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In Vitro Thermal Requirements of Some *Beauveria* sp. Isolates Under Constant Conditions

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Abstract

The thermal tolerance of four isolates of *Beauveria bassiana* and one isolate of *Beauveria pseudobassiana* was evaluated in vitro, by measuring the colonial diameters, on PDA medium, at temperatures between 5 and 35 ° C, during 14 days. The data obtained were used to calculate the growth rate of fungal colonies (mm / day), using linear regression. The representation of the values corresponding to the minimum, optimal and maximum temperature for vegetative growth was a curve described by a modified beta (ß) mathematical function. The minimum growth temperature of these isolates varied between 3.4 and 4.5 °C, the optimum temperature varied between 21.8 and 22.9 °C, except for one isolate of which optimal temperature was 26.8 °C, while the maximum temperature was varied for all isolates between 35.0 and 35.7 °C.

Keywords: *Beauveria*, entomopathogenic fungi, thermal tolerance

Introduction

Beauveria bassiana is a cosmopolitan entomopathogenic fungus which causes white muscadine disease in a range of arthropods, including agricultural pests. *B. bassiana* is being exploited worldwide for insect pest management because of the ease with which it can be produced as biopesticide. Biocontrol strategies, which are based on the use of entomopathogens, dependent not only on the interaction between the pathogen and the host, but also on the environmental conditions to which they might interfere (Mishra et al., 2015). Many abiotic factors are known to have an influence on mycelial growth of entomopathogenic fungi, while also producing fundamental changes in the process of conidia production (Hu et al., 2021) and certain characteristics could restrain their use, in particular, temperature sensitivity, ultraviolet radiation or different abiotic factors (Han et al., 2021). Identification of the thermal profile of entomopathogenic fungi has a major importance in their selection as future candidates in the development of a mico-insecticide. Fluctuations in sensitivity to environmental factors depends on the isolate or specific strain, although the importance of temperature and relative humidity is known to influence the pathogenicity of entomopathogenic fungi (Bugeme et al., 2008). Thus, in this context, the identification of abiotic factors, which confer optimal biological characteristics of different strains, becomes extremely important, insofar as they denote virulence on the target insects. Since pathogenic fungi are living organisms, the percentage in which they penetrate and grow inside a host is also influenced by temperature (Klass et al., 2007).

Fungi have advantages being easy to handle good adaptation to different environmental conditions, specificity and infect target insects by contact (Lecuona et al., 1996). However, in order to obtain successful results, the environmental conditions to which the fungi will be exposed must be taken into account because temperature and humidity are the most important factors that influence the adaptability of entomopathogenic fungi. Although there is a belief in the scientific world that for the effective use of fungi in microbial control programs, moisture conditions are essential, some studies have shown that this ability of fungi to germinate and infect the host is attributed to ambient humidity in microhabitats. In contrast, temperature is a key factor because it influences metabolism by modifying the enzyme and toxins production processes, spore germination, germ tube development, penetration, colonization and reproduction (Alves, 1998). As a result, it is very important that thermal tolerance of one fungal isolate to overlap with climatic conditions in the environment of the target organism. Conidia become more sensitive in the context in which they are exposed to high temperatures during

product distribution and exposure during field treatment, considering that they are sensitive microorganisms (Burges, 1998). Given these, the need to identify the thermal profile has a major importance in the selection of entomopathogenic fungi as future candidates in the development of a micro-insecticide. This experiment can also be useful in predicting mycosis of soil insects, in field conditions.

Materials and Methods

To identify the thermal profile of the fungal isolates, five *Beauveria* fungal isolates from various regions of Romania were used (Table 1).

Table 1. Origin of fungal isolates (Romania, Europe)

Code	Species	Host insect	Romanian County of isolation
BbTd1	*Beauveria bassiana*	*Tanymecus dilaticollis* (adult)	Tulcea
BbTd2	*Beauveria bassiana*	*Tanymecus dilaticollis* (adult)	Ilfov
BbS1/07	*Beauveria bassiana*	*Leptinotarsa decemlineata* (adult)	Ialomita
BpLy	*Beauveria pseudobassiana*	*Lymantria dispar* (pupae)	Vrancea
BbId	*Beauveria bassiana*	*Ips duplicatus* (adult)	Botosani

In this experiment, monosporal isolates were used, which were obtained as follows: a suspension of spores diluted to a titer of 10 - 20 CFU / ml was sown on PDA medium poured in a very thin layer (12-15 ml) in a Petri dish. After an incubation period of 18 hours at 25 ° C, the Petri dish was placed on the microscope plate and examined under an optical microscope using the 10 x objective. Once the propagule was localized, the platinum was adjusted so that the conidia were in the center of the microscopic field. Then, the lens was moved to one side and the microscope aperture was closed until only the area of interest remained illuminated.

