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IN VITRO THERMAL REQUIREMENTS OF TWO ISOLATES OF *METARHIZIUM ANISOPLIAE* (METCH.) SOROK. UNDER CONSTANT CONDITIONS

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Abstract: The effect of temperature on the mycelial growth of two isolates of *Metarhizium anisopliae* (Metch.) Sorok. (Hypocreales, Clavicipitaceae) was evaluated in vitro on PDA growth medium, by measuring the diameter of the fungal colonies, at the following temperature values: 5, 10, 15, 20, 25, 30, 35 and 40 °C, over 14 days under constant conditions. The data obtained were used for the calculation of the growth rate (mm/day) using linear regression. The representation of the values corresponding to the minimum, optimal and maximum temperature of vegetative growth was made by a curve described by a mathematical function, a modified beta (ß) function. The lowest temperatures at which growth occurs are 7 and 10°C, the highest is 35.7°C; temperature at which the most growth occurs, as indicated by the measurements, was distributed over a range of temperatures from 22.9 to 31°C.

Key words: *Metarhizium anisopliae, entomopathogenic, growth rate, temperature*

INTRODUCTION

Entomopathogenic fungi are the most common reported natural enemies in agricultural fields, causing regular epizootics (Rios-Velasco et al., 2010). A very small number of them are used as biological control agents. *Metarhizium anisopliae* is one of the most studied and used species of entomopathogenic fungi in microbial control (Zimmermann, 2007).

Fungi are affected by many biotic and abiotic factors in their environment (Roy et al., 2006). Temperature is a primary factor affecting the rate of conidial germination and mycelial development which is closely related to the rate of infection and virulence against the target pests (Nussenbaum et al., 2013; Vidal et al., 1997). The ability to persist in the target habitat following application is one of the most important and necessary characteristic of a fungal isolate. Temperature and moisture are two essential ecological factors that influence fungal survival, vegetative growth, sporulation and host defenses. The different fungus species and isolates within each species vary in their tolerances to these ecological factors. This is why intra-specific differences in the germination response must be taken into account when selecting isolates for development as myco-insecticides (Hywel-Jones & Gillespie, 1990).

In Romania, studies on the effect of temperature on the biology and virulence of entomopathogenic fungi were made by Andrei (1998) and Fătu et al. (2015).

In this paper are presented the results of laboratory tests performed with two *M. anisopliae* isolates, in order to assess the thermal regimes which are a prerequisite in selection of isolates for the application in biological control.

MATERIALS AND METHODS

Fungal isolates

Two isolates of *M. anisopliae* were used in experiments, one autochthonous isolate (MaF) originated from an infected larva of *Anoxia* sp. and the other (DSM 1490) from German Collection of Microorganisms and Cell Culture GMBH. Monosporal cultures were obtained on PDA medium.

Determination of temperature requirements

From nonsporulated monosporal cultures, 0.5 cm diameter discs were cut with a corke-borer and a single disc was placed in the center of a new dish of PDA medium. The dishes were sealed with Parafilm and incubated in dark at different temperatures ranging from 5 to 40ºC with temperature intervals of 5ºC. For each temperature and isolate, 5 Petri dishes were placed. The diameter of the fungus colonies was measured every 2 days until day 14.

Because a linear relationship between colony diameter and time was observed, the growth rate of the fungal mycelium (mm / day) was calculated by linear regression (y = a + bx) using GraphPad Prism version 7.00 biostatistics software for Windows. Thus, for each isolate, at each temperature ranging from 10 to 35ºC 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).

To estimate the optimal temperature, maximum growth rate and maximum temperature for growth, a nonlinear regression were performed for each isolate by fitting the following modified ß (beta) function (Bassanezi et al., 1998) to the growth rate data:

$$Y=Yo*[(T-T_{min})/(T_{opt}-T_{min})]^{[B_3*(T_{opt}-T_{min})/(T_{max}-T_{opt})]}*[(T_{max}-T)/(T_{max}-T_{opt})]^{B_3}$$

where Y is the growth rate at T, where T is the incubation temperature. T_{min}, T_{max} and T_{opt} are, the lowest, the highest and the optimal temperature for fungal growth respectively. Yo is the fungal growth at the optimal temperature T_{opt}. B_3 is a shape parameter of the growth curve ranging from 0 to 3: the higher values, the sharper decline of the curve around the peak.

