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Original article

Pathogenicity and thermal biology of mitosporic fungi as potential microbial control agents of *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of honey bee, *Apis mellifera* (Hymenoptera: Apidae)*

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Abstract – Pathogenicity and thermal biology (tb) were investigated for entomopahogenic fungi isolates (EFI), which were examined as potential biocontrol agents of the ectoparasite *Varroa destructor*, the major disease of honey bees in Andalusia (Spain). All 16 of the assayed EFI were pathogenic to *V. destructor*, while two of them were exceptionally pathogenic to the mite: *Metarhizium anisopliae* 01/121-Su [AST 35.0 h, 100% mortality at 72 h post infection (p.i.)] and *Beauveria bassiana* 01/103-Su (AST 39.4 h, 96.0% mortality at 72 h p.i.). The tb of the five most virulent isolates against the mite was investigated at seven temperatures (10–40 °C) by using a modified generalized beta function that accounted for 79.8–96.4% of the data variance. Optimum temperatures and maximum temperatures for growth ranged from 24.9 to 31.2 °C and from 30.1 to 35.0 °C respectively. On the basis of their pathogenicity, thermal requirements and safety to honey bees, two isolates showed promise as candidates for *V. destructor* control.

***Varroa destructor* / *Apis mellifera* / entomopathogenic fungi / virulence / biological control / *Beauveria* / *Metarhizium* / *Lecanicillium* / *Hirsutell*a / mite / parasites/ mycoacaricides**

1. INTRODUCTION

Varroa destructor Anderson and Trueman (2000) is a parasitic mite of western Asia and is responsible for the highest mortality of colonies of *Apis mellifera* L. in Andalusia (Spain). Its expansion into Europe, northern Africa, and North and South America began in the last quarter of the 20th century. *V. destructor* has devastating consequences in bee colonies and is the most important parasite of honey bee in Andalusia (Spain) (García-Fernández et al., 1995, 2001). The parasite's adult females feed on their host's haemolymph and transmit viruses that can lead to deformities (Todd et al., 2007) and that are manifested at all stages of development, from larvae to adults (Todd et al., 2007).

Currently, *V. destructor* is mainly controlled with synthetic acaricides, the pyrethroids, fluvalinate (Apistan®) and flumethrin (Bayvarol®), and the amitraz neurotoxin (Apivar®) and with organic acids such as formic acid, lactic acid, and oxalic acid. The supposed harmlessness of the chemical treatments has come into question. Repeated use of the same active ingredients has promoted the development of resistant mite populations (Milani, 1999; Anon, 2001;

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Luzón and García Fernández, 2002), and acaricide residues can contaminate honeybee products (Wallner, 1999) In addition, the organic acids require careful handling because sudden release can cause the death of the brood or the substitution of the queen in Spain's weather conditions.

The use of natural compounds, particularly essential oils from aromatic plants such as thymol (Apiguard®) have limited drawbacks, but their utilization has not been well accepted by Spanish beekeepers because they are difficult to apply and dependent on ambient temperature (> 15 °C and < 40 °C).

Biological control by the natural enemies of mites provides an option that overcomes the drawbacks of controlling *V. destructor* with chemicals (Chandler et al., 2001). Among entomopathogenic microorganisms, the fungal pathogens show the highest potential for biocontrol of the mite as they are the only ones that show contact action, via integument (Chandler et al., 2001). The potential use of mitosporic Ascomycetes such as *Metarhizium*, *Beauveria, Lecanicillium* and *Hirsutella* has been pointed out in particular (Kanga and James, 2002; Kanga et al., 2002, 2003; Meikle et al., 2006, 2007; Shaw et al., 2002). To date, only a limited number of *B. bassiana* isolates have been isolated from *V. destructor* cadavers, by Meikle et al. (2006) in southern France and by us in southern Spain. The mite's susceptibility to *B. bassiana* (Meikle et al., 2007), to *Metarhizium anisopliae* (Kanga et al., 2003) and to *Hirsutella thompsonii* Fisher (Kanga and James, 2002; Kanga et al., 2002, 2003; Shaw et al., 2002) has been observed in previous laboratory assays.

The broad spectrum of hosts shown by these fungal species and the enormous differences in virulence and heat requirements for growth among isolates (Zimmermann, 2007a, b) raises the hope of finding an isolate with selective activity against *V. destructor*, with thermal requirements adapted to the environmental conditions found in beehives. Considering the high humidity conditions within the bee hive's interior environment, the major constraint to fungal effectiveness may instead be the high temperatures maintained within some areas of the honey bee colony (Davidson et al., 2003). The temperature regime within honey bee colonies is determined by ambient conditions and the absence or presence of brood. Temperatures within broodless areas of the colony are around 25 °C, while those in brood areas are maintained around 34–35 °C, with 32 °C in the periphery of the brood (Le Conte et al., 1990). Thus, fungal isolates selected from laboratory bioassays to be evaluated in future experiments within honey bee colonies should tolerate the thermal conditions inside the bee hive (Davidson et al., 2003).