An agar disk (approx. 4-5m) was cut and aseptically transferred to another culture vessel using a seed needle tip. Microscopic observations were made at various intervals until the development of the colony was observed, to ensure that only one propagule was transferred, it was not affected during the manipulations and also no contaminants emerged. Discs (0.5 cm diameter) were cut from unsporulated monosporal cultures for each fungal isolate, using a cork borer and transferred to Petri dishes with PDA medium. They were sealed with Parafilm and incubated in dark conditions at the following temperatures 5, 10, 15, 20, 25, 30, 35 ± 0.1º C. For each isolate and temperature value, 5 repetitions were set up. The radial growth of the colonies was recorded by measuring two perpendicular diameters previously marked on the back of the box, every 2 days for 14 days. Because a linear relationship between colony diameter and time was observed, the growth rate of the fungal mycelium (mm / day) was calculated using linear regression (y = a + bx) using GraphPad Prism v. 7.00 biostatistics software for Windows. Thus, for each isolate, at all temperature values and for each repetition, the growth rate of the fungal mycelium was estimated by the regression coefficient b, after previously testing the significance of b, using the F test (Ceapoiu, 1968). For the estimation of the optimal, maximum values and estimation of the maximum growth rate of the vegetative mycelium, a nonlinear regression model represented by the modified ß (beta) function after Bassanezi (1998) was used. The beta function is given by the equation:

$$Y = Yo * \left[\frac{T - Tmin}{Topt - Tmin}\right]^{\left[B3 * \frac{Topt - Tmin}{Tmax - Topt}\right]} * \left[\frac{Tmax - T}{Tmax - Topt}\right]^{B3},$$

where Y represents the growth rate of the fungus (mm / day) at the incubation temperature, T.

T_{min}, T_{opt} and T_{max} represent the value of minimum, optimal and maximum fungal growth temperatures. Y_o represents the growth rate at the optimum temperature and T_{opt}. B_3 is the curve equation parameter, without biological significance.

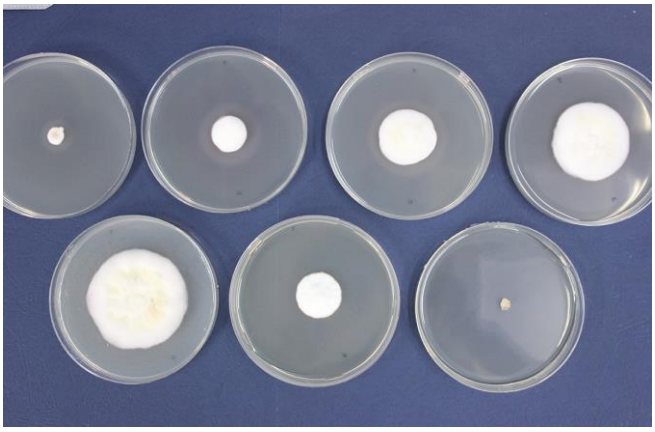
This parameter can have values between 0 and 3, so that the higher its value, the steeper the curve around the maximum value of Yo (Bassanezi, 1998). The adjustment of the parameters T_{min}, T_{opt}, T_{max}, Y_o and B_3 to make the regression curve approach the established points, was done by the Marquardt and Levenberg method. The

validation of the model was performed based on the coefficient of determination r^2 and the SD (standard deviation). The estimation of the parameters using the beta function was performed for each repetition of each temperature value and for each fungal isolate. The difference between isolates was analyzed by applying the t test. All statistical analysis was done using GraphPad Prism v.7 software for Windows.