RESULTS AND DISCUSSIONS

The temperature influences *in vitro* mycelial growth of *M. anisopliae*. Both fungal isolates grew at 10, 15, 20, 25, 30, 35 and 40ºC. For the first two days, both isolates had a radial growth at 40ºC, and then stopped and no other development was noticed throughout the 14-day observation period. However, MaF strain slowly expanded its hyphal growth at 40ºC, so that 27 days after the last observation (day 14-th), the colony diameter was 4.2 times larger than in the first two days. No radial growth was observed at 5ºC. A linear relationship between colony diameter and the time needed for growth at the tested temperatures 15, 20, 25, 30 and 35 ºC was observed. The colony extension rate varied from 0.22 to 0.49 mm/day at 15 ºC, from 0.65 to 1.38 mm/day at 20 ºC, from 1.04 to 2 mm/day at 25 ºC, from 0.37 to 1.34 mm/day at 30 ºC, from 0.59 to 0.69 mm/day at 35 ºC. The effect of temperatures on mycelial growth of the fungal is presented in Figures 2 and 3.

Applying the nonlinear regression model, for estimating the minimum, optimal and maximum growth parameters, growth curves were obtained with a determination coefficient that varied between 0.72 and 0.99, using a modified ß function, modified according to Bassanezi et al. (1998) (Figure 1 and Table 1). The shape parameter for MaF isolate was high (3.46) which mean that the temperature ranges around T_{opt}, in which the curve stays near to Yo is very narrow. The optimum growth temperature for the Romanian isolate (MaF) was lower than the other, probably because of the different geographic origin. The MaF isolate was obtained from a mycosed *Anoxia* sp. larva, founded in the south region of Romania (Ialomita County), an area where air temperatures could reach 40 ºC.

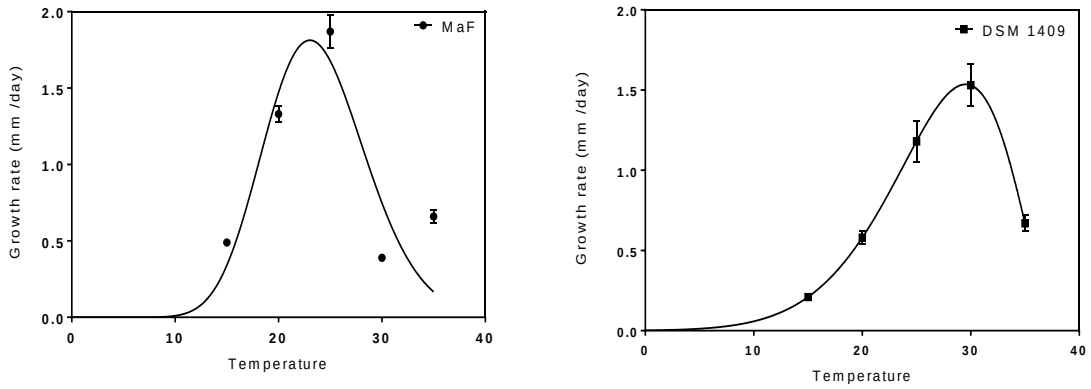

Figure 1.Growth curves of *Metarhizium anisopliae* isolates at different temperatures obtained using the generalized ß function modified according to Bassanezi et al. (1998)

Table 1. Estimated parameters and coefficients of determination r^2 of *Metarhizium* isolates calculated using the function ß modified in accordance with Bassanezi et al. (1998)

Isolate	Optimal growth rate (mm/ day)	Minimum growth temperature (ºC)	Optimum growth temperature (ºC)	Maximum growth temperature (ºC)	B_3	r^2
Ma F	1.48±0.04	10.00±0.00	22.95±0.26	43.83±1.28	3.46±0.67	0.72±0.04
DSM 1409	1.63±0.23	7.24±2.86	31.06±2.08	35.51±0.49	0.52±0.34	0.99±0.01

