Pathogenicity of entomopathogenic fungi to *Ornithodoros erraticus* and *Ornithodoros moubata* (Acari: Argasidae)

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Abstract

The argasid ticks *O. erraticus* and *O. moubata* are of great medical and veterinary importance because they are vectors of the African swine fever virus and several species of human relapsing fever borreliae. Biocontrol of these ticks using entomopathogenic fungi has not been previously reported. We examined the pathogenicity to different developmental stages of these two argasids of six strains of the fungal species *Beauveria bassiana* (strains Bb1764 and Bb2157), *Lecanicillium lecanii* (strains Ll586, Ll618 and Ll3047) and *Tolypocladium cylindrosporum* (strain Tc3398). Three strains, Bb1764, Bb2157, and Tc3398, caused in Spanish *O. erraticus* mean mortality rates between 34.4% and 62% in 14-28 days post-inoculation. Additionally, Bb2157 also induced in African *O. moubata* mean mortality rates of 31.9%. The remaining strains caused lower mortality rates and were not considered effective. This is the first study in which some strains of entomopathogenic fungi are found to be effective against argasid ticks of the genus *Ornithodoros,* and its results might justify further efforts towards the application of entomopathogenic fungal strains as anti-argasid biocontrol agents

Keywords: tick biocontrol; *Ornithodoros*; entomopathogenic fungi; *Beauveria bassiana*; *Lecanicillium lecanii*; *Tolypocladium cylindrosporum*

1. Introduction

The argasid ticks *Ornithodoros erraticus* and *O. moubata* are reservoirs and vectors of important animal and human pathogens. In the Iberian Peninsula *O. erraticus* transmits the African swine fever virus (ASFv) (Basto et al., 2006), and several species of tickborne relapsing fever borreliae, such as *Borrelia hispanica* and *B. crocidurae* (Piesman and Gage, 2004). In Africa *O. moubata* is an important vector of the ASFv (Rennie et al., 2001) and of the causal agent of the African human relapsing fever, *Borrelia duttoni* (Piesman and Gage, 2004). Accordingly, control of these ticks would greatly improve the control of such diseases.

Current tick control is based on the use of acaricides, but these chemicals have serious drawbacks, including the development of resistance in ticks, toxicity, contamination of food products, and environmental pollution (Graf et al., 2004; Ostfeld et al., 2006). These disadvantages have stimulated the search for alternative methods to control ticks.

Biological control based on entomopathogenic fungi is a promising option. The ability of entomopathogenic fungi to penetrate ticks through their cuticle, thus avoiding the need to be ingested, the capability of a single fungal species or strain to kill several stages of the same tick, and the specific virulence of each fungal strain to one or a small group of ticks make them good candidates as biocontrol agents (Samish et al., 2001; Gindin et al., 2002; Samish et al., 2004; Pirali-Kheirabadi et al., 2007).

Among the entomopathogenic fungal species examined for pathogenicity against ticks in diverse laboratory and field studies, the most pathogenic were found to be *Beauveria bassiana* and *Metarhizium anisopliae* (Samish et al., 2004 and Ostfeld et al., 2006). Consequently, these two fungal species have received major attention and have been the object of subsequent studies (Alvares-Campos et al., 2005; Arruda et al., 2005; Hornbostel et al., 2005a, 2005b; Polar et al., 2005a, 2005b; Bahiense et al., 2006; Pirali-Kheirabadi et al., 2007). However, these studies have been focussed almost exclusively on the control of ixodid ticks, and have neglected the control of argasid ticks. The only exception seems to be the work by Sewify and Habib (2001), which studied the pathogenic effect of *M. anisopliae* on the argasid

tick *Argas persicus*. These authors sprayed heavily infested poultry houses with a fungal spore suspension and observed that the argasid population disappeared in 3 weeks. Despite this interesting result, no further studies on the control of argasids with *M. anisopliae, B. bassiana,* or other entomopathogenic fungi have been published.

The objective of the present work was to test the pathogenicity of several strains of three entomopathogenic fungal species, *Beauveria bassiana*, *Lecanicillium lecanii,* and *Tolypocladium cylindrosporum,* to different developmental stages of the argasid ticks *O. erraticus* and *O. moubata*.

Lecanicillium lecanii (=*Verticillium lecanii*) is an entomopathogen with a wide host range which has been used as a biological control agent against agricultural insect pests (Shah and Pell, 2003). *L. lecanii* is known to naturally infect the ixodid ticks *Ixodes ricinus* and *I. scapularis* (Kalsbeek et al., 1995; Zhioua et al., 1999). However, its effect on argasid ticks is unknown.

Tolypocladium cylindrosporum is pathogenic to larvae of several mosquito genera, including *Anopheles* and *Aedes,* which contain vectors of human parasites causing important diseases such as malaria, yellow fever and dengue (Scholte et al., 2004). In addition, this fungus is known to be pathogenic to other insects such as black flies (*Simulium vittatum*), and *Galleria mellonella,* a honey bee pest (Bandani, 2004; Nadeau and Boisvert, 1994). However, it has not been previously reported as a tick pathogen.

