, 2007; Meyling, Hajek, 2010; Kryukov, 2012). A significant constraint to using fungi in biocontrol is their sensitivity to environmental conditions (Devi *et al*., 2005). Recent studies revealed the adaptation of *Beauveria bassiana* to environmental conditions (i.e., temperature, moisture) rather than to its hosts (Bidochka *et al*., 2002). Substantial implications for the efficiency of an entomopathogen as a biocontrol agent has its adaptation to a specific environment. Moreover, crucial for selecting environmentally well-adapted pathogens is climatic pressure (Fargues, Bon, 2004).

Amongst others, temperature is one of the major limiting factors that influences the growth and development of an entomopathogen. The ability to infect the host insect is also directly influenced by the temperature tolerance (Mietkiewski *et al*., 1994; Mishra, Kumar, Malik, 2015; Athanassiou *et al*., 2017). Increased temperatures have a major impact on fungus sporulation and spore viability, while extreme temperatures can completely inhibit the production of spores (Piatkowski,

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Krzyzewska, 2007; Mwamburi, Laing, Miller, 2015). It is also known that temperatures above 45°C induce the synthesis of heat shock proteins (Xavier, Khachatourians, 2011). Therefore, thermal tolerance is essential for the stability and efficiency of the mycoinsecticide under field conditions (Tong, Feng, 2020).

The fungal strain *Beauveria bassiana* CNMN-FE-01, isolated and registered in the Republic of Moldova, has demonstrated a potential to be used as a biological control agent against curculionid pests (Moldovan, Toderas, Munteanu-Molotievskiy, 2018; Moldovan, Munteanu-Molotievskiy, 2019). However, for the further preparation of local biological insecticides based on this strain, further investigations are needed to reveal the effect of abiotic factors on its growth and development.

The study aimed to evaluate the effects of temperature on vegetative growth, sporulation, and viability of *Beauveria bassiana* strain CNMN-FE-01. In this context, the following tasks were setup: 1) to assess radial growth within 15-35°C temperature range, 2) quantification of conidia produced after 30 days of cultivation at each tested temperature, 3) estimation of conidia viability after 90 days storage in incubator at 15°C, 20°C, 25°C, 30°C, and 35°C.

2. MATERIALS AND METHODS

Fungal isolate. In this study, *Beauveria bassiana* CNMN-FE-01, a fungal strain with high insecticidal activity against Curculionidae pests (Moldovan, Munteanu-Molotievskiy, Toderas, 2018), was tested to determine the temperature effect on its vegetative growth, sporulation, and conidia viability.

Inoculum preparation. Conidia were harvested from the nutrient medium with a sterile microbiological loop and transferred into a 1.5 ml tube containing 0.5 ml pure distilled water. The mix was shaken gently to prevent the release of conidia into the air. Mechanical methods were used to suspend hydrophobic conidia in water evenly and without cell damage. Subsequently, the suspension was vigorously shaken for 30 seconds by moving vertically and horizontally the pistil.

Conidia quantification. The number of conidia per unit of volume was quantified using the hemocytometer (Goryaev chamber) and visualized with Meiji Techno MT5310H stereomicroscope. The aqueous suspension with propagules was centrifuged, and 10 µl were transferred to the hemocytometer. For conidia sedimentation, the hemocytometer was kept horizontally for about 5 minutes. The conidia were counted in 5 large squares divided into 16 small ones (arranged diagonally), with a 0.00025 µl volume. To avoid repeated counting of the same conidia in one square, only those intersecting or touching the top and left sides of the square were considered. The number of conidia was calculated according to the formula: $C = (N \times d \times 4 \times 10^6)/80$, where: C - the concentration of propagules/ml, N - the number of propagules in 80 small squares of the hemocytometer, d - the dilution of the suspension. The arithmetic mean and standard deviation were calculated. The quantification was considered valid if the standard deviation was equal to 10-15% of the mean value.

Study of temperature influence on vegetative growth. A suspension of conidia in sterile distilled water with a concentration of 10^5 conidia/ml was prepared, by 100 µl of conidia suspension was plated on PDA (Potato Dextrose Agar, Merck) and incubated for three days at 25°C. Further mycelium discs (d = 5 mm) were excised, transferred aseptically to the center of a new PDA plate, and cultured at 15°C, 20°C, 25°C, 30°C, and 35°C (4 repetitions). The radial growth of the fungi was recorded daily for two weeks, measuring all four radii on two mutually perpendicular axes.