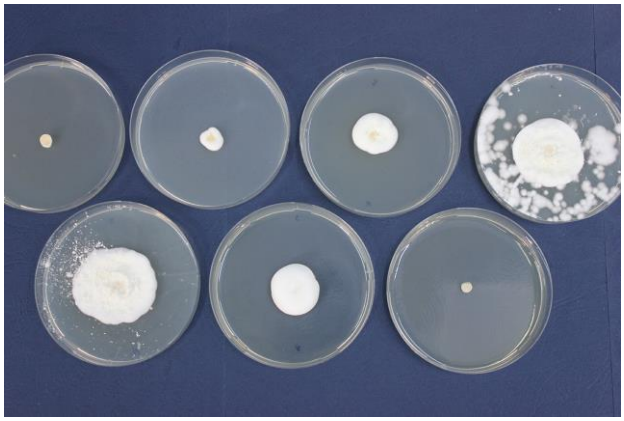
Results and Discussion

The temperature had a significant effect on the radial growth in vitro for all analyzed strains. It was observed that the measurements recorded during the observation period (14 days) at test temperatures of 10, 15, 20, 25, 30 ºC, correspond to linear regression models. The coefficients of determination for regression lines were between 0.843 and 0.999. All fungal isolates developed at temperatures of 10, 15, 20, 25, 30 ºC (Fig. 1). During the observation period, no development of vegetative mycelium was recorded at the temperature of 5 ºC and 35 ºC but in all isolates they developed at a temperature of 5 ºC outside the observation period (after 14 days).

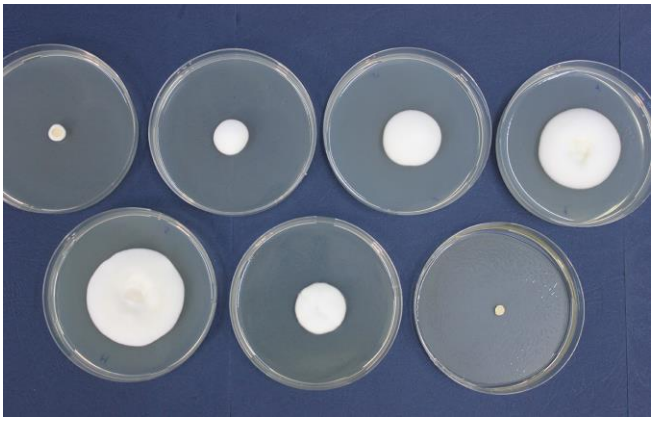
The growth rate of the fungus indicated by the linear regression curve was the main parameter for evaluating the influence of temperature on vegetative growth. Growth rate of the colonies varied from 0.29 to 0.60 mm/day at 10 ºC, from 0.48 to 1.04 mm/day at 15 ºC, from 0.9 la 1.4 mm/day at 20 ºC, from 0.95 to 1.6 mm/ day at 25 ºC and from 0.52 to 1.2 mm/ day at 30 ºC.



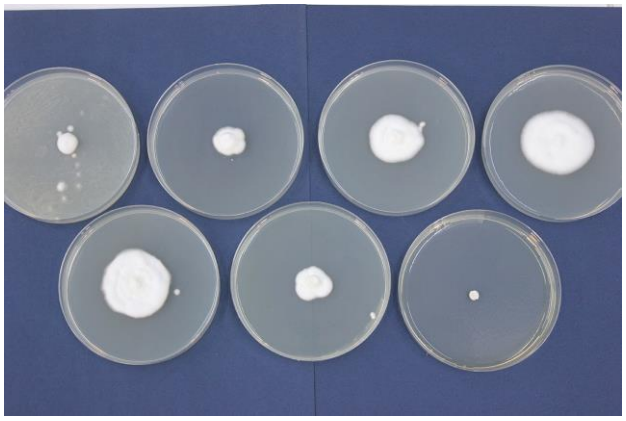
BbTd1



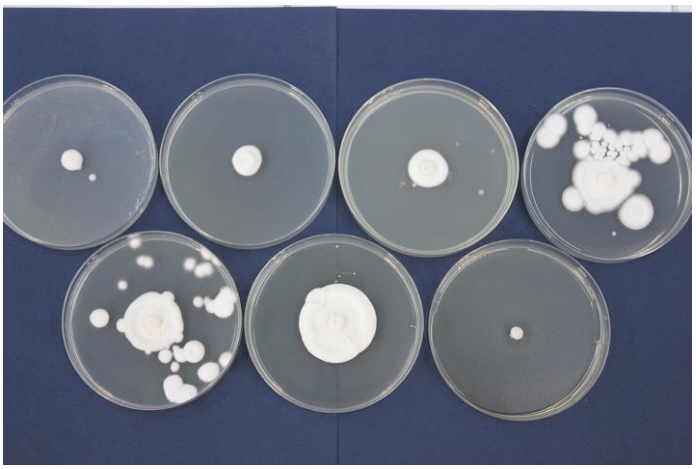
BbTd2



BpLy



BbId



Bbs1/07

Figure 1. Appearance of *Beauveria* isolates that were incubated under different temperature conditions (5, 10, 15, 20, 25, 30, 35 ºC), on PDA medium, after 15 days

The nonlinear regression model was applied to estimate the minimum, optimal and maximum growth parameters. Growth curves with a coefficient of determination ranging between 0.92 and 0.98, were obtained using a modified ß function according to Bassanezi et al. (1998) (Fig. 2 and Table 2).