The studies described here were undertaken with the goal of identifying autochthonous isolates of mitosposporic fungi that possess good pathogenicity against *V. destructor*. The fungal isolates were obtained from the University of Cordoba Agricultural Entomology Research group's collection. The thermal biology of the fungal pathogens was assessed to determine whether or not these isolates could be potential microbial control agents for *V. destructor*.

2. MATERIALS AND METHODS

2.1. Fungi culture and preservation

Sixteen isolates were used in this experiment: seven belonged to *Beauveria bassiana* (Balsamo) Vuillemin, five to *Metarhizium anisopliae* (Metsch) Sorok, one to *Lecanicillium lecanii* Zimmermann (Gams & Zare), one to *Hirsutella kirchneri* (Rostrop) Mister, one to *H. nodulosa* Petch and one to *Hirsutella* sp. (Tab. I). Two of the *B. bassiana* isolates have been obtained from naturally infected mites at Granada and Jerez (Andalusia, Spain) after surveying natural populations of the mite during the period 2005–2007.

All isolated were maintained in Malt Extract Agar medium (MEA) at 4 °C, For the susceptibility assays, subcultures were made in Sabouraud Dextrose Agar (SDA) in Petri dishes and incubated at 27 °C for 14 d for *B. bassiana*, *M. anisopliae* and *L. lecanii* and incubated for 21 days for the *Hirsutella* species to ensure proper sporulation. The concentration of conidia was assessed by the colony forming unit method (CFU) (Goettel and Inglis, 1997).

Table I. Identity of the fungal isolates from the culture collection at A.F.S.R. Department of the University of Cordoba assayed against *Varroa destructor*.

No.	Fungal species[1]	Isolate	Insect host or substrate (habitat)	Site and date of origin
1	*Beauveria bassiana*	EABb 04/01-Tip	*Timaspis papaveris* (Hymenoptera; Cynipidae)	Sevilla (Spain), 2004
2		EABb 01/110-Su	Soil (oak)	Sevilla (Spain), 2001
3		Bb-1333	*Bactrocera oleae* (Diptera; Tephritidae)	Grece, 1961
4		EABb 01/103-Su	Soil (forest)	Sevilla (Spain), 2001
5		EABb 01/33-Su	Soil (olive tree orchard)	Cádiz (Spain), 2001
6		EABb 07/05-Vd	*Varroa destructor*	Cádiz (Spain), 2007
7		EABb 07/07-Vd	*Varroa destructor*	Granada (Spain), 2007
8	*Metarhizium anisopliae*	EAMa 92/9-Dm	*Dociostaurus maroccanus* (Orthoptera; Acrididae)	Badajoz (Spain), 1992
9		EAMa 01/44-Su	Soil (non-cultivated)	Jaen (Spain), 2001
10		EAMa 01/58-Su	Soil (wheat crop)	Córdoba (Spain), 2001
11		EAMa 01/121-Su	Soil (cotton crop)l	Sevilla (Spain), 2001
12		EAMa 01/152-Su	Soil (cotton)	Sevilla (Spain), 2001
13	*Lecanicillium lecanii*	L1	Predator mite	Poland 2006
14	*Hirsutella kirchneri*	H1	*Abacarus hystrix*	Poland 2006
15	*Hirsutella* sp.	H2	Predator mite	Poland 2006
16	*Hirsutella nodulosa*	H3	Tarsonemid	Poland 2006

[1] Isolates of *Lecanicillium* and *Hirsutella* used in this study were obtained from Dr. Cezary Tkaczuk, Department of Plant Protection, University of University of Podlasie (Poland).

2.2. Bioassays for *Varroa destructor* susceptibility to entomopathogenic fungi

Young white-eyed *Apis mellifera* pupae were taken from a comb from a beehive in an experimental apiary at the IFAPA Centro Camino del Purchil (Institute for Training in Agriculture and Fishing) in Lanjarón, Granada (Spain) and were placed separately in eppendorf tubes closed with absorbent cotton wrapped in sterile gauze.

Adult *V. destructor* females, collected from an infested beehive were allowed to walk for 5 min on each of the subcultures (24 mites per subculture) (Peng et al., 2002). Subsequently, they were transferred by pairs to eppendorf tubes containing a white-eyed pupa at the bottom. They were incubated at 27 °C and 70% RH. Twelve eppendorf tubes were used for each isolate. As a control, *V. destructor* females were allowed to walk on MEA medium in a Petri dish.

To quantify the number of conidia taken up by the mites from the two best isolates (determined from experiment outlined above), additional groups of 20 inoculated *V. destructor* females were transferred individually to 2-mL cryogenic tubes containing 1 mL of sterile distilled water plus 0.2% Tween. The tube was vortexed for 2–3 min to dislodge conidia from the mite, and the concentration of conidia was determined using a haemocytometer.

Mite mortality was recorded every 24 h and dead mites were removed daily, immediately surface sterilized with 1% sodium hypochlorite for 5 minutes, and washed twice consecutively for 1 min in sterile distilled water. They were then placed on sterile wet filter paper in sterile Petri dishes sealed with parafilm® and kept at 27 °C and 70% RH to be inspected for the presence of mycelium in the cadavers. The fungal species was verified microscopically based on morphological characteristics using taxonomic keys (Barnett and Hunter, 1987; Humber, 1997). During the observation period, the pupae that died while the female mites were still alive were replaced by live pupae.