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Study of temperature influence on sporulation. The fungal culture was grown in Petri dishes for 30 days at temperatures of 15°C, 20°C, 25°C, 30°C, and 35°C. From each Petri dish with sporulated culture, four mycelium discs (d = 5 mm) were taken, placed in a tube with 1 ml sterile distilled water, and vortexed at 1000 rpm. Subsequently, 100 µl of suspension from each tube was transferred to a new one with 300 µl of pure distilled water and vortexed at 1000 rpm. Thus, a four-times diluted mix was obtained. The number of conidia was quantified using the hemocytometer according to the method described above. The number of conidia produced per cm^2 was determined.
Study of temperature influence on the viability of conidia. The viability of the conidia was estimated after 90 days of culturing the fungal strain at 15°C, 20°C, 25°C, 30°C, and 35°C. By 100 µl suspensions with approximately 10^7 conidia/ml, for each tested temperature, were evenly spread on sterile PDA plate, and incubated in darkness at 25°C for 24 h. Conspicuous swelling of the conidia was considered a sign of viability (Inglis, Enkerli, Goettel, 2012). Altogether, 200 conidia per plate were checked. The viability of the conidia was determined according to the following formula: R = (N / T) × 100%, where: R - viability/germination rate, N - number of germinated conidia, T - total number of conidia recorded. The method was repeated three times.
Data analysis. The following statistical parameters were used for data processing and analysis: the arithmetic mean (x_m), variance (σ^2), standard deviation (σ), and standard error (SE). Also, the confidence intervals were calculated, and the unifactorial dispersion analysis (ANOVA) was performed. The MS Office Excel 2010 application was used to perform mathematical calculations and graphical interpretation of the results.

3. RESULTS AND DISCUSSIONS

As a result of the research, the effects on vegetative growth, sporulation, and germination of conidia of the entomopathogenic fungal strain *Beauveria bassiana* CNMN-FE-01 at five different temperatures (15°C, 20°C, 25°C, 30°C, and 35°C) were determined. The analysis of the data regarding the vegetative growth, the measurements all four radii per colony in four repetitions of the investigated strain at different temperatures allowed to reveal the growth pattern (Figure 1).

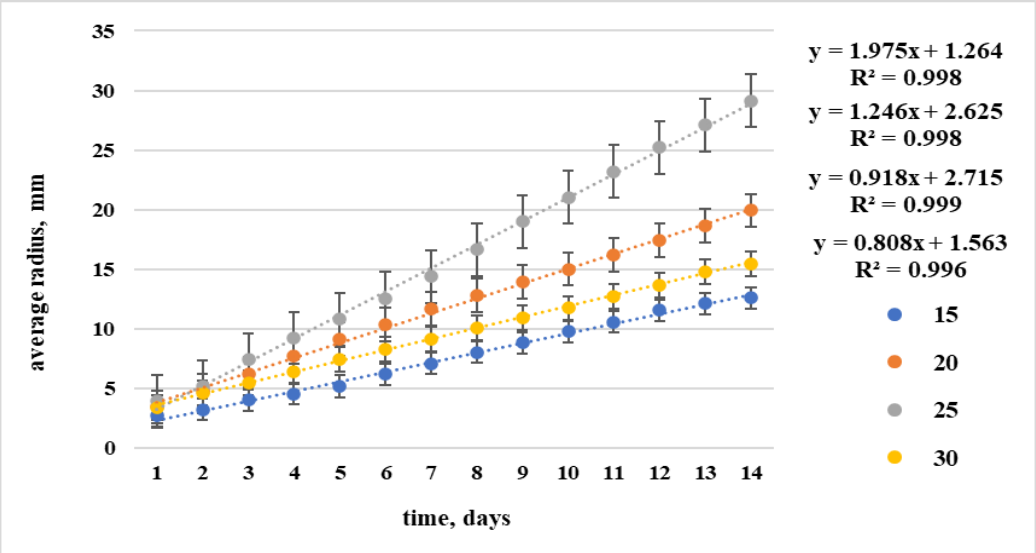

Figure 1. Vegetative growth of Beauveria bassiana strain CNMN-FE-01 at different temperatures.
Average colony radius (mm) is expressed as function of cultivation time (days). Linear equations and R^2 values displayed on graph

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Thus, the radial growth of the strain *B. bassiana* CNMN-FE-01 follows a linear model. From the equation of the line, the average radial growth rate was deduced, which is equal to the slope. The lowest growth rate was documented at 15°C (0.808 ± 0.015 mm/day), followed by a higher rate of 1.246 ± 0.017 mm/day at 20°C. The optimum growth temperature for the investigated strain with a rate of 1.975 ± 0.026 mm/day was observed at 25°C, a decrease in growth rate was recorded at 30°C (0.918 ± 0.007 mm/day), and at 35°C the growth of the strain stopped (Figure 2).