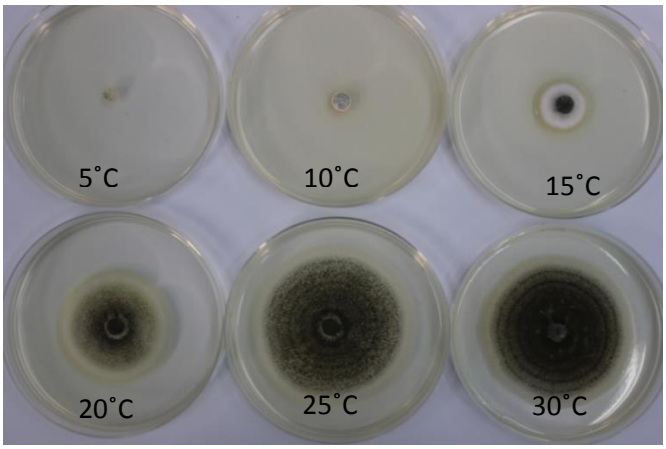

Figure 2. Growth of MaF fungus colonies on PDA medium at different temperatures, after 17 days

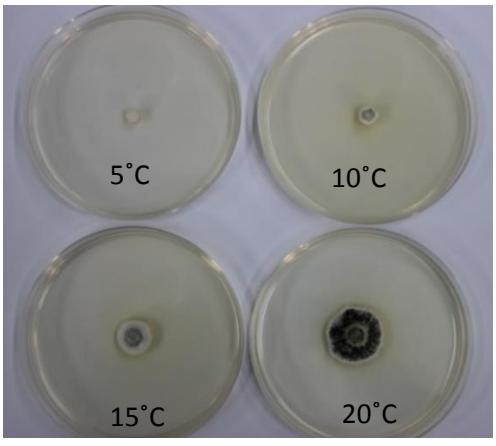

Figure 3. Growth of DSM 1409 fungus colonies on PDA medium at different temperatures, after 17 days

Optimal germination and growth rates of fungal entomopathogens generally range between 23 and 28°C and fail at 34-37°C (Jaronski, 2009). *Metarhizium* sp. is an exception from the rule. The thermal death point for *M. anisopliae* conidia has been found to be between 49 and 60°C (Ment et al., 2011) and some authors stated that the maximal temperature for conidial germination and mycelial growth is around 35-37°C (Polar et al., 2005). Our findings confirm that these two strains also have the ability to grow at temperatures higher than 35ºC.

There are also laboratory studies on a *M. anisopliae* strain at which the fastest growth rate was recorded at 25-30ºC (Ekesi et al., 1999). Laboratory evaluation of temperature effects on the germination and growth of some entomopathogenic fungi revealed that the rate of in vitro conidial germination of all isolates was slower at 10 and 15ºC than at 20 and 25ºC; the greatest reduction at 10ºC in rates of conidial germination and colony growth, compared with other temperatures, was for *M. anisopliae* isolates (Yeo et al., 2003). When growth rates of different isolates of *M. anisopliae* were compared, Brooks et al. (2004) found that fungal growth was higher at the lower temperatures and none of the isolates grew at 40ºC; the growth of an isolate declined markedly with temperature and, in contrast, other isolates grew almost as well at 32 and 35ºC, as at 28 and 30ºC; some isolates showed some growth at 37.5ºC. High thermotolerance of *M. anisopliae* isolates evaluated by measuring its growth on an artificial medium kept between 25 and 37.5ºC is mentioned by Lekimme et al. (2008) which found that although all isolates of different entomopathogenic fungi were able to grow up to 30ºC, only *M. anisopliae* and *Paecilomyces farinosus* tolerated temperatures up to 35ºC. In Romania, a thermotolerant strain of entomopathogenic fungus was also identified; is about a *Beauveria bassiana* strain, which, in conditions of high temperature and drought caused mass disease in a population of short-horned grasshoppers in the family Acrididae which infested an alfalfa crop in Constanta County (Andrei & Galani, 1996).

CONCLUSIONS

MaF strain, the autochthonous isolate originated from an infected larva of *Anoxia* sp., could be considered a good candidate for mycoinsecticide production. Further research is needed to evaluate *in vivo* thermal requirements of *M. anisopliae* strains, in order to determine the temperature range of insecticidal activity against pests, considering the importance of host-pathogen interactions in pathogenicity and virulence; germination, vegetative growth and sporulation of entomopathogens are dependent on host defense mechanism.

The isolation of MaF strain from Ialomita County, a region of Romania meteorologically characterized by dry periods and high temperatures during the summer, proves the capacity of this fungal strain to survive in such condition.

Resistance of conidia to high temperatures ensures the stability of the bioproduct under high temperatures and represents an asset for use of fungi in biological control of pest insects from semi-arid areas, or during severe weather conditions, with hot days and high temperatures on the ground and in the air.

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