

SELECTION OF ENTOMOPATHOGENIC FUNGI TO CONTROL *Varroa destructor* (Acari: Varroidae)

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ABSTRACT

The aim of this work was select entomopathogenic fungi tolerant to temperatures inside the brood area of honey bees (*Apis mellifera*) for to control *Varroa destructor*. For this purpose, 50 *Beauveria bassiana* (Balsamo) Vuillemin and 48 *Metarhizium anisopliae* (Metschn.) Sorokin isolates were evaluated at 30 and 35 °C. For each isolate, colony discs of 5 mm with mycelium were placed in the center of a Petri dish with Sabouraud dextrose agar (SDA) medium. The dishes were incubated at 30 and 35 °C, without light. Radial growth of each colony was measured daily. All the *B. bassiana* and *M. anisopliae* isolates presented a lineal growth rate at a temperature of 30 °C. However, at 35 °C, most of the isolates did not grow, except three *B. bassiana* and 14 *M. anisopliae* isolates ($P < 0.001$). These isolates were selected to evaluate the pathogenicity on *V. destructor* by applying a suspension of 10^7 conidia mL⁻¹. The most effective isolate was *M. anisopliae* Qu-M845 ($P = 0.0033$) which produced 85% mortality. The pathogenic capacity of this isolate on *V. destructor* and its tolerance to hive conditions allows us to consider this strain as an alternative to control this pest.

Key words: biological control, entomopathogenic fungi, *Varroa destructor*, varroasis, entomopathology.

INTRODUCTION

The *Varroa destructor* (Acari: Varroidae) (Anderson and Trueman, 2000) mite, an ectoparasite of the honey bee (*Apis mellifera*; Hymenoptera: Apidae), is considered as a very serious pest affecting apiarists worldwide, and if not controlled can cause losses of up to 100% of the bee colonies (Calderone, 1999). Until now, control of this pest has been based on chemical acaricides which in turn affect production costs, toxic residue content, and resistance development (Melathopoulos *et al.*, 2000).

A partial or total alternative to the use of chemical acaricides is the biological control of varroa which could be part of an integrated management system of the pest. Research of natural enemies against mites phylogenetically related with varroa point out the use of entomopathogenic fungi as a promising alternative (Chandler *et al.*, 2001). Indeed, Chandler *et al.* (2000) reported the susceptibility of *V. destructor* to infection by *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschn.) Sorokin fungi when

the mites were placed on these colonies. Kanga *et al.* (2003) found a highly pathogenic *M. anisopliae* isolate for *V. destructor*, reaching control levels in field assays similar to those obtained with fluvolate acaricide 42 days after its application.

Although the fungi presented the advantages of easy manipulation, adaptation to different environments, specificity, and penetration directly through the tegument of the host (Lecuona *et al.*, 1996), to achieve successful results, environmental conditions to which they will be exposed must be considered since these can influence germination and subsequent host colonization by the pathogen (Alves, 1998). One of the most important abiotic factors for the entomopathogenic fungi is temperature since it affects its metabolism by altering the production processes of enzymes, toxins, spore germination, development of the germinative tube, penetration, colonization, and reproduction (Alves, 1998).

Temperatures in bee colonies can be a determinant in the effectiveness of any biological control agent for varroa. Summer temperatures in the colony brood area are usually maintained between 32 and 37 °C while in the rest of the beehive vary from 28 to 33 °C. In winter, in temperate regions, temperature is maintained between 20 and 30 °C (Chandler *et al.*, 2001). Le Conte *et al.* (1990) point out that optimal temperature for the development of *V.*

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destructor is between 32.5 and 33.4 °C which corresponds to the bee brood temperature. On the other hand, the optimal temperature for growth of most Hyphomycetes fungi, order in which the *M. anisopliae* and *B. bassiana* species belong, is between 20 and 25 °C with infections and pathogenesis that occur at temperatures between 15 and 30 °C. Over 30 °C, vegetative growth of most Hyphomycetes species is inhibited and usually ceases at 37 °C (Inglis *et al.*, 2006).