Table 2. Estimated parameters and coefficients of determination r^2 of *Beauveria* isolates calculated using the modified ß function, according to Bassanezi et al. (1998) - each value represents the mean ± standard deviation of five repetitions

Isolate	Y_o	T_{min}	T_{opt}	T_{max}	B_3	r^2
BbTd1	1.30±0.01	3.85±0.74	22.91±0.05	35.00±0.00	1.73±0.09	0.92±0.01
BbTd2	1.4±0.10	4.51±0.58	22.39±0.58	35.72±0.85	1.65±0.43	0.98±0.01
BpLy	1.41±0.05	4.48±0.33	22.56±0.38	35.00±0.00	0.91±0.06	0.95±0.01
BbId	1.13±0.11	4.54±1.01	21.85±1.29	35.14±0.25	1.38±0.29	0.97±0.01
BbS1/07	1.11±0.04	3.44±0.51	26.79±0.30	35.00±0.00	0.58±0.02	0.94±0.02

The value of parameter B3 varied between 0.58 and 1.73, the highest being calculated for BbTd1 isolate. This result reveals that there is a very narrow range of temperature values at which the growth rate of fungal mycelium is close to the maximum value.

The most important advantage in using the modified beta function according to Bassanezi et al. (1998) is represented by the fact that all parameters have biological significance, while other models do not provide parameters with these characteristics (Quesada M. et al., 2005). Analysis of the variant (test t) showed that there were no significant differences between the isolates in terms of the relative value of the minimum temperature. It ranged from 3.4 ± 0.5 ºC (Bbs1 / 07) to 4.5 ± 1.0 ºC (BbId). The optimum growth temperature exceeded 25 ºC only for Bbs1 / 07 isolate. This isolate differs significantly from the others, at which the optimal temperature varies between 21.8 ± 1.2 ºC (BbId) and 22.9 ± 0.05 ºC (BbTd1). The results of comparing the growth rates at the optimum temperature demonstrated significant differences between isolates. The growth rate at the optimum temperature varied between 1.1 ± 0.04 mm / day (Bbs1 / 07) and 1.4 ± 0.05 mm / day (BbLy). No significant differences were observed between BbTd1 and BbTd2, between Bbs1 / 07 and BbId, nor between BbLy and BbTd isolates.