The entire experiment was repeated with new collections of mites, honey bee adults and pupae, and new fungal suspensions from new cultures.

The cumulative mortality response across the assessment period was analyzed with Kaplan-Meier survival analysis, a non-parametrical method of estimating the probabilities of survival according to time, and a comparison was made of the average survival times (AST) (Bates and Watts, 1988). The average values per bee pupa were used to determine the survivorship rate for the experiment. Statistical analyses were performed using SPSS 8.0 for Windows (SPSS, 1997).

Post-treatment pupae that died during the assay period were also surface sterilized as described above and placed on sterile wet filter paper in sterile Petri dishes that were then sealed with parafilm® and kept at 27 °C and 70% RH in order to be inspected for the possible presence of mycelium in the cadavers.

2.3. *Apis mellifera* susceptibility to entomopathogenic fungi

Adult worker bees were assayed for susceptibility to four of the entomopathogenic fungi isolates tested against *V. destructor*, EABb 04/01-Tip, EABb 01/110-Su, Bb-1333 and, EABb 01/103-Su. An airbrush (Holding Air Brush Kit Mod. AB-124) connected to a regulator valve (Atlantis PT3500) providing a constant airflow of 10 L/min was used to spray 1 mL of a watery suspension containing 1.0×10^8 conidia/mL and 0.03% tween 80. The spray deposition at the level of the target surface was approximately 0.1 μL/mm^2 (resulting from spraying of a 1 mL aliquot). Sets of 30 bees were sprayed with the airbrush and then placed in experimental boxes containing water and food under laboratory conditions (22 °C, 50% RH). By using the CFU method, we estimate that the used suspension produced a deposition of 10 000 viable conidia/mm^2. The controls were treated with water solution containing only 0.03% tween 80. There were three replicates with 30 bees each per treatment, and the experiment was repeated with new fungal inoculum. The experiment was monitored for 21 days. Post-treatment adult bees that died during the assay period were immediately surface sterilized with 1% sodium hypochlorite for 5 min, with two consecutive washes for 1 min in sterile distilled water. They were then placed on sterile wet filter paper in sterile Petri dishes sealed with parafilm® and kept at 27 °C and 70% RH in order to be inspected for the presence of mycelium in the cadavers.

2.4. Effect of temperature on in vitro radial colony growth of fungi

For each selected isolate, circular plugs (5 mm diameter) were cut from non-sporulating mycelia

of 7-day-old culture Petri dishes using a cork-borer, and a single plug was placed upside down in the center of a new dish of MEA medium. Dishes were sealed with parafilm® and incubated in the dark in separate incubators at 10, 15, 20, 25, 30 and 35 ± 1 °C. Five replicate dishes were prepared for each isolate and temperature combination. Surface radial growth was recorded daily using two cardinal diameters previously drawn on the bottom of the dish. The experiment was run for 10 days or until the fungal colony had covered the Petri dish.

Radial growth data were fitted by regression analysis using SPSS 8.0 for Windows. Because radial measurements (from the 2nd to the 10th day) were fit by a linear model (y = vt + b), the linear regression slope (v), which indicated the growth rates (velocity in mm d^{-1}), was used as the main parameter to evaluate the influence of temperature on fungal growth (Fargues et al., 1992; Ouedraogo et al., 1997; Yeo et al., 2003; Davidson et al., 2003). To this end, a generalized beta function, modified according to Bassanezi et al. (1998) was fitted to the average growth rates under different temperatures. The generalized beta function is given by: $Y(T) = TY_{opt}[(T - T_{min})/(T_{opt} - T_{min})]\exp[TB_3(T_{opt} - T_{min})/(T_{max} - T_{opt})][(T_{max} - T)/(T_{max} - T_{opt})]\exp TB_3$, where Y(T) is the fungal growth in mm d^{-1} (dependent variable) and T is the incubation temperature (independent variable). T_{min}, T_{max} and T_{opt} are the lowest, highest and optimal temperatures for fungal growth, respectively. TY_{opt} is the fungal growth at the optimal temperature T_{opt}. TB_3 is the shape parameter that influences the temperature range around T_{opt} in which the curve stays close to TY_{opt}; for low values such as 0.1, a broad temperature range exits, while, for high values such as 3.0, the curve sharply declines when temperature differs only slightly from T_{opt} (Bassanezi et al., 1998). Based on our preliminary experiments, T_{min} was fixed at 5 °C.

TY_{opt}, T_{opt}, T_{max} and TB_3 values were estimated by the method of Levenberg-Marquardt (Bates and Watts, 1988). The validation of the model was based on the coefficient of determination values and on the significance of the standard errors of the parameters (Campbell and Madden, 1990). A separate beta function analysis was conducted on the data from each of the five replicates (one from each temperature), which finally yielded five replicates of each parameter. Multivariate analysis of variance (MANOVA) was used to compare parameters. When the model was shown to be significantly different for different isolates, one-way analysis of variance (ANOVA) was performed for each parameter (Campbell and Madden, 1990). Analyses of variance were followed by comparison of means using least significant difference (LSD). Statistical analyses were performed using SPSS 8.0 for Windows (SPSS, 1997).