2. Materials and methods

2.1. Ticks

The *O. erraticus* and *O. moubata* ticks used in this work came from two colonies maintained in our laboratory. The colony of *O. erraticus* was established from specimens captured in Salamanca, western Spain, and the colony of *O. moubata* was established from

specimens obtained from the Institute for Animal Health, Pirbright, Surrey, UK. These ticks are fed regularly on rabbits, and kept at 28 ºC and 80 % relative humidity (RH).

2.2. Fungal strains and preparation of conidial suspensions

The isolates of the three species of fungi used for the experiments were endophytes obtained from asymptomatic grasses. The strains of *Beauveria bassiana* (Bb1764 and Bb2157), and *Lecanicillium lecanii* (Ll586, Ll618, and Ll3047) were isolated from plants of *Dactylis glomerata* (Sánchez Márquez et al., 2007), and the strain of *Tolypocladium cylindrosporum* (Tc3398) was obtained from a plant of *Holcus lanatus*.

To obtain conidia, fungal cultures grown on potato dextrose agar Petri plates were maintained in the dark at room temperature (22-25º C). Conidia from three-week old cultures were released from the mycelium with a glass rod, after adding 5 ml of sterile water containing 0.01% Tween 80 to each plate. The conidial suspension from three plates was collected and centrifuged at 2000 g for 5 min. The pellet was resuspended in 1 ml of sterile water and the concentration of conidia was estimated with a Bürker chamber. Suspensions of 10^7 or 10^8 conidia/ml were prepared in sterile water containing 0.01% Tween 80.

2.3. Bioassays

Two bioassays were carried out using a methodology adapted from that described by Samish et al. (2001) and Fernandes et al. (2003).

2.3.1. Bioassay 1.

Five developmental stages (newly-moulted unfed males, females, nymphs-4, nymphs-3, and nymphs-2) from both *Ornithodoros* species were treated with the two strains of *B. bassiana* (Table 1). Each treatment group was placed in a vial containing 2 ml of the corresponding conidial suspension (10^7 conidia/ml in 0.01% Tween 80). After 5 minutes, the excess

suspension was removed, and the ticks were incubated for 28 days at 28 ºC and 80% RH. Simultaneously, similar groups of each species and developmental stage were treated with a 0.01% Tween 80 aqueous solution, without conidia, and used as controls. Mortality was recorded for every group on 7, 14, 21 and 28 days post-inoculation (p.i.), and the percentage of cumulative mortality calculated.

2.3.2. Bioassay 2.

Three strains of *L. lecanii* and one of *T. cylindrosporum* were tested against the same developmental stages of O. *erraticus* and *O. moubata* used in bioassay 1. The number of ticks in some treatment groups was different from their equivalent groups in bioassay 1, owing to specimen availability (Table 1). Each treatment group was inoculated in a similar way to that described for bioassay 1, but with a higher conidial dose (10^8 conidia/ml, 2 ml). Similar groups of each tick species and developmental stage were included as controls. Ticks were incubated for 62 days at 28 ºC and 80% RH. Mortality was recorded for every group at 7, 14, 21, 28, and 62 days p.i.

2.3.3. Statistics

The percentages of mortality of all developmental stages for each fungal treatment were analysed using one way ANOVA, followed by the LSD test for comparisons between the control and treatment means. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Pathogenicity of B. bassiana to O. erraticus and O. moubata.

All the developmental stages of *O. erraticus* and *O. moubata* included in this study were susceptible to both strains of *B. bassiana*, although their degree of susceptibility varied considerably (Fig. 1). As a general rule, mortality of ticks began around 7 days p.i., after that, it

156 increased slowly reaching a maximum, or a plateau, at 14 to 28 days p.i. The exception to this
157 rule were the adults and nymphs-3 of *O. erraticus* infected with Bb2157, whose mortality
158 experienced a sharp increase between 21 and 28 days p.i.

159 The cumulative mortality of ticks at 28 days p.i. is shown in Fig. 2 and Table 2. In *O.*
160 *erraticus* (Fig. 2A), strain Bb2157 caused higher and more uniform mortality rates (40% to
161 90%) than Bb1764 (5% to 67%). In *O. moubata* (Fig. 2B), strain Bb2157 also caused higher
162 mortality rates (8% to 79%,) than *strain* Bb1764 (5% to 50%). As a whole (Fig. 2C and Table
163 2), strain Bb2157 caused mean mortality rates significantly higher than the control ($p < 0.05$) in
164 both argasids, 62% for *O. erraticus* and 31.9% for *O. moubata*. In contrast, Bb1764 caused less
165 mortality in both argasids, 34.4% ($p < 0.05$) for *O. erraticus*, and 18.5% for *O. moubata*.