The objective of this research study was to select *Metarhizium anisopliae* var. *anisopliae* and *Beauveria bassiana* isolates for their capacity to grow at temperatures between 30 and 35 °C and evaluate the pathogenicity of the selected isolates on *Varroa destructor* to control this pest.

MATERIALS AND METHODS

Isolate selection and multiplication

Isolates, 50 *B. bassiana* and 48 *M. anisopliae* var. *anisopliae*, were selected from the entomopathogenic collection of fungi from the Centro Tecnológico de Control Biológico del Instituto de Investigaciones Agropecuarias INIA, Chillán, Chile which are stored as monospore cultures at -198 °C in liquid nitrogen. Each isolate was sown by taking conidia with a bacteriological loop. Conidia were superficially scattered onto the nutritive medium of Sabouraud dextrose agar (SDA) contained in Petri dishes 90 mm in diameter. The dishes were incubated at 25 °C for 2 or 3 days.

Evaluation of radial growth

Cultures were carried out and, before observing conidia development, 5 mm diameter discs with agar plus mycelium were extracted from the colony edge growth with a lab scoop. These discs were placed in the center of the Petri dishes with SDA medium and the mycelium in contact with the nutritive medium. Dishes were incubated in incubation chambers in the dark at temperatures of 30 and 35 °C. Colony radial growth was recorded periodically for 18 days, measuring the growth radius from the center of the colony in four equidistant radii previously marked on the bottom of each dish.

The experimental unit corresponded to a Petri dish. The experimental design was completely randomized with three replicates for each isolate and temperature treatment. Analysis of variance and medium separation was carried out with the Tukey multiple comparison test.

Growth rate

Radial growth data were expressed as growth (mm) over time. Growth rate for each isolate and temperature was calculated from the linear regressions of each

replicate which corresponded to the slope of the regression (Ouedraogo *et al.*, 1997). Different growth rates were compared on day 18 postsowing.

Pathogenicity tests

Isolates were selected for their growth at temperatures of 30 and 35 °C (Figures 1 and 2). In this way, 14 *M. anisopliae* and three *B. bassiana* isolates were cultured by means of sowings in Petri dishes with papa dextrose agar, and were incubated at 25 °C until fungal sporulation. Conidia were harvested from the surface of the culture; a suspension was carried out in sterile distilled water with 1% Tween 80 at 0.1% and the conidia were disintegrated with a gyratory shaker. Conidia concentration was determined with a Neubauer (BOECO, Neubauer, Germany) counting chamber.

