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Natural Tolerance to UV-B and Assessment of Photoprotectants in Conidia of Six Native Isolates of *Beauveria bassiana* (Bals-Criv) Vuillemin

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Abstract: Ultraviolet radiation (UV-B) causes a deleterious effect on conidia of entomopathogenic fungi in the field. Conidia of six isolates of *Beauveria bassiana* selected for their virulence against *Rhipicephalus (Boophilus) microplus* ticks were used to evaluate the natural tolerance of the fungal isolates to UV-B (λ=312 nm) and the ability of oils and oxybenzone to protect conidia from UV-B radiation. Also, the performance of the UV-B protectant selected was evaluated against tick eggs. There was a high variability in the natural tolerance to UV-B. Isolate Bb259 was the most tolerant, reaching 100% and 78.5% of relative conidial germination after 2 and 4h, respectively, of exposure to UV-B. Sunflower, corn and soybean oils were the most effective UV-B protectants. Sunflower oil was selected for its effectiveness and availability in the market to perform tick egg bioassays. The six isolates formulated with 5% (v/v) sunflower oil remained virulent after 4h of exposure to UV-B radiation, suggesting that the formulation protected the viability of conidia. Thus, the most UV-B-tolerant fungal isolate as well as the most virulent fungal isolate formulated in appropriate emulsifiable oil have a great potential to control pests and to be included in programs of integrated management of *R. microplus* ticks.

Key words: UV-B radiation · Microbial control · *Beauveria bassiana* · *Rhipicephalus (Boophilus) microplus*

INTRODUCTION

The tick *Rhipicephalus (Boophilus) microplus* Canestrini, 1887 (Acari: Ixodidae) [1] is one of the most important bovine ectoparasites in several countries worldwide [2]. Entomopathogenic fungi have been investigated as promising biological control agents of ticks [3-5]. The genus *Beauveria* (Bals-Criv) Vuillemin (Ascomycota: Hypocreales) is one of the most commonly studied fungi, primarily due to its cosmopolitan geographical distribution, wide host range and capacity to cause enzootic and epizootic outbreaks in several arthropod pests [6, 7]. The deleterious effects of UV radiation have been demonstrated in several genera of entomopathogenic fungi such as *Metarhizium* spp., *Beauveria* spp. and *Paecilomyces* spp. [8-13]. However, the tolerance to this radiation is highly variable even within the same fungal species [14]. In general, a few hours of direct exposure to summer midday sunlight in temperate and subtropical locations is sufficient to fully inactivate the conidia of almost all entomopathogenic fungi studied to date [14].

Both the conidial inactivation and the delay in the germination of the survivors are caused mainly by DNA damage induced by UV radiation. While UVA usually causes only indirect damage to DNA by catalyzing the formation of chemical intermediates such as sensitizer radicals or reactive oxygen species, UV-B acts directly on DNA, inducing the formation of several photoproducts [15, 16]. The most common photoproduct induced by UV-B is the cyclobutane pyrimidine dimer [17].

The reduction of inocula due to conidial inactivation and the delay in germination are expected to reduce the efficiency of these organisms as bioinsecticides in situations with strong solar radiation [18]. However, through the selection of the most UV-B-resistant isolates and the incorporation of UV protectants in formulations, it is possible to significantly prolong the persistence of these fungi in highly insolated habitats and thereby significantly increase the efficacy of fungi against target pests [9].

With the purpose to develop a mycoacaricide to be used in integrated tick management programs, in the current study, we investigated the natural tolerance to

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UV-B radiation in conidia of six native isolates of *Beauveria bassiana* previously selected for their virulence against *R. microplus* and evaluated the effectiveness of the compound oxybenzone and several oils as UV-B protectants. In addition, the performance of the formulation selected was evaluated against *R. microplus* eggs in a laboratory bioassay.

MATERIAL AND METHODS

Fungal Culture: Six *B. bassiana* native isolates previously selected for their virulence against *R. microplus* [5] were used (Table 1). The isolates were kept on agar slants at 8 °C in a refrigerator, then freeze-dried and deposited in the collection of the Entomopathogenic Fungi Laboratory (Instituto de Microbiologia y Zoologia Agrícola, Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina). For preserving purposes, isolates were cultured on Completed Medium Agar (CMA), which is composed of (g/L): potassium phosphate monobasic, 0.4; sodium phosphate, 1.4; magnesium sulfate, 0.6; potassium chloride, 1; ammonium nitrate, 0.7; glucose, 10; yeast extract, 5; agar, 15, chloramphenicol, 0.5.