166 Dead ticks appeared swollen and with reddish areas in their body and legs. Four to
167 seven days after death, white fungal mycelium started to emerge and sporulate on the tick
168 surface. When observed with the microscope the fungus growing on dead tick bodies was
169 identified as *B. bassiana*.

170

171 *3.2. Pathogenicity of L. lecanii and T. cylindrosporum to O. erraticus and O. moubata*

172

173 As shown in Fig. 3, the three *L. lecanii* strains examined caused low mortality rates in
174 all the treatment groups of *O. erraticus,* except in nymphs-3, where the mortality rates ranged
175 from 21% to 49%. On the other hand, *T. cylindrosporum* Tc3398 induced higher mortality rates
176 than *L. lecanii* in all developmental stages of *O. erraticus*. In general, mortality of *O. erraticus*
177 ticks began around 7 days p.i. and reached a plateau between 14 and 28 days p.i. Little or no
178 additional mortality took place after this period.

179 Regarding *O. moubata* (Fig. 3), the three strains of *L. lecanii* and the one of *T.*
180 *cylindrosporum* caused low mortality in all treatment groups; in some groups the mortality rate
181 barely differed from that of the controls. In general, most deaths occurred between 7 and 28
182 days p.i. although, in several treatment groups some additional deaths occurred afterwards.

As observed with *B. bassiana*, dead specimens appeared slightly swollen and with their cuticle and legs stained in red. Seven days after death, fungal mycelium showing morphological characteristics of *L. lecanii* or *T. cylindrosporum* was observed on the surface of dead ticks.

As shown in Fig. 4A, *O. erraticus* was the species most affected by the fungi. *L. lecanii* strains Ll586, Ll618 and Ll3047 induced, in the different treatment groups, mortality rates that ranged from 0 to 49%. The mean mortality rates observed in *O. erraticus* with these three fungal strains were 9.8%, 14.0%, and 16.8%, respectively (Fig. 4C). In contrast, in the groups treated with *T. cylindrosporum* Tc3398 the mortality rates were higher and ranged from 20% to 72% (Fig 4A). The mean mortality rate observed in *O. erraticus* with this strain was 41.2% ($p < 0.05$) (Fig. 4C and Table 2).

In *O. moubata*, groups treated with *L. lecanii* Ll586 showed very low mortality rates (from 0% to 5%), whereas groups treated with Ll618, Ll3047 and *T. cylindrosporum* Tc3398 showed slightly higher mortality rates, ranging from 0% to 23% (Fig. 4B). The mean mortality rates caused in *O. moubata* by the four fungal strains were 2.5%, 9.6%, 13.9%, and 10.4%, respectively (Fig. 4C).

4. Discussion

Despite the increasing interest in entomopathogenic fungi as biocontrol agents in ixodid ticks, studies on the use of these fungi to control argasid ticks are almost inexistent (Sewify and Habib, 2001). In the search for anti-argasid fungal agents, in this study we assessed the pathogenicity of several fungal isolates for *O. erraticus* and *O. moubata* two argasids of great medical and veterinary importance. The fungi tested were six Spanish isolates of *B. bassiana*, *L. lecanii*, and *T. cylindrosporum.*

We examined two strains of *B. bassiana* (Bb1764 and Bb2157), a species whose pathogenicity to argasid ticks had never been studied (Bioassay 1). The results of this experiment showed that the two strains were pathogenic to both argasid species, although their degree of virulence varied noticeably among species and developmental stages. Both strains

were more virulent to the Spanish tick *O. erraticus*, and caused mean mortality rates significantly higher than the controls. However, for the African tick *O. moubata*, only the Bb2157 strain caused a mortality rate significantly higher than the control. This experiment also showed that Bb2157 was more virulent to both tick species than Bb1764 (Fig. 2C and Table 2), and that the pathogenicity of Bb2157 was more uniform for the different developmental stages included in the study, at least for *O. erraticus* (Fig. 2A). These results indicate that there is variation in pathogenicity to argasids among strains of *B. bassiana*.

The mortality rates observed in this bioassay were comparable to those observed by other authors on ixodid ticks using similar methodology and inoculum doses (in the range of 10^7 conidia/ml). For example, Samish et al. (2001) observed that the mortality rates of unfed *Rhipicephalus sanguineus* adult ticks inoculated with different strains of *M. anisopliae* ranged from 20% to 100%. More recently, Fernandes et al. (2003) observed mortalities of 63% to 85% in *Boophilus microplus* larvae inoculated with different isolates of *B. bassiana*.

Likewise, the mortality curves observed by us in bioassay 1 (Fig. 1), showed that most tick deaths occurred in the second week after inoculation. This result is comparable to that reported by Samish et al. (2001), which observed that all deaths in unfed adults and engorged females of *R. sanguineus* infected with *M. anisopliae* took place between 5 and 14 days p.i.