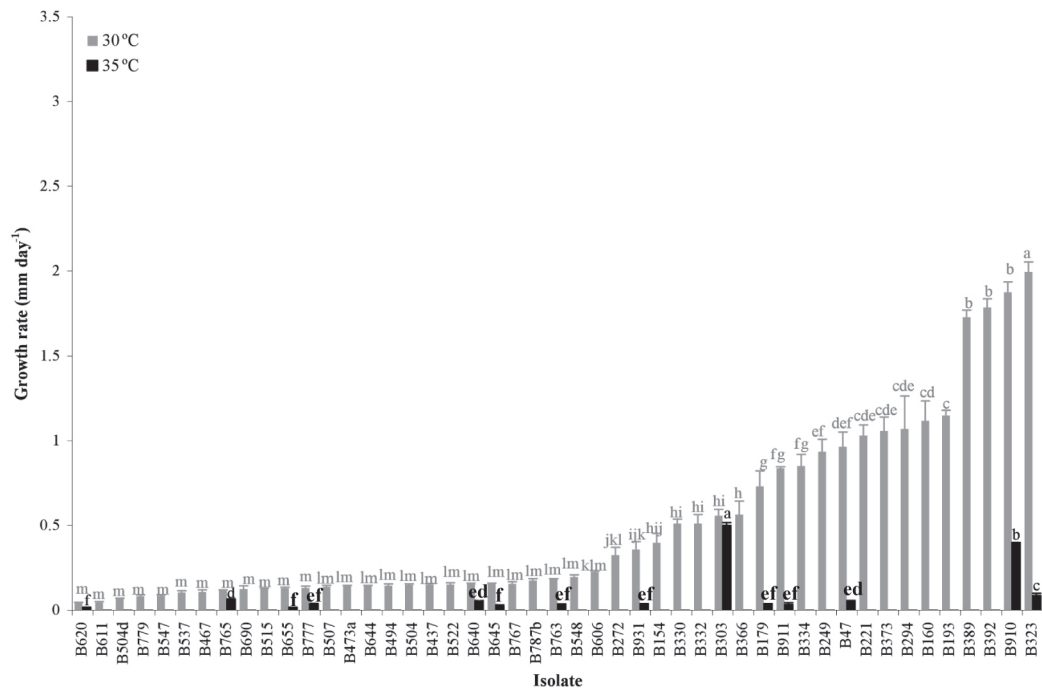
Varroa females were collected from the infested beehives and transferred to plastic chambers with blotting paper at the bottom. Suspensions of 10^7 conidia mL⁻¹ from each isolate were applied to the chambers by means of a Potter pulverization tower. *Varroa* treated only with the distilled water solution and Tween 80 made up the control. Immediately after treatment, *varroa* were placed on drone pupas maintained in 1 mL plastic tubes with cotton wool in the top, and then incubated at 25-30 °C in the dark for 10 days. Mortality of the mites and mycosis were recorded daily. Dead *varroa* were transferred to humid chambers and incubated at 25 °C to observe the possible development of mycelia and conidia.

The experimental design was completely randomized with 17 isolates as treatments, three replicates, and an experimental unit consisted of 10 *varroa*. Results were subjected to analysis of variance after angular transformation, and the means compared with the Tukey test ($P \leq 0.01$).

RESULTS AND DISCUSSION

Evaluation of radial growth

All evaluated *B. bassiana* isolates presented growth at 30 °C. The colony extension rate varied from 0.043 to 1.99 mm d⁻¹ with the greatest development in the Qu-B323 isolate followed by Qu-B910, Qu-B392, and Qu-B389 in which there were no differences ($P < 0.0001$) (Figure 1). At 35 °C, 14 of the 50 isolates presented some degree of development in their colonies, of which Qu-B303 was the best ($P < 0.0001$) followed by Qu-910 and Qu-B323 (Figure 1). In spite of the fact that the growth rate of the best isolate at 35 °C did not exceed 0.5 mm d⁻¹, it was similar to that observed at 30 °C, thus indicating tolerance in this temperature range. Meanwhile, in the other isolates, a marked reduction at a greater temperature were observed, characteristic that concurs with Tefera and



Distinct letters over the bars indicate differences according to Tukey test ($P < 0.05$).

Figure 1. Colony growth rates of *Beauveria bassiana* isolates incubated at 30 and 35 °C.

Pringle (2003) who observed a decrease in radial growth of *Beauveria* isolates at 35 °C.

M. anisopliae presented development in all the colonies at 30 °C, temperature at which the rates varied from 0.14 to 3.39 mm d⁻¹, and with the greatest growth for Qu-M38 and Qu-M285 isolates ($P < 0.0001$) (Figure 2). Incubation at 35 °C indicated that only 24 isolates could develop, with growth rates that varied from 0.017 to 2.17 mm d⁻¹. The Qu-M845 isolate showed the greatest radius (2.17 mm d⁻¹) and was also one of those that best reacted to both temperatures. Qu-M984 and Qu-M256 were not affected by the rise in temperature ($P < 0.0001$) while the rest statistically decreased their growth (Figure 2).