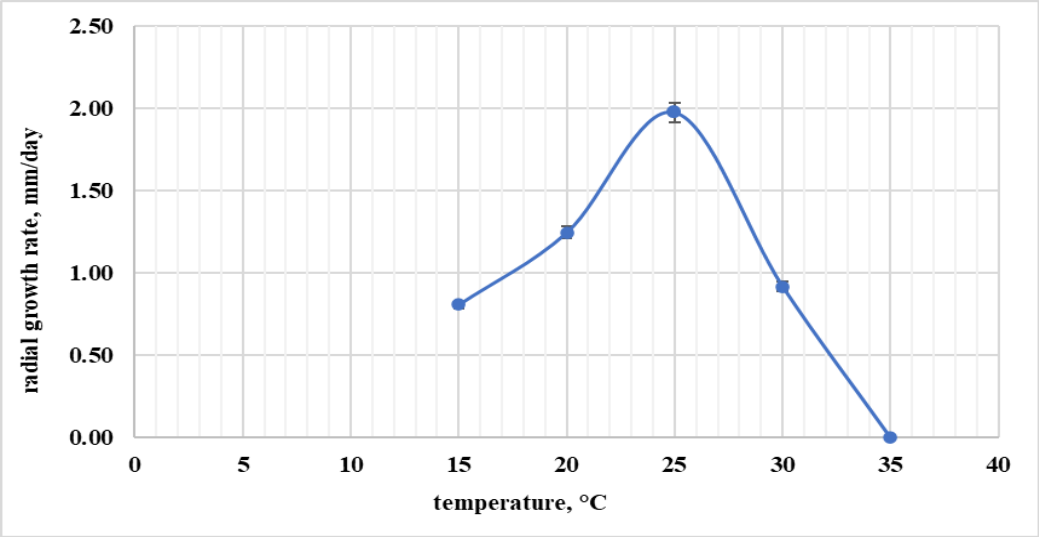

Figure 2. Radial growth rate, mm/day for B. bassiana CNMN-FE-01 strain at 15-35°C

Depending on the temperature tested, the number of spores produced by the *Beauveria bassiana* CNMN-FE-01 strain varied between 3.46 × 10^7 conidia/cm^2 at 15°C and 3.44 × 10^7 conidia/cm^2 at 30°C. The optimal number of spores, 9.78 × 10^7 conidia/cm^2, was recorded at 25°C. Due to vegetative growth inhibition at 35°C, no sporulation was observed (Figure 3).

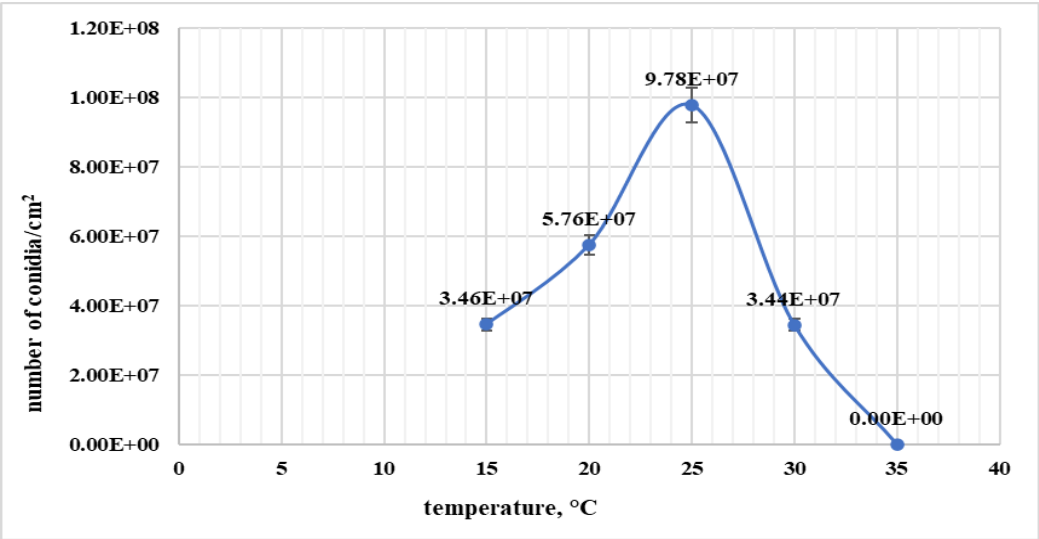

Figure 3. Number of conidia/cm^2 produced by B. bassiana strain CNMN-FE-01 at 15-35°C

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Statistically, the radial growth rate of investigated *B. bassiana* CNMN-FE-01 strain at five different temperatures differs significantly ($F(3.52) = 6.073$, $p = 0.001$).
The germination rate of the investigated strain conidia, after 90 days, varied between 40% at 15°C, the lowest temperature tested, and 7% at 30°C tested, the highest value of 86.5% was recorded at 25°C. Temperatures above 25°C significantly reduce the viability of spores (Figure 4).