3. RESULTS

3.1. Pathogenicity of entomopathogenic fungi to *V. destructor*

Female mites were allowed to walk on fungal cultures for 5 min, resulting in 22 000 ± 1250 (mean ± EE) conidia attached to each mite for *B. bassiana*. All *B. bassiana* isolates assessed against *Varroa destructor* were pathogenic and caused 96.0–100.0% mortality within 72 h after inoculation (Fig. 1A,B). In the first group of *B. bassiana* isolates, there were significant differences in the average survival time (AST) of the inoculated mites compared to the control mites (Tab. II). However, only the isolate Bb-1333, 50.0 h, had significantly lower AST compared to the remaining *B. bassiana* isolates EABb 01/103-Su, EABb 01/33-Su, EABb 04/01-Tip and EABb 01/110-Su that had AST values ranging between 39.4 and 45.0 h (Tab. II). Median survival time (MST) values were all around 48 h, with the exception of one isolate, EABb 01/103-Su, which caused the death of half of the treated mite population within 24 h after inoculation. In the second group of *B. bassiana* isolates obtained from *Varroa destructor*, AST of EABb 07/05-Su isolate (45.0 d) had significantly lower AST compared to the isolate EABb 07/07-Vd (56.0 d), as shown in Table II.

The female mites were allowed to walk on fungal cultures of *M. anisopliae* for 5 min, resulting in 2600 ± 250 (mean ± EE) conidia attached on each mite. The five *M. anisopliae* isolates assessed against *V. destructor* were also pathogenic (Fig. 2), whereas mortality rates after 72 h did not reach 100%, except for isolates EAMa 01/58-Su and EAMa 01/121-Su (Fig. 1C). However, 100% mortality rates were caused by all of the assayed isolates within 120 h. Significant differences were also detected in the AST of the inoculated

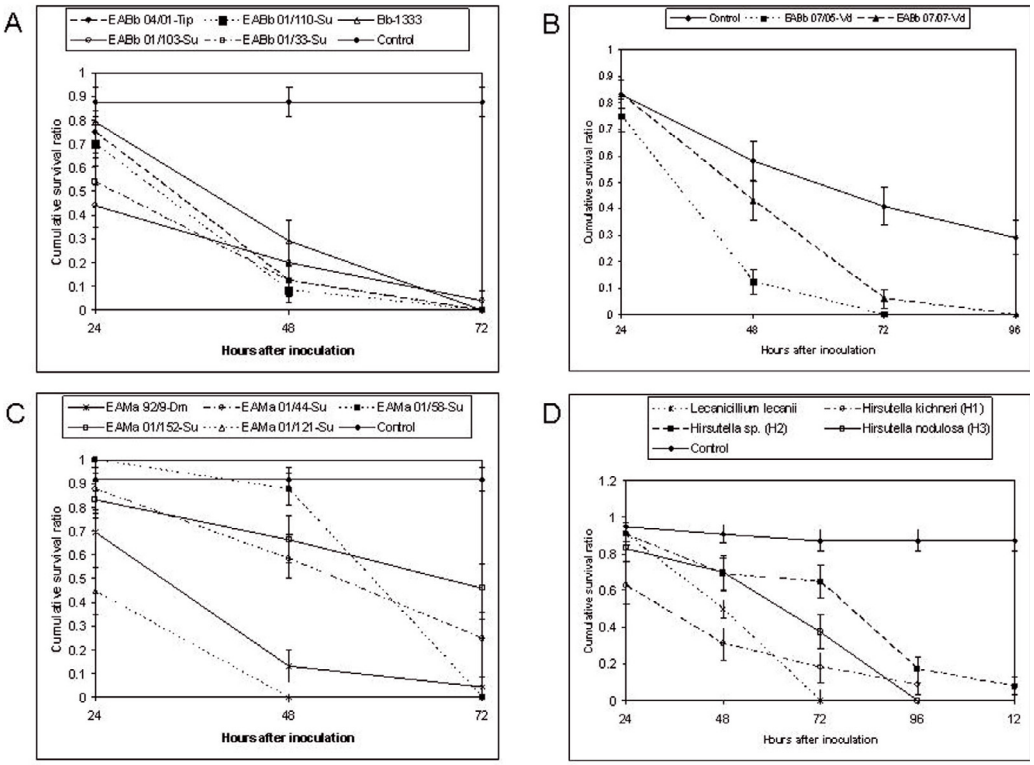

Figure 1. Cumulative survival ratio of *Varroa destructor* inoculated with entomopathogenic fungi. (1A *Beauveria bassiana* isolates obtained form the soil and from insect host, 1B *Beauveria bassiana* isolates obtained from *Varroa destructor*, 1C *Metarhizium anisopliae*, 1D *Lecanicillium lecanii* and *Hirsutella species*.)

mites as compared to the control. Thus, the mites inoculated with the most virulent EAMa 01/121-Su isolate showed an AST of 35.0 h and MST of 24 h (Tab. II).