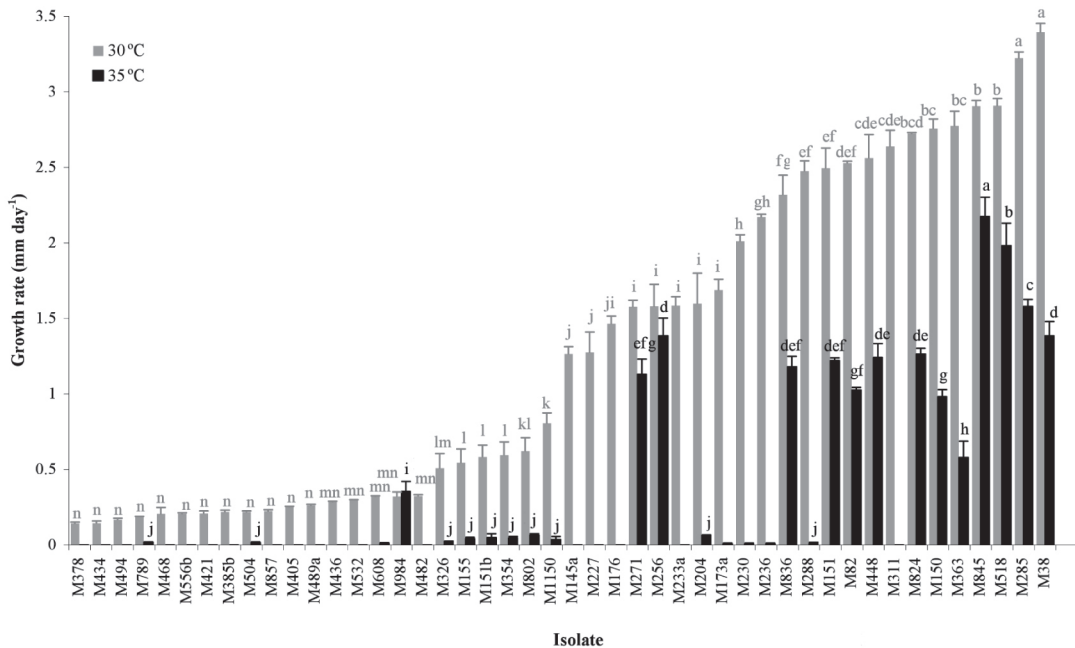
In this study, the growth response under the two evaluated temperatures varied considerably between isolates and species, this is how there was a greater quantity of *Metarhizium* than *Beauveria* isolates that grew at 30 and 35 °C. Ouedraogo *et al.* (1997) and Hallsworth and Magan (1999) also found that *M. anisopliae* has a wider growth range and a higher optimal temperature (30 °C) than *B. bassiana*. Milner *et al.* (2002) found *M. anisopliae* isolates with rapid growth between 20 and 30 °C, with the greatest growth at 30 °C and without growth at 35 °C.

Ouedraogo *et al.* (1997) point out that using pathogenic fungi as micopesticides needs various selection criteria, among them tolerance to different climatic contrasts and the possible effect of the pathogen-host interaction. In spite

of the fact that thermal requirements vary with the fungal species and its ecological niche, optimal temperatures for development, pathogenicity, and survival are found between 20 and 30 °C (Tanada and Kaya, 1993). Only a limited number of species have an optimal development temperature greater than 35 °C (Davidson *et al.*, 2003).

In this case, thermal requirements of the studied fungal isolates as potential controllers of *V. destructor* are the temperatures existing in the brood area of the bee colonies. Just as Davidson *et al.* (2003) pointed out, our results indicate that the selected isolates grow at 35 °C, and as a result could also function at peripheral temperatures of the brood area (32.5-33.4 °C) where the mites preferably reproduce (Le Conte *et al.*, 1990).

Bee colony temperature changes markedly between summer and winter. In the summer, temperatures depend more on the environment. The brood area is usually maintained from 32 to 37 °C while the rest of the beehive shows temperatures from 28 to 33 °C. In winter, bees remain active to generate heat and stay together to reduce the rate of energy loss. In temperate regions, the winter temperature of the bee nest is normally maintained between 20 and 30 °C (Shaw *et al.*, 2002). Although the evaluated temperatures have been based on the summer beehive conditions, it is considered that the selected isolates will also function under winter temperature conditions.