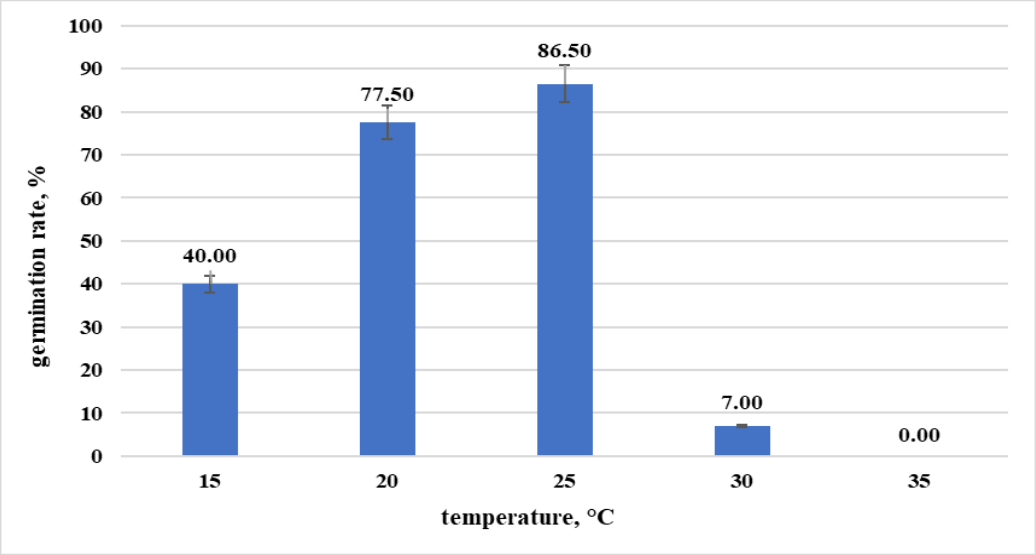

Figure 4. Conidia viability for B. bassiana strain CNMN-FE-01 after long-term culturing

As previously reported by Roberts and Campbell (1977) *Beauveria bassiana sensu lato*, is a mesophilic fungus able to growth between 6 and 35°C with optima between 20 and 30°C. Previous studies showed that the optimal growth temperatures of the entomopathogenic fungi may significantly vary between species and within species. For example, according to Hallsworth and Magan (1999), for species from *Beauveria*, *Metarhizium*, and *Paecilomyces* fungal genera, the optimum temperature for growth and development stands between 24°C and 27°C. In this study the investigated strain *B. bassiana* CNMN-FE-01, falls within the same temperature range in term of vegetative growth with an optimum at 25°C. Although, the lack of growth and development at temperatures close to the human body represents a significant advantage when developing insecticides based on entomopathogenic fungi (Butt, 2002; Fargues *et al.*, 1997). At the same time, the lack of high temperature tolerance can significantly impact the pathogenesis process in case of a substantial increase in the temperature at the surface of the soil (Arthurs *et al*., 2001; Rangel *et al*., 2005; Vega *et al*., 2012).
Most of the fungi are successfully grown in laboratory conditions. Currently, the research studies aim to find the fungal strains able to grow on available nutrients and produce a high quantity of spores. Another research topic aims to characterize the metabolites produced by fungal strains and identify those substances that play a vital role in the pathogenesis process. However, significant points should be addressed for the development of fungal-based biopesticides. In this context, more studies are needed regarding the response of fungal strains to various physical and chemical factors. It is known that in field conditions, temperature, humidity, radiation, and salinity significantly influence fungi's vegetative growth, sporulation, and viability (Jaronski, 2010; Vega *et al.*, 2012).

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However, few studies in recent years have sought to characterize the physiological aspects of biological control agents from this point of view (Piatkowski, Krzyzewska, 2007).
In the present study, the gradual inhibition of vegetative growth and conidia production at ≥30°C temperature was attested together with a significant reduction of conidia viability after long-term exposure at 30°C temperature. These patterns suggest that if the temperature above ground level hits 30°C, repeated application of *Beauveria bassiana* CNMN-FE-01 strain foliar spray is required. Also, treatments should be correlated with pest activity to maximize the probability of immediate contact between conidia and insect cuticle. Furthermore, alternative application strategies could be considered for this strain to be more effective such as seed treatment or investigation of endophytic potential.

4. CONCLUSIONS

Studies regarding the effect of various physical factors on the growth and development of fungal strains with high insecticidal potential are essential for predicting the efficacy of the selected strain in field conditions, biopesticide formulation, development of treatment recommendations, and adjustment of the doses required.

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