The only isolate of *Lecanicillium lecanii* assessed was pathogenic to *Varroa destructor*, causing 100% mortality in 72 h, with an AST of 58.0 h (Tab. II; Fig. 1C).

The *Hirsutella* isolates assessed were also pathogenic, although they showed significant differences in pathogenicity. The *Hirsutella kirchneri* isolate was the most virulent, with both the lowest AST (58.9 h) and MST (48 h) (Tab. II; Fig. 1D).

3.2. Effect of temperature on in vitro radial colony growth of fungi

We selected a set of the five most virulent isolates against *V. destructor* to study their thermal biology. Temperature had significant effects on in vitro radial colony growth of all fungal isolates (Tab. III). The radial measurements from the 2nd day to the 10th were fitted to a linear model, with the coefficients of determination of regression lines for all isolates and all temperatures varying from 0.89 to 0.98. All the fungal isolates grew at 10, 15, 20, 25 and 30 °C, whereas isolates EAMa 01/58-Su, EAMa 01/121-Su and EABb 01/103-Su also grew at 35 °C. Fungal growth was not observed at 40 °C for any of the isolates. Colony extension rates varied from

Table II. Kaplan-Meier survival analysis of *Varroa destructor* adults females inoculated with different fungal isolates by walking for 5 minutes on sporulated cultures.

Fungal species	Isolate	Kaplan-Meier survival analysis			
		Average Survival Time (mean ± SE h)[1]	95% confidence interval	Median Survival Time (mean ± SE h)	95% confidence interval
Beauveria bassiana	Control	72.0 ± 0.0 a			
	EABb 04/01-Tip	45.0 ± 3.0 c	39.1–50.8	48.0 ± 2.6	42.9–53.1
	EABb 01/110-Su	43.0 ± 2.9 c	37.4–48.7	48.0 ± 2.2	43.8–52.3
	Bb-1333-Su	50.0 ± 3.5 b	43.1–56.9	48.0 ± 4.5	39.3–56.7
	EABb 01/103-Su	39.4 ± 3.9 c	26.8–39.2	24.0	-
	EABb 01/33-Su	40.0 ± 3.4 c	33.3–46.7	48.0	-
Beauveria bassiana[2]	Control	75.0 ± 5.1 a	64.9–85.1	72.0 ± 10.3	51.9–92.1
	EABb 07/05-Vd	45.0 ± 2.1 c	40.9–49.1	48.0 ± 2.1	40.9–49.1
	EABb 07/07-Vd	56.0 ± 2.9 b	50.3–61.6	48.0 ± 4.3	39.5–56.5
Metarhizium anisopliae	Control	72.0 ± 1.4 a			
	EAMa 92/9-Dm	43.8 ± 3.2 c	37.4–50.2	48.0 ± 3.0	42.1–53.8
	EAMa 01/44-Su	59.0 ± 3.5 b	52.0–66.0	72.0 ± 6.3	59.5–84.5
	EAMa 01/58-Su	69.0 ± 1.7 b	65.6–72.2	72.0 ± 0.0	-
	EAMa 01/152-Su	60.0 ± 3.9 b	52.4–67.6	72.0	-
	EAMa 01/121-Su	35.0 ± 2.5 d	30.1–39.9	24.0	
Lecanicilliun lecanii	Control	112.0 ± 4.8 a	102.5–121.5	-	-
	Ll	58.0 ± 3.2 b	51.7–64.3	48.0 ± 5.3	37.5–58.5
Hirsutella spp.	Control	112.0 ± 4.8 a	102.5–121.5	-	-
	H1	58.9 ± 8.0 c	40.9–57.1	48.0 ± 7.5	33.3–62.7
	H2	82.4 ± 6.4 a	69.9–95.0	96.0 ± 3.9	87.5–104.5
	H3	70.0 ± 5.4 b	59.4–80.5	72.0 ± 7.1	58.05–86.0

[1] For each fungal species, data followed by the same letter in each column indicate no significant differences (Log rank test, $\alpha = 0.05$). AST limited to 72 h for *Beauveria bassiana* and *Metarhizium anisopliae* assys and 120 h for *Lecanicillium lecanii* and *Hirsutella* spp. Assays.

[2] *Beauveria bassiana* isolates obtained from natural *Varroa destructor* populations.

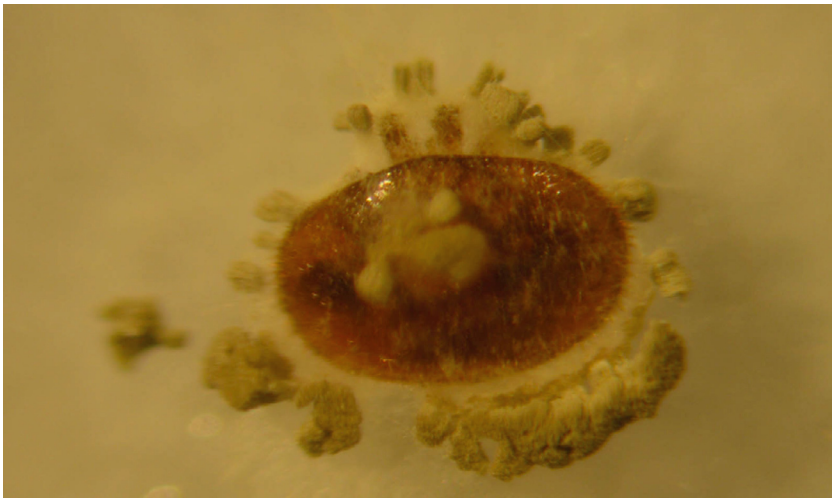

Figure 2. Female *Varroa destructor* mycosed by *Metarhizium anisopliae* (72 h after incubation).