Distinct letters over the bars indicate differences according to Tukey test ($P < 0.05$).

Figure 2. Colony growth rates of *Metarhizium anisopliae* var. *anisopliae* isolates incubated at 30 and 35 °C.

Pathogenicity tests

Two *B. bassiana* isolates caused varroa mortality (20 to 30%) after 6 days of having been treated (Figure 3). Of the 14 *M. anisopliae* isolates, 11 presented some degree of mortality in adult varroa. Only the Qu-M845 isolate achieved the highest sporulation percentage (85%), followed by Qu-M271 and Qu-M488 with 60 and 50%, respectively ($P = 0.0033$) (Figure 3).

Differences in degrees of virulence of strains belonging to the same fungal species could be due to genetic variations arising from specialization toward a determined host and the geographical distribution of the strains (Alves, 1998; Coates *et al.*, 2002). In this case, isolates belonged to soil samples of different geographical origins and were collected with *Galleria mellonella* L. (Lepidoptera: Pyrellidae) as insect bait (Goettel and Inglis, 1997) (Table 1).

Sporulation capacity of the fungus over its host is fundamental for the dissemination of the disease in field conditions allowing reinfestations from parasitized insects (Gottwald and Tedders, 1984). In accordance with the above-mentioned, the most promising isolate to control the pest could be *M. anisopliae* Qu-M845.

A period of mite mortality of 5 to 12 days was also observed for *B. bassiana* and 4 to 7 days for *M. anisopliae*. Meikle *et al.* (2006) reported a death period for mites between 5 and 10 days for *B. bassiana* and 4 to 7 days for *M.*

anisopliae by using the same spore concentration as in this study. Meanwhile, Shaw *et al.* (2002) reported values of 2 to 3 days when using a concentration of 10^8 conidia mL⁻¹ and the immersion of the mites in this suspension; this application method can produce greater effectiveness of the fungus.

Numerous pathogens are known to infect mites and those found most frequently belong to the order Entomophorales. Common mite pathogens are of the *Hirsutella* and *M. anisopliae* genus (James, 2009). In the case of varroa control, a lot of research has been based on the use of pathogens of other arthropods, with the object of finding an appropriate fungal species or isolate that present high virulence in varroa. There is background information about an important group of *Hirsutella thompsonii* strains isolated from citrus mites, and many *M. anisopliae* isolates that have been isolated and stored, providing an important bank of potential strains to test on varroa (James, 2009). However, in their selection, as above-mentioned, these organisms must, in addition, adapt to the environmental conditions of the beehive in which they will be used (Sun *et al.*, 2003).

Meikle *et al.* (2006; 2007) searching for varroa pathogens in beehives collected six *B. bassiana* isolates. However, subsequent tests confirmed that temperatures in the 30 to 32 °C range presented survival and germination problems of *B. bassiana* when the spore are applied in the beehives (Meikle *et al.*, 2008).

Although it is possible that the thermal requirements of the fungus change according to the development state such as conidia germination, formation of apresorio, penetration, vegetative growth, and host colonization, selection of the *M. anisopliae* Qu-M845 isolate based only on its vegetative growth at high temperatures (30 and 35 °C) might not be sufficient to ensure its functioning in the conditions inside the beehive, and consequently, its development as a microbial control agent requires future studies.

However, this isolate could have advantages if you consider that fungal spores can work directly through the cuticle. This is important since varroa is an ectoparasite that feeds by mouth suction for which it would be difficult to apply any pathogenic agent or toxin that need to pass through the intestine such as bacteria and virus. Furthermore, these fungi are easy to produce in artificial

medium which is crucial to carry out a biological pest control strategy (James, 2009).

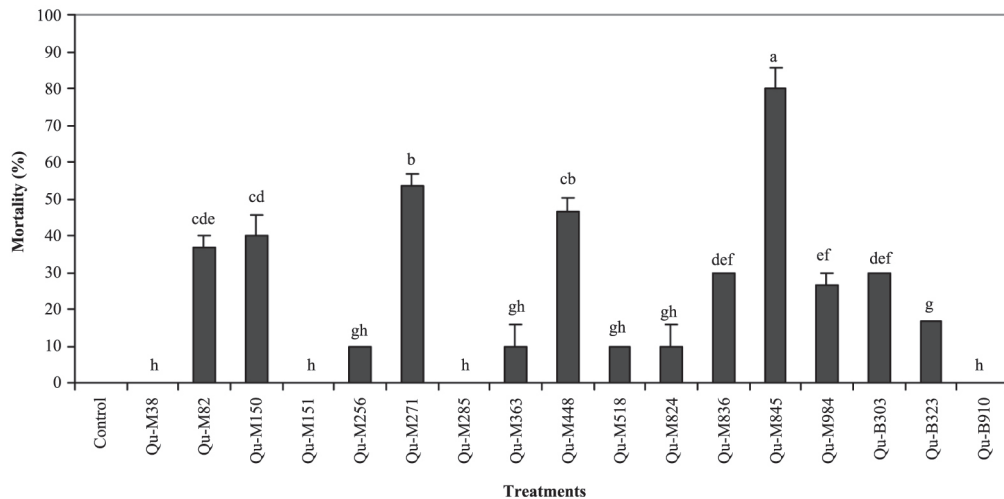
CONCLUSIONS

All the evaluated *Beauveria bassiana* and *Metarhizium anisopliae* var. *anisopliae* isolates showed growth at a temperature of 30 °C. However, at 35 °C, a reduction of the growth rates was observed in the isolates of both species, with the exception of three strains. Qu-M845 was the *M. anisopliae* strain with the greatest development at 35 °C, as well as reaching high varroa mortality levels, fact which allows us to consider it as a potential controller of *Varroa destructor*. Pathogenic capacity and tolerance to environmental conditions to which it would be exposed inside the beehive allow us to consider it as a new alternative to control this pest. Nevertheless, new research studies are required to ensure its effectiveness in the field.

Table 1. Growth rates at 30 and 35 °C and geographical origin of *Metarhizium anisopliae* var. *anisopliae* (Qu-M) and *Beauveria bassiana* (Qu-B) isolates selected for pathogenicity tests on *Varroa destructor*.

Isolate	30 °C	35 °C	Origin	
			Location	Geographical coordinates
	mm d ⁻¹			
Qu-M38	2.59 ± 0.81 ¹ (0.97) ²	1.38 ± 0.1 (0.9)	Unknown	-
Qu-M82	2.53 ± 0.01 (0.97)	1.03 ± 0.02 (0.99)	El Carmen, Bío Bío Region	36°48' S, 72°02' W
Qu-M150	2.76 ± 0.06 (0.92)	0.98 ± 0.04 (0.98)	Lago Chapo, Los Lagos Region	41°25' S, 72°31' W
Qu-M151	2.49 ± 0.13 (0.95)	1.22 ± 0.02 (0.97)	Río Chamiza, Aysén Region	45°23' S, 72°42' W
Qu-M256	1.03 ± 0.42 (0.98)	1.38 ± 0.12 (0.92)	Cañete, Bío Bío Region	37°48' S, 72°23' W
		1.24 ± 0.07c-f		
Qu-M271	1.19 ± 0.39 (0.97)	1.11 ± 0.13 (0.9)	Coihueco, Bío Bío Region	36°37' S, 71°49' W
Qu-M285	3.67 ± 0.39 (0.94)	1.58 ± 0.05 (0.91)	Lago Puyehue, Los Lagos Region	40° 43' S, 72°19' W
		0.97 ± 0.11efg		
Qu-M363	2.77 ± 0.094 (0.97)	0.58 ± 0.11(0.7)	Pinto, Bío Bío Region	36°41' S, 71°53' W
Qu-M448	2.56 ± 0.15 (0.97)	1.24 ± 0.09 (0.9)	Huape, Bío Bío Region	36°36' S, 71°15' W
		1.38 ± 0.12b-e		
Qu-M518	2.91 ± 0.05 (0.97)	1.98 ± 0.15 (0.93)	Santa Bárbara, Bío Bío Region	37°39' S, 72°01' W
Qu-M824	2.73 ± 0.00 (0.96)	1.26 ± 0.04 (0.98)	El Carmen, Bío Bío Region	36°48' S, 72°02' W
		1.58 ± 0.05a-e		
Qu-M836	2.02 ± 0.33 (0.96)	1.18 ± 0.11 (0.96)	Villa Alegre, Maule Region	35°39' S, 71°44' W
Qu-M845	2.91 ± 0.04 (0.97)	2.17 ± 0.13 (0.9)	Antuco, Bío Bío Region	37°19' S, 71°40' W
		1.24 ± 0.09c-f		
Qu-M984	0.32 ± 0.03 (0.9)	0.35 ± 0.07 (0.9)	Quillota, Valparaíso Region	32°51' S, 71°15' W
Qu-B303	0.56 ± 0.04 (0.6)	0.5 ± 0.02 (0.98)	Valle de Lluta, Arica and Parinacota Region	17°14' S, 70°07' W
Qu-B323	1.99 ± 0.06 (0.97)	0.086 ± 0.01 (0.92)	Río Chaca, Arica and Parinacota Region	18°26' S, 70°20' W
Qu-B910	1.87 ± 0.059 (0.97)	0.4 ± 0.0002 (0.6)	Easter Island	27°07' S, 109°20' W

¹Standard error (P < 0.05); ²Regression correlation coefficient (R²).



Distinct letters over the bars indicate differences according to Tukey test ($P < 0.01$).

Figure 3. Mortality of *Varroa destructor* inoculated with different *Metarhizium anisopliae* var. *anisopliae* (Qu-M) and *Beauveria bassiana* (Qu-B) isolates.

RESUMEN

Selección de hongos entomopatógenos para el control de *Varroa destructor* (Acari: Varroidae). El objetivo de este trabajo fue seleccionar hongos entomopatógenos tolerantes a las temperaturas del nido de cría de las abejas (*Apis mellifera*), para ser utilizados en el control de *Varroa destructor*. Se evaluaron 50 aislamientos de *Beauveria bassiana* (Balsamo) Vuillemin y 48 de *Metarhizium anisopliae* (Metschn.) Sorokin a temperaturas de 30 y 35 °C. Discos de agar de 5 mm de diámetro con micelio de colonias de cada aislamiento, se depositaron en el centro de placas Petri con medio agar Sabouraud dextrosa (ASD). Las placas fueron incubadas a 30 y 35 °C y oscuridad y diariamente se midió el radio de cada colonia. Todos los aislamientos de *B. bassiana* y *M. anisopliae* var. *anisopliae* presentaron una tendencia lineal a través del tiempo a temperaturas de incubación de 30 °C. A 35 °C la mayoría de los aislamientos no crecieron, excepto tres aislamientos de *B. bassiana* y 14 de *M. anisopliae* ($p < 0,001$). Estos aislamientos fueron seleccionados para realizar pruebas de patogenicidad sobre *V. destructor*, aplicando una suspensión de 10^7 conidias mL^{-1} . El aislamiento más efectivo fue Qu-M845 de *M. anisopliae* ($p = 0,0033$), produjo una mortalidad de 85%. La capacidad patogénica de este aislamiento en *V. destructor* y su tolerancia a las condiciones ambientales a las que sería expuesto, permite considerar esta cepa como una alternativa de control para esta plaga.

Palabras clave: control biológico, temperatura, hongo entomopatógeno, *Varroa destructor*, varroasis.

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