Natural UV-B Tolerance Assays: To test the natural tolerance of *B. bassiana* isolates to UV-B radiation, conidial suspensions were performed in Tween 80 (0.05%) and adjusted to 2 x 10^7 conidia/ml using an improved Neubauer chamber. Aliquots of 100 µl were spread over the surface of Petri plates containing CMA plus 0.002 % (w/v) benomyl with 25 % active ingredient (Punch Química SA, Argentina). Benomyl has little effect on germination, but severely inhibits the growth of germ tubes, thereby preventing overgrowth of mycelium and allowing germination to be monitored for up to 24 h [19]. Three replicates were done for each treatment and fungal isolate. All plates were opened and placed in a laminar flow chamber (ESCO AC2-4A1) and exposed to UV-B irradiance provided by a PL-L 36 W/01 (λ= 312 nm wavelength peak, 4.8 W/cm^2/s) fluorescent lamp (Philips, Holland), for 0, 1, 2, 3 and 4 h. Control plates were covered with aluminum foil to block all UV radiation. The distance between the exposed plates and the UV-B source was 0.3 m. To achieve a stable level of radiation during the different trials, lamps were aged prior to the start of the experiments. After irradiation, the plates were incubated in darkness at 25 ± 1 °C and after 24 and 48 h of incubation, one drop of lactophenol blue (Sigma, USA) and a coverslip were placed on the plates and germination was observed at 400 x magnification in an optical microscope. For each plate, four microscopic fields, each containing a minimum of 100 conidia, were evaluated. The relative germination percentage of treated conidia, in relation to conidia not exposed to UV radiation, was calculated as described elsewhere [11, 12].

Values of relative germination from different exposure times and fungal isolates were compared by means of ANOVA. Tuckey´s tests were performed to post hoc comparisons. Experiment was repeated twice.

UV-B Protectants: Three plant-based oils, one mineral oil and the compound oxybenzone were evaluated as UV-B protectants. The plant-based oils used were soybean oil, sunflower oil and corn oil. The mineral oil evaluated was liquid petrolatum (Cicarelli, Argentina). Oxybenzone (2-hydroxy-4-methoxybenzophenone) was purchased from Sigma-Aldrich Chemical Co., USA.

Assessment of UV-B Protectants: Six fungal suspensions of each isolate were prepared in 10 ml Tween 80 (0.05%) and adjusted to 2 x 10^7 conidia/ml using an improved Neubauer chamber. UV-B protectants were incorporated to the fungal suspensions. Oils were added at 5 % (v/v), whereas oxybenzone was added at 0.5 % (w/v). The control was obtained by adding 5 % (v/v) of distilled water. Aliquots of 100 µl of each suspension were spread over the surface of Petri plates containing CMA plus 0.002 % (w/v) benomyl with 25 % active ingredient. Three replicates were done for each treatment and fungal isolate.

Table 1: Origin of the isolates used and their values of LC_{50} (Lethal concentration; virulence) against *Rhipicephalus (Boophilus) microplus*

Isolate	LC_{50} [a] (95% CL)	Host/Source	Locality
Bb 26	8.48 x 10^8 (7-12)	*Diatraea saccharalis* (Lep:Pyralidae)	Colón / Buenos Aires/ Argentina
Bb 98	1.15 x 10^7 (0.15-2.43)	*Cyclocephala signaticollis* (Col:Scarabaeidae)	Balcarce / Buenos Aires / Argentina
Bb 132	1 x 10^7 (0.32-2.35)	*Deuterocampta quadrijuga* (Col:Crisomelidae)	Córdoba / Cordoba / Argentina
Bb 175	2.48 x 10^8 (1.02-5.65)	*Rh. (Bo) microplus* (Acari: Ixodidae)	Castelar / Buenos Aires / Argentina
Bb 238	1.34 x 10^9 (1-3.43)	*Acromyrmex lundi* (Hym: Formicidae)	Castelar / Buenos Aires / Argentina
Bb 259	6.58 x 10^7 (4.38-8.89)	Soil	Castelar / Buenos Aires / Argentina

a-CL, 95% confidence intervals for LC_{50} values (Posadas and Lecuona, 2009).

All plates were exposed to UV-B for 4 h as described above. After irradiation, the plates were incubated in darkness at 25 ± 1°C and 48 h later one drop of lactophenol cotton blue and a coverslip were placed on the plates and germination was observed at 400 x magnification in an optical microscope. For each plate, four microscopic fields, each containing a minimum of 100 conidia, were evaluated. The percentage of germination of treated conidia, in relation to conidia not exposed to UV-B radiation, was calculated and differences among the protectants were compared by means of ANOVA. Tuckey´s tests were performed to post hoc comparisons.