0.1 mm/d to 5.0 mm/d, with a direct relationship between temperature and extension rate in the range of 10–30 °C. Linear regression slopes indicating the growth rates in mm/d at each temperature were used as the main parameter to evaluate the influence of temperature on fungal growth. The fungal growth rate-temperature curves were well described overall by the generalized beta function used in these studies (Fig. 3). The shape parameter ranged between 0.20 and 0.72 for all isolates.

MANOVA showed significant differences among isolates in their beta model (λ de Wilks = 0.002; $P < 0.001$) (Tab. III). Optimum temperatures were significantly different among *B. bassiana* isolates. They varied between 24.93 ± 0.4 °C (EABb 01/103-Su) and 31.24 ± 2.5 °C (EAMa 01/121-Su). There were significant differences among isolates in maximum temperatures for fungal growth, which varied from 30.15 ± 0.3 °C (EABb 01/33-Su) to 35.02 ± 0.2 °C (EABb 01/103-Su). Comparisons of relative growth rates at the optimum temperature indicated that there were also significant differences among isolates (Tab. III). The growth rates at the optimum temperature varied from 2.1 ± 0.5 mm/d (EABb 04/01-Tip) to 5.8 ± 0.1 mm/d (EAMa 01/58-Su).

3.3. Results of honey bee susceptibility to entomopathogenic fungi

3.3.1. Pupae

In general, pupae that died during the assays showed no mycosis due to entomopathogenic fungi after being surface sterilized and placed on sterile wet filter paper in sterile Petri dishes. However, they did show the presence of saprophytic *Aspergillus* and *Penicillium*. For isolates *B. bassiana* EABb 01/110-Su and *M. anisopliae* EAMa 01/58-Su, fungal growth was observed in a few pupae, in which cases cross-sections revealed that infection was on the surface and did not affect the internal organs of the pupae. Isolate Bb-1333, obtained from a dipteran species [*Batrocera oleae* (Gmelin)], was the only isolate that caused an early mycosis in the pupae, which became red in color 3 days after treatment.

3.3.2. Adult bees

No adult bee mortality was attributed to *B. bassiana* isolates EABb 04/01-Tip, EABb 01/110-Su, Bb-1333 and EABb 01/103-Su during the 21 days after treatment. After being inspected for fungal outgrowth, we found *Aspergillus* sp. in only four cadavers.

Table III. Estimated parameters (± standard error) and coefficients of determination r^2 of the generalized beta function modified according to Bassanezi et al. (1998)[a] fitted to data of the vegetative growth of different *Beauveria bassiana* and *Metarhizium anisopliae* isolates.

Isolate	TY_{opt}	T_{opt}	T_{max}	TB_3	r^2
EAMa 01/58-Su	5.8280 a	26.3151 e	35.0022 a	0.6959 ab	0.9510
EAMa 01/121-Su	4.2400 b	31.2478 a	35.0093 a	0.2181 b	0.9638
EABb 01/103-Su	2.5595 c	24.9388 f	35.0281 a	0.7250 a	0.8813
EABb 01/33-Su	4.1656 b	27.2808 d	30.1563 c	0.2028 b	0.9592
EaBb 04/01-Tip	2.0679 d	27.6703 c	33.3375 b	0.2862 b	0.7986

[a] The generalized beta function is given by, $P = TY_{opt}[(T - T_{min})/(T_{opt} - T_{min})] \exp [TB_3 (T_{opt} - T_{min})/(T_{max} - T)] [(T_{max} - T)/(T_{max} - T_{opt})] \exp TB_3$, where Y(T) is the fungal growth in mm d^{-1} (dependent variable) and T is the incubation temperature (independent variable). T_{min}, T_{max} and T_{opt} are respectively the lowest, the highest and the optimal temperature for fungal growth. TY_{opt} is the fungal growth at the optimal temperature T_{opt} and TB_3 is the shape parameter. Means within columns with the same letter are not significantly different ($P < 0.05$) according to the least significant difference (LSD) test.

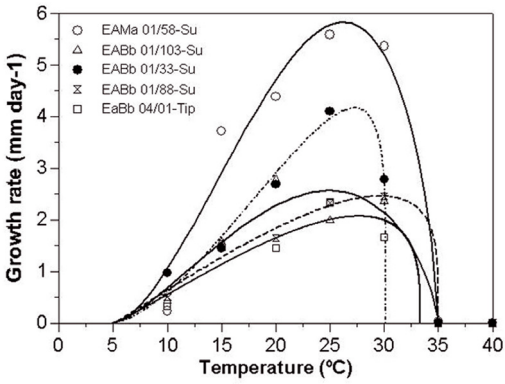

Figure 3. Model predictions for the effect of temperature on the growth rate of candidate *Beauveria bassiana* and *Metarhizium anisopliae* colony isolates for development of biocontrol against *Varroa destructor*. Lines represent fitted curves obtained using the generalized beta function modified according to Bassanezi et al. (1998).