Thus, bioassay 1 showed that the *B. bassiana* strains can infect and kill two argasid tick species, a result that justifies further studies on the pathogenicity of *B. bassiana* strains Bb1764 and Bb2157 to *O. erraticus, O. moubata* and perhaps other *Ornithodoros* species. These studies would have to assess the effect of these fungi on the developmental stages not included in the present work, such as larvae and eggs, and also their effect on the rates of survival, moult (immatures), and fecundity (females) of engorged specimens.

In bioassay 2 we tested three strains of *L. lecanii* and one of *T. cylindrosporum*; two fungal species that have been never tested against ticks. In an attempt to improve the tick mortality rates obtained in bioassay 1, in bioassay 2 we used higher conidial doses (10^8 conidia/ml). Despite this, the tick mortality rates recorded in bioassay 2 were lower than in bioassay 1, suggesting that the above species are less pathogenic than *B. bassiana*. In this assay

239 most tick deaths also occurred in the second week p.i., and the prolongation of the incubation
240 period to 62 days hardly increased the mortality rates.

241 The results of bioassay 2 showed that the three strains of *L. lecanii* were not effective
242 against *O. moubata,* and only slightly effective against *O. erraticus,* causing moderate to low
243 mortality among nymphs-3 (Figs. 3 and 4). Accordingly we consider that these three strains of
244 *L. lecanii* are not good candidates as biocontrol agents against *O. erraticus* or *O. moubata*.

245 The Tc3398 isolate of *T. cylindrosporum* was also ineffective against *O. moubata*;
246 however it was quite pathogenic to *O. erraticus,* since it affected all its developmental stages
247 included in the study, and caused moderate to high mortality rates, ranging from 20% to 72%
248 (Fig. 4A and Table 2). The mean mortality rate induced by Tc3398 at 28 days. p.i. in *O.*
249 *erraticus* was significantly higher than the control (41.2%). Although this mortality rate is
250 similar to that obtained with the strains of *B. bassiana*, *T. cylindrosporum* might not be as
251 virulent, because its inoculum dose was higher than that used with *B. bassiana*. Despite this, our
252 results suggest that *T. cylindrosporum* Tc3398 could be a good candidate as a biocontrol agent
253 for *O. erraticus.*

254

255 **5. Conclusions**

256

257 We have conducted a preliminary screening to evaluate entomopathogenic fungal
258 isolates that can be used in the biocontrol of two argasid ticks of medical and veterinary
259 importance, *O. erraticus* and *O. moubata*. We found that three fungal strains (*B. bassiana*
260 Bb1764 and Bb2157 and *T. cylindrosporum* Tc3398) were effective against *O. erraticus,* and
261 one of them (*B. bassiana* Bb2157) was also effective against *O. moubata*. To our knowledge
262 this is the first study in which entomopathogenic fungi have been found to be pathogenic for
263 argasid ticks of the genus *Ornithodoros*. Additionally, this study represents one of the few
264 studies aimed at the biocontrol of ticks of the family *Argasidae.* The results justify further
265 efforts towards the application of entomopathogenic fungi as anti-argasid biocontrol agents.

266

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345 **Figure captions**

346

347 Figure 1. Bioassay 1. Cumulative mortality (%) in 5 developmental stages of *O. erraticus* and
348 *O. moubata* at 7, 14, 21 and 28 days post-inoculation with *B. bassiana* strains Bb1764 and
349 Bb2157. Control: tick specimens treated with 0.01% Tween 80 without fungal conidia. M,
350 males; F, females; N4, nymphs-4; N3, nymphs-3; N2, nymphs-2.

351

352 Figure 2. Bioassay 1. Percentage of mortality in each developmental stage of *O. erraticus* (A)
353 and *O. moubata* (B) at 28 days post-inoculation with *B. bassiana* strains Bb1764 and Bb2157.
354 Control: tick specimens treated with 0.01% Tween 80 without fungal conidia. M, males; F,
355 females; N4, nymphs-4; N3, nymphs-3; N2, nymphs-2. (C) Mean percentage of mortality ±
356 standard error for all developmental stages of each argasid species at 28 days post-inoculation.

357

358 Figure 3. Bioassay 2. Cumulative mortality (%) in 5 developmental stages from *O. erraticus* and
359 *O. moubata* at 7, 14, 21, 28 and 62 days post-inoculation with *L. lecanii* strains Ll586, Ll618,
360 and Ll3047, and *T. cylindrosporum* strain Tc3398. Control: tick specimens treated with 0.01%
361 Tween 80 without fungal conidia. M, males; F, females; N4, nymphs-4; N3, nymphs-3; N2,
362 nymphs-2.

363

364 Figure 4. Bioassay 2. Percentage of mortality in each developmental stage of *O. erraticus* (A)
365 and *O. moubata* (B) at 28 days post-inoculation with *L. lecanii* strains Ll586, Ll618, and Ll3047
366 and *T. cylindrosporum* strain Tc3398. Control: tick specimens treated with 0.01% Tween 80
367 without fungal conidia. M, males; F, females; N4, nymphs-4; N3, nymphs-3; N2, nymphs-2. (C)
368 Mean percentage of mortality ± standard error for all developmental stages of each argasid
369 species at 28 days post-inoculation.

370

Figure 1

Figure 1

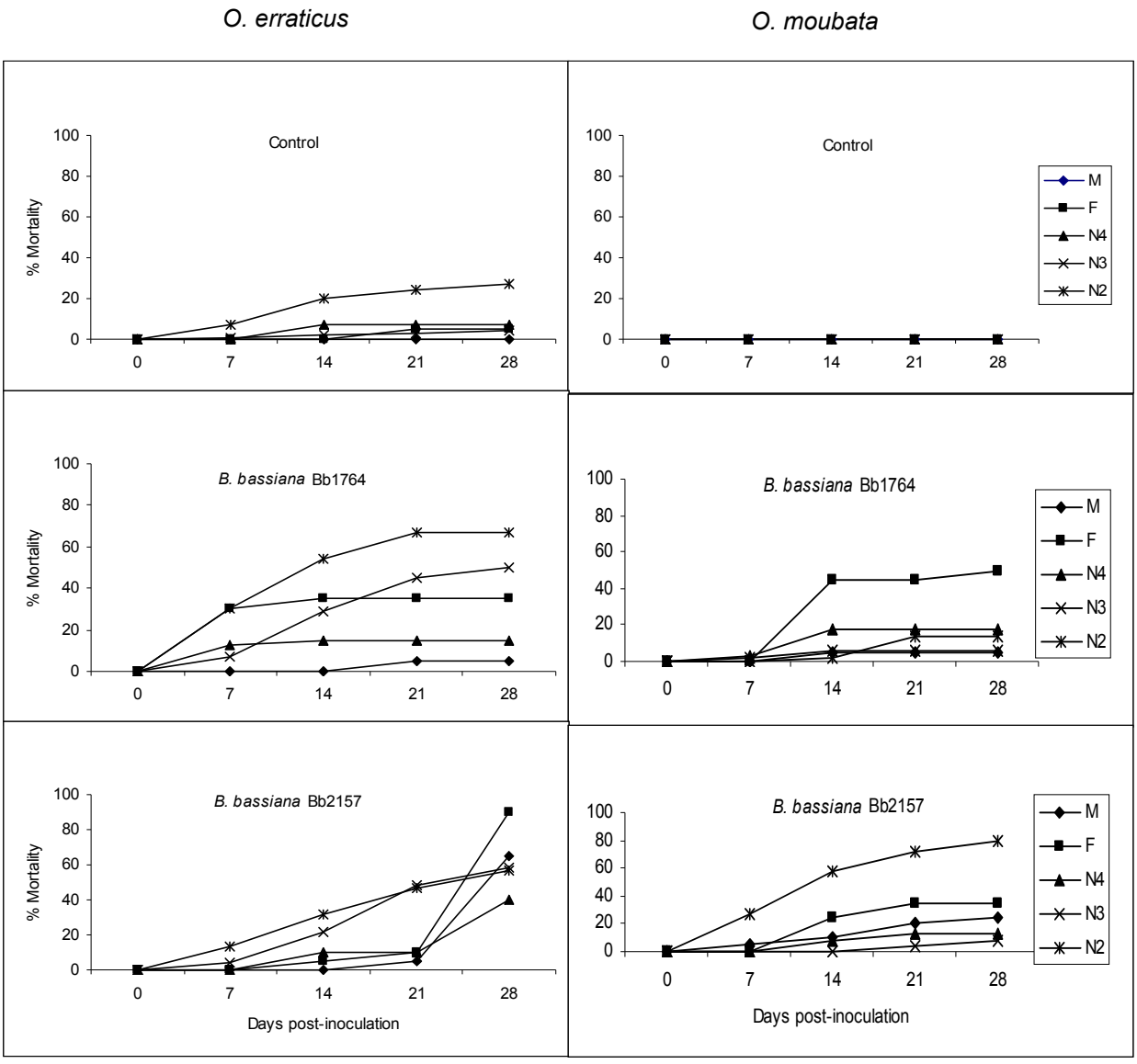

Figure 2

Figure 2

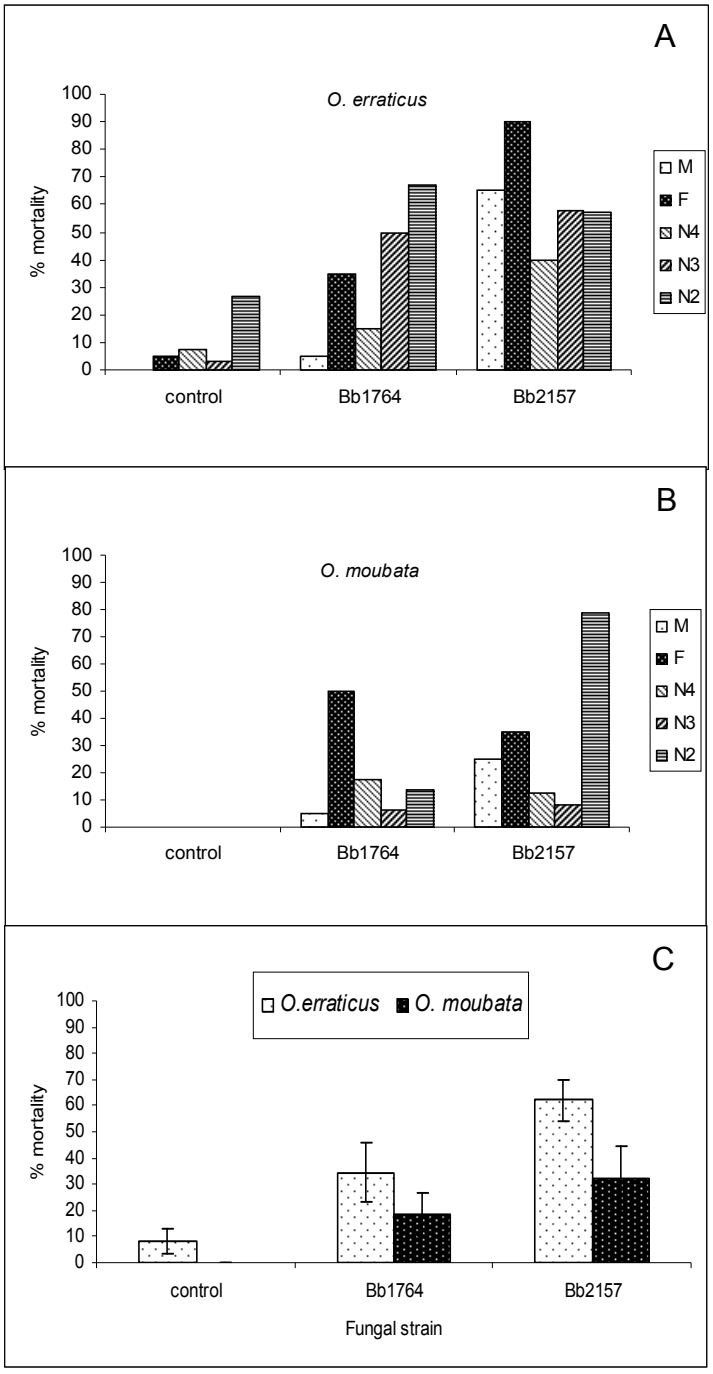

Figure 3

Figure 3

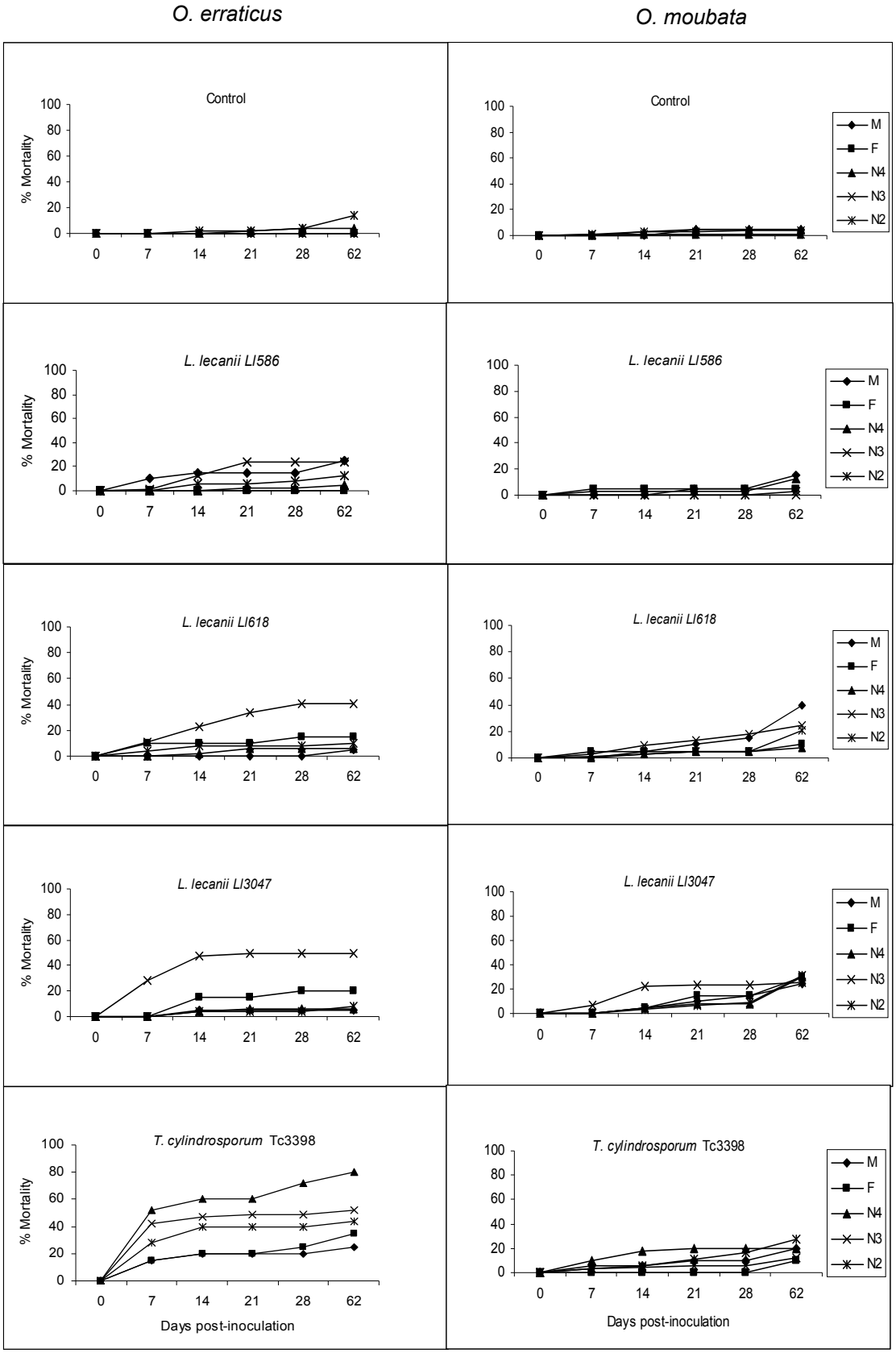

Figure 4

Figure 4

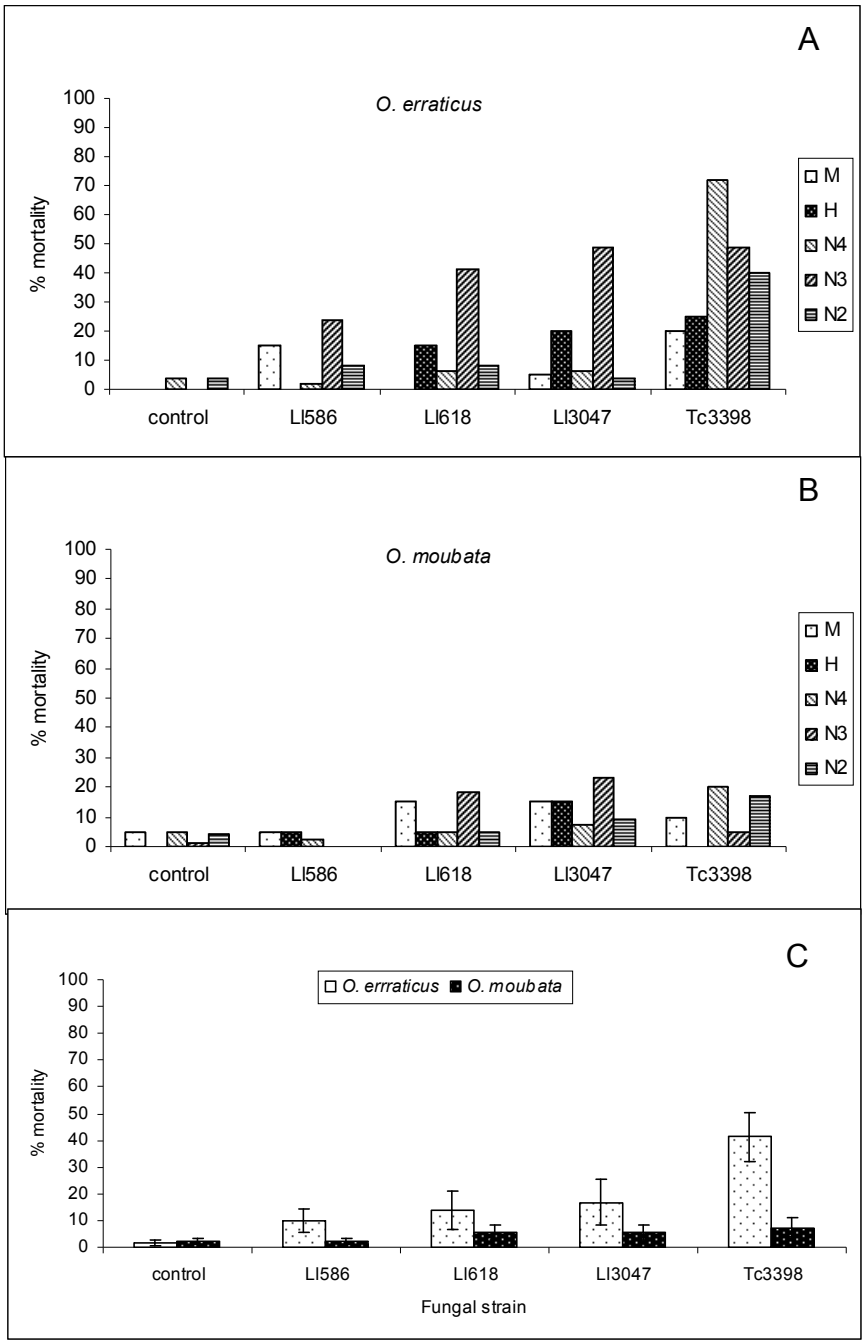

Table 1

Table 1. Description of the fungi, inoculum doses, and number of specimens of each tick species and developmental stage used for each treatment in bioassays 1 and 2.

	Treatment	***Fungal species and strain***	**Inoculum dose**	***O. erraticus* treatment groups**	***O. moubata* treatment groups**
Bioassay 1	Control	none	-		
	1	*B. bassiana* Bb1764	10^7 conidia/ml, 2 ml	20 males	20 males
	2	*B. bassiana* Bb2175	10^7 conidia/ml, 2 ml	20 females	20 females
				40 nymph-4	40 nymph-4
				100 nymph-3	50 nymph-3
				100 nymph-2	100 nymph-2
Bioassay 2	Control	none	-	20 males	20 males
	1	*L. lecanii* Ll586	10^8 conidia/ml, 2 ml	20 females	20 females
	2	*L. lecanii* Ll618	10^8 conidia/ml, 2 ml	50 nymph-4	40 nymph-4
	3	*L. lecanii* Ll3047	10^8 conidia/ml, 2 ml	100 nymph-3	100 nymph-3
	4	*T. cylindrosporum* Tc3398	10^8 conidia/ml, 2 ml	50 nymph-2	100 nymph-2

Table 2

Table 2. Mortality of *O. erraticus* and *O. moubata* at 28 days post-inoculation with each of the six fungal strains examined in bioassays 1 and 2: dead ticks/inoculated ticks (calculated %). *B. bassiana* strains Bb1764 and Bb2157, *L. lecanii* strains Ll586, Ll618 and LL3047, and *T. cylindrosporum* strain Tc3398. Control: tick specimens treated with 0.01% Tween 80 without fungal conidia.

Tick species	**Developmental stage**	**Control**[b]	**Fungal strains**					
			Bioassay 1		**Bioassay 2**			
			Bb1764	**Bb2157**	**Ll586**	**Ll618**	**Ll3047**	**Tc3398**
O. erraticus	Males	0/40 (0%)	1/20 (5%)	13/20 (65%)	3/20 (15%)	0/20 (0%)	1/20 (5%)	4/20 (20%)
	Females	1/40 (2.5%)	7/20 (35%)	18/20 (90%)	0/20 (0%)	3/20 (15%)	4/20 (20%)	5/20 (25%)
	Nymphs-4	5/90 (5.6%)	6/40 (15%)	16/40 (40%)	1/50 (2%)	3/50 (6%)	3/50 (6%)	36/50 (72%)
	Nymphs-3	4/200 (2%)	50/100 (50%)	58/100 (58%)	24/100 (24%)	41/100 (41%)	49/100 (49%)	49/100 (49%)
	Nymphs-2	29/150 (19.3%)	67/100 (67%)	57/100 (57%)	4/50 (8%)	4/50 (8%)	2/50 (4%)	20/50 (40%)
	Mean ± SE[a]	5.9 ± 3.4	34.4 ± 11.3*	62.0 ± 8.1*	9.8 ± 4.4	14.0 ± 7.1	16.8 ± 8.6	41.2 ± 9.3*
O. moubata	Males	1/40 (2.5%)	1/20 (5%)	5/20 (25%)	1/20 (5%)	3/20 (15%)	3/20 (15%)	2/20 (10%)
	Females	0/40 (0%)	10/20 (50%)	7/20 (35%)	1/20 (5%)	1/20 (5%)	3/20 (15%)	2/20 (0%)
	Nymphs-4	2/80 (2.5%)	7/40 (17.5%)	5/40 (12.5%)	1/40 (2.5%)	2/40 (5%)	3/40 (7.5%)	8/40 (20%)
	Nymphs-3	1/150 (0.7%)	3/50 (6%)	4/50 (8%)	0/100 (0%)	18/100 (18%)	23/100 (23%)	5/100 (5%)
	Nymphs-2	4/200 (2%)	14/100 (14%)	79/100 (79%)	0/100 (0%)	5/100 (5%)	9/100 (9%)	17/100 (17%)
	Mean ± SE[a]	1.5 ± 0.5	18.5 ± 8.2	31.9 ± 12.7*	2.5 ± 1.2	9.6 ± 2.9	13.9 ± 2.7	10.4 ± 3.7

[a] Mean percentage of mortality ± standard error for all the developmental stages for each fungal treatment.

[b] Mean mortality in control groups of both bioassays.

* $p < 0.05$ respect to the control groups