Tick Colonies: The tick species used in this work was *R. microplus*. Tick colonies were kept at the Parasitology Laboratory (Instituto de Patobiología, INTA, Castelar, Argentina). Tick eggs and larvae were incubated in a controlled environment chamber at 27 °C, relative humidity (RH) = 80 % and a 14 h: 10 h cycle of light/dark. Ticks developed in a 12-month-old Holstein bovine (*Bos taurus L.,* Bovidae) infested every 15 d with tick larvae. After 21 d, engorged females were collected from the stable soil and placed into a controlled environment chamber at 27 °C and RH = 80% until the end of the egg-laying period.

Tick Egg Bioassays: To evaluate the performance of the formulation selected, engorged females were held in Petri dishes and incubated at 27 °C and RH = 80 % for oviposition. The eggs laid until the tenth day of oviposition were used in this bioassay. Egg aliquots of 50 mg were placed in test tubes (16 x 100 mm) sealed with cotton plugs. Each treatment was formed by eight test tubes. Four treatments were evaluated for each fungal isolate: the fungal suspension (5 x 10^8 conidia/ml) in Tween 80 (0.05%) before and after 4 h of exposure to UV-B and the formulation (fungal suspension plus 5 % (v/v) sunflower oil) before and after 4 h of exposure to UV-B. The control was without the fungus. The eggs were immersed in 1 ml of conidial suspension or formulation for 3 min. The test tubes were then turned upside down to remove the excess of conidial suspension/formulation through absorption by the cotton plug. Tubes were kept in a controlled environment chamber at 27 °C and RH = 80 % and the percentage of larvae hatching was evaluated after 15 d. The percentage of egg mortality was calculated as 100-% larvae hatching. This parameter was compared between treatments before and after UV-B exposure by a *t*-test [20]. Also, for the same fungal isolate, the percentage of egg mortality between the suspension and the formulation were compared by means of a *t*-test. The unhatched eggs were inoculated in Petri dishes containing OA-CTAB medium [21] and kept in a controlled environment chamber to confirm fungal infection.

RESULTS

Natural UV-B Tolerance Assays: There was variability in the tolerance to UV-B radiation among the six *B. bassiana* isolates. Values of relative germination after 24 and 48 h of incubation are shown in Table 2. Generally, all isolates presented a significant decrease ($p<0.05$) in conidial germination after UV-B exposure. This tendency to decrease is directly related to UV-B exposure time. After 24 and 48 h of incubation, Bb 259 was the only isolate which presented a percentage of relative germination greater than 75 % after 4 h of exposure to UV-B light. This isolate was the most tolerant to UV-B radiation ($F = 64.74$; $df = 5$; $p<0.001$); it showed 100 % of relative germination after 1 and 2 h of UV-B exposure and 48 h of incubation and 87.7 % and 78.5 % after 3 and 4 h of exposure respectively. Isolates Bb 26 and 132 were the most sensitive ($F = 64.74$; $df= 5$; $p<0.001$), with values of relative germination after 4 h of exposure to UV-B and 48 h of incubation of 35.6 % and 34.1 % respectively. A high variability in the natural tolerance among the isolates was observed both at 24 and 48 h of incubation.

Assessment of UV-B Protectants: All UV-B protectants significantly ($p<0.001$) increased the percentage of conidial germination when compared with the control after 4 h of exposure to UV-B (Table 3). As a general tendency, sunflower, corn and soybean oils were the most effective protectants, reaching values of percentage of conidial germination between 80.48 % and 100 %. The protectant oxybenzone presented the lowest values of percentage of conidial germination, which ranged between 58.89 % (isolate Bb 175) and 88.39 % (isolate Bb 259). Also, this protectant showed no significant differences ($F = 126.95$, $df = 5$, $p<0.001$) with the control in isolate Bb 175.

Isolate Bb 259, which presented the highest natural tolerance to UV-B, was the most protected, reaching 100 % of photoprotection with sunflower, corn and soybean oils. Sunflower oil was selected as UV-B protectant for the tick egg bioassays, based on its high performance, low cost and availability in the market.