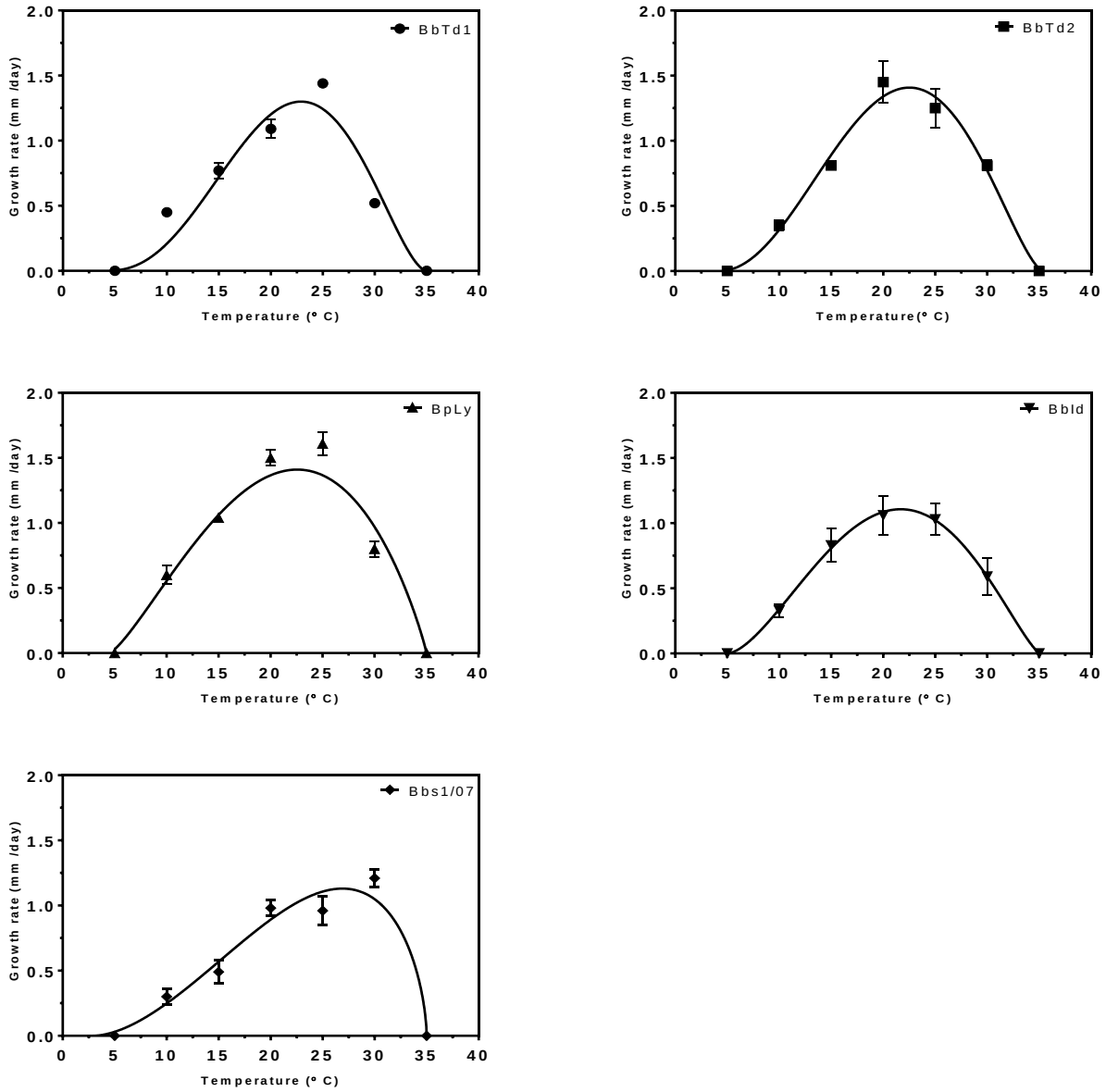


Figure 2. Prediction of the effect of temperature on growth rate of *Beauveria* colonies. The lines represent curves estimated by using the beta function (ß) modified according to Bassanezi et al. (1998).

Analyzing the data resulting from the tests performed in the laboratory, and comparing them with those in the literature, both similarities and slight differences were observed. Respectively, according to the premise that the temperature influences the rate of radial growth, it was observed that for all variants exposed to different temperature conditions from 5 to 35 ºC, the entomopathogenic fungus showed or not a radial growth. Thus, only for the 10-30 ºC interval a vegetative growth of the mycelium was noticed, some studies (Rodríguez et al., 2009) presenting, however, data according to which the fungus increased even at 5 and 35 ºC, but the growth being weaker at 20 and 35 ºC. Rodríguez et al. (2009) also present in their study the fact that, although for the variant with a temperature of 30 ºC a linear growth rate was registered, for the variant with a temperature of 35 ºC most of the isolates did not grow, except for three isolates of *B. bassiana* (P>0.0001). Other studies have shown, that exposure to 35 ºC, for 24 h, determined no fungal germination (Lizzy et al., 2015), and at 37 ºC the impossibility of the fungus to tolerate such of high temperature conditions (Borisade et al., 2014). It is relevant, that in the case of tests performed for the temperature of 5 ºC, outside the observation period, there was

observed a growth for all analyzed isolates. Shimazu (2004) found that even after exposure at high temperatures, the fungus was able to recover and grow after transfer to 25 ºC. In the case of the optimum growth temperature, except for the Bbs1 / 07 isolate at which it exceeded 25 degrees C, most isolates preferred temperature conditions between 21.8 ± 1.2 ºC (BbId) and 22.9 ± 0.05 ºC (BbTd1). Comparison with other results showed a slight difference, in other studies the optimal temperature being 24.5-25.5 ºC (Qiu et al., 2019). A higher thermotolerance of entomopathogenic fungus is indispensable for a better practical application (Yu et al., 2020).

Conclusions

The temperature had a significant effect on the radial growth in vitro for all analyzed strains. No development of vegetative mycelium was recorded at the temperature of 5 ºC and 35 ºC but in all isolates they developed at a temperature of 5 ºC after 14 days. There is a very narrow range of temperature values at which the growth rate of fungal mycelium is close to the maximum value. The experiments will be continued with studies focused on the influence of culture medium on thermal tolerance of fungal strains.

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Conflicts of Interest

The authors have no conflict of interest to declare.

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