4. DISCUSSION

In this paper, the pathogenicity to *Varroa destructor* of 16 isolates of entomopathogenic fungi was studied. This is the first report of this kind in Spain. From our extensive work on the occurrence and distribution of entomopathogenic fungi in natural and cultivated areas in Spain (Quesada-Moraga et al., 2007), it has been shown that the only two species present are *B. bassiana* and *M. anisopliae*. Moreover, we have recently found that *B. bassiana* is the only species found on natural populations of *V. destructor* in southern Spain in hives originating in Granada and Jerez (data included in this paper). In order to assay a larger number of fungal genera, we obtained one isolate of *Lecanicillium lecanii* and three isolates of the University of Podlasie (Poland). Our work with *M. anisopliae* is based on previous studies in the USA by Kanga et al. (2003).

Previous research on the use of entomopathogenic fungi for *V. destructor* control has been performed with *Beauveria*, *Metarhizium*, *Lecanicillium*, *Hirsutella*, *Paecilomyces*, and *Tolypocladium* genera (Shaw et al., 2002), *M. anisopliae* (Kanga et al., 2003) and *Hirsutella thompsonii* (Peng et al., 2002).

We selected the infection method described by Peng et al. (2002) for the pathogenicity tests, which involved making female *Varroa destructor* walk on a fungal isolate for 5 minutes. The method gave excellent results because 100% of the *V. destructor* had mycosis 7 days after treatment.

As revealed by the mortality rates, AST values and MST values, our best isolates belonged to the species *Metarhizium* and *Beauveria*. In particular, two isolates, *M. anisopliae* EAMa 01/121-Su and *B. bassiana* EABb 01/103-Su, stood out from the rest, with 96.0% mortality rates and ASTs of 35.0 and 39.4 h, respectively. The two isolates obtained from *V. destructor* were also very pathogenic, whereas isolate EABb 07/05-Vd showed a lower AST value of 45.0 d, which was in the same range of

isolates EABb 01/103-Su and EAMa 01/121-Su. Conversely, in previous studies Peng et al. (2002) found *Hirsutella thomsonii* isolates to be the most virulent against *V. destructor* using the same bioassay method that we employed here.

The colony extension technique described here is widely used to estimate the optimum temperatures for fungal growth, and it is a valuable tool for screening entomopathogenic fungi that have already demonstrated high virulence against the target pest (Fargues et al., 1992; Ouedraogo et al., 1997; Davidson et al., 2003; Yeo et al., 2003). The response of fungi to temperature is a characteristic bell-shaped curve, skewed to the lower temperatures. However, non-linear regression models have not routinely been used to analyze the effects of temperature on fungal growth. Here, we used for the first time the generalized beta function modified according to Bassanezi et al. (1998) to study temperature-growth interactions of entomopathogenic fungi (Quesada-Moraga et al., 2006). In their study of thermal biology of fungal isolates for control of *V. destructor,* Davidson et al. (2003) used the Schoolfield et al. (1981) re-formulation of the Sharpe and DeMichele model. By using this model, Davidson et al. (2003) found optimum temperatures for growth in the range of 22.9 to 31.2 °C, even though only three isolates out of 37 showed optimum temperature above 30 °C. However, their model did not provide the maximum temperature for growth but instead used a different parameter named the super-optimum temperature, which ranged between 32 and 36 °C, with two isolates showing super-optimum temperatures above 40 °C.

The thermal requirements of our best candidate *Beauveria* and *Metarhizium* isolates for development of a mycoacaricide for *Varroa* control were well matched to the temperatures of the broodless areas of bee hives in temperate regions in September-October (ca. 25 °C) (Simpson, 1961). However, it is urgent to study the thermal biology of the two isolates obtained from *V. destructor*, particularly isolate EABb 07/05-Vd as it also shows promise for development as a mycoacaricide for the control of the mite. In addition, all isolates also provide optimum mite mortality under the temperature conditions on the periphery of the brood nest, where the mites preferentially reproduce (32.5–33.4 °C), and even in the central areas of the brood nest, where temperatures range from 33 to 36 °C (Southwick and Heldmaier, 1987). However, in central areas of the brood nest, the efficacy could by highly impaired by small increases in temperature above the maximum temperature for growth.

Pupal susceptibility to infection with the assayed isolates was very low, with none of the pupae kept in humid chambers observed showing signs of fungal infection during the first 120 h, except for the Bb-1333 isolate, which interestingly was not autochthonous. In addition, the results obtained on the susceptibility of adult bees to *B. bassiana* isolates were satisfactory because we tested five isolates, none of which were pathogenic after 21 days post infection. However, the assays must still be extended and replicated in subsequent experiments.

In our work, we have found that *V. destructor* showed a very high mortality rate within 72 h, while adult and pupal honey bees were not susceptible to the fungal isolates assayed. These laboratory results suggest that our best isolates may be useful for future biological control studies of the mites under field conditions. In addition to determining whether the thermal regimes of honey bee hives in our Mediterranean area favour infection of *V. destructor* by our candidate fungal isolates, we also plan to study the best application techniques for optimizing efficacy and economic cost.

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Pouvoir pathogène et biologie thermique de champignons mitosporiques comme agents microbiens potentiels pour lutter contre *Varroa destructor* (Acari : Mesostigmata), acarien

ectoparasite de l'Abeille domestique *Apis mellifera* (Hymenoptera : Apidae).

champignon entomopathogène / *Beauveria* / *Metarhizium* / *Lecanicillium* / *Hirsutella* / lutte biologique / *Varroa destructor* / *Apis mellifera* / mortalité / virulence

Zusammenfassung – Pathogenität und Thermobiologie mitosporischer Pilze als potentielle Behandlungsmittel gegen *Varroa destructor* (Acari: Mesostigmata), eine ektoparasitische Milbe der Honigbiene *Apis mellifera* (Hymenoptera: Apidae). Ziel der Untersuchung war, die Pathogenität und Thermobiologie von insektenpathogenen Pilzen als potentielle mikrobische Behandlungsmittel gegen den Ektoparasiten *Varroa destructor*, den schädlichsten Parasiten der Honigbienen (*Apis mellifera iberiensis*) in Andalusien (Spanien) zu ermitteln. Darüber hinaus selektierten wir Isolate mit begrenzter Wirkung gegen adulte Honigbienen und deren Puppen. Wir untersuchten 16 Pilzisolate aus den vier Arten *B. bassiana* [7 Isolate, von denen zwei aus am Boden von Versuchsbienenvölkern in Granada und Jerez (Spain) gefundenen *V. destructor* Milben stammten], *M. anisopliae* (fünf Isolate), *L. lecanii* (ein Isolat) und *Hirsutella* sp. (drei Isolate). Die meisten der 16 Isolate entstammten der Sammlung autochthoner Kulturen des C.R.A.F. Department der Universität von Cordoba.
In dem Pathogenitätstest ließen wir adulte, aus einem befallenen Volk gesammelte weibliche *V. destructor* Milben jeweils 5 Minuten lang über die 16 Pilzisolate laufen. Diese wurden dann paarweise in Eppendorfröhrchen verbracht, die eine weißäugigen Puppe enthielten. Die Röhrchen wurden bei 27 °C und 70 % RH inkubiert. Alle der untersuchten Pilzarten waren pathogen für die Milben, hierbei waren die Isolate von *B. bassiana* und *M. anisopliae* am virulentesten. Von den 16 untersuchten Isolaten hoben sich zwei auf Grund ihrer Pathogenität ab: *M. anisopliae* EAMa 01/121-Su Isolat (AST 35,0 h, 100 % Mortalität 72 h nach Infektion) und *B. bassiana* EABb 01/103-Su Isolat (AST 39,4 h, 96,0 % Mortalität 72 h nach Infektion). Generell wiesen Puppen, die während des Tests starben, nach einer 7 Tage dauernden Inkubierung in einer feuchten Kammer keine durch die insektenpathogenen Pilze hervorgerufenen Mykosen auf, keines der vier untersuchten Isolate war für adulte Bienen pathogen.
Die thermische Biologie der fünf Isolate mit höchter Pathogenität für *V. destructor* wurden bei sieben verschiedenen Temperaturen untersucht (10–40 °C). Die radiale Wachstumsrate der Kolonien wurde aus der Steigung der linearen Regression über die Zeit abgeschätzt, und die Daten wurden dann an eine modifizierte generalisierte Betafunktion angepasst, durch die 79,8–96,4 % der Datenvarianz erfasst wurde. Die für die Ausbreitungsrate optimalen Temperaturen lagen zwischen 24,9 bis 31,2 °C, während die Maximaltemperaturen für das Pilzwachstum von 3,1 bis 35,0 °C variierten.
Auf Grund ihrer Pathogenität und ihrer Temperaturbedürfnisse, zusammen mit dem Fehlen einer Pathogenität für Honigbienen erwiesen sich zwei der Isolate als viel versprechende Kandidaten für die Bekämpfung von *V. destructor* in mediterranen Honigbienenvölkern. Auf Basis dieser Ergebnisse planen wir die Untersuchung von experimentellen Formulierungen dieser zwei Isolate in Freilandversuchen zur Bekämpfung der Varroose in Spanien. Wir beabsichtigen weiterhin die thermische Biologie der beiden aus *V. destructor* gewonnen *B. bassiana* Isolate, insbesondere Isolat EABb 07/05-Su, zu untersuchen, mit dem Ziel diese in unsere Freilanduntersuchungen einzubeziehen.

***Varroa destructor* / *Apis mellifera* / entomopathogene Pilze / Virulenz / Biologische Bekämpfung / *Beauveria* / *Metarhizium* / *Lecanicillium* / *Hirsutella* / Milben / Parasiten/ Mycoakaricide**

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