Table 2: Relative germination of *Beauveria bassiana* conidia after 24 and 48 h of incubation and four exposure times to UVB radiation

Relative Germination after 24 h of incubation (%)				
Isolate/ Exposure Time (h.)	1	2	3	4
Bb 26	92.5 a, B	81.4 b, B	73.3 b, B	53.4 c, B
Bb 98	98.6 a, AB	87.6 b, B	79.9 b, B	43.6 c, B
Bb 132	95.2 a, AB	71.1 b, A	59.9 c, AB	39.8 d, A
Bb 175	84.4 b, AB	80.2 b, B	59.44 c, AB	51.46 c, B
Bb 238	90.9 b, A	63.3 c, A	59.3 c, AB	48.9 c, B
Bb 259	92.2 a, C	95 a, C	84.6 b, C	76.1 c, C
Relative Germination after 48 h of incubation (%)				
Bb 26	92.8 a, B	80.7 b, B	59.1 c, A	35.6 d, B
Bb 98	92.4 a, B	73.7 b, A	69.8 b, A	40.5 c, A
Bb 132	87.4 b, A	81.7 b, B	56.4 c, A	34.1 d, B
Bb 175	85.4 b, A	82.4 b, B	63 c, A	42.5 d, A
Bb 238	85.8 b, A	63.1 c, A	66.1 c, A	49.2 d, A
Bb 259	100 a, C	100 a, C	87.7 b, B	78.5 c, C

Data followed by different lowercase letters within each line are significantly different (ANOVA and Tuckey test; $p<0.05$).

Data followed by different uppercase letters within each column are significantly different (ANOVA and Tuckey test; $p<0.05$).

Table 3: Percentage of conidia germination formulated with several UVB protectants after 4h of UVB exposure

	UVB protectants					
Isolate	Control (5 % (v/v))	Liquid petrolatum (5 % (v/v))	Sunflower oil (5 % (v/v))	Corn oil (5 % (v/v))	Soybean oil (5 % (v/v))	Oxybenzone (5 % (w/v))
Bb 26	36.61a	87.49 b	93.94 c	91.16 c	93.27 c	78.57 b
Bb 98	37.61a	85 b	96.35 c	86 b	98.42 c	88 b
Bb 132	29.55 a	94.08 c	95.42 c	95.71 c	98.23 c	62.95 b
Bb 175	47.94 a	87.72 b	95.54 c	93.07 c	96.34 c	58.89 a
Bb 238	42.16 a	89.85 c	96.17 c	80.48 c	92.26 c	65.16 b
Bb 259	82.15 a	84 a	100 b	100 b	100 b	88.39 a

Data followed by different letters within each line are significantly different (ANOVA and Tuckey test; $p<0.05$).

Table 4: Values of percentage of egg mortality, before and after exposing fungal suspensions / formulations to UVB radiation for 4 h.

Fungal Isolate	Treatment	% egg mortality	% egg mortality after UVB exposure
Control*	Suspension	2.53 a, A	2.61 a, A
	Formulation	2.35 a, A	3.13 a, A
Bb 26	Suspension	81.77 a, A	13.92 b, B
	Formulation	79.21 a, A	87.34 a, A
Bb 98	Suspension	87.17 a, A	10.9 b, B
	Formulation	84.89 a, A	89.6 a, A
Bb 132	Suspension	83.83 a, A	17.94 b, B
	Formulation	82.5 a, A	88.63 a, A
Bb 175	Suspension	75.77 a, A	28.27 b, B
	Formulation	85.03 a, A	84.74 a, A
Bb 238	Suspension	75.2 a, A	28.22 b, B
	Formulation	74.16 a, A	75.37 a, A
Bb 259	Suspension	73.87 a, A	60.81 a, A
	Formulation	71.37 a, A	69.93 a, A

Data followed by different lowercase letters within each line are significantly different (*t*-test; $p<0.05$).

For the same fungal isolate, data followed by different uppercase letters within each column are significantly different (*t*-test; $p<0.05$).

Formulation: Fungal suspension plus 5 % (v/v) sunflower oil.

Suspension: fungal conidia suspended in Tween 80 (0.05 %).

*Formulation control: Tween 80 (0.05%) plus 5 % (v/v) sunflower oil without fungal conidia.

*Suspension control: Tween 80 (0.05 %) without fungal conidia.

Tick Egg Bioassays: The values of percentage of egg mortality before and after 4 h of exposing fungal suspensions / formulation to UV-B radiation are shown in Table 4. The fungal formulation did not affect the virulence of conidia, so that the percentage of egg mortality did not show significant differences ($p<0.05$) when compared to the same isolate unformulated before UV-B exposure. With the exception of Bb 259, which presented the highest natural tolerance to UV-B, conidia formulated in oil exposed for 4 h to UV-B radiation killed more tick eggs ($P<0.05$) than the unformulated fungal suspensions. The values of egg mortality in conidia formulated in oil were between 75.37 % and 89.6 %, while those in unformulated suspensions ranged between 10.9 % and 28.22 %.

DISCUSSION

Relative germination in most isolates was reduced after only a few hours of direct exposure to UV-B. The variability in UV-B tolerance found among our six *B. bassiana* isolates was similar to that found by Fernandes *et al*. (2007) [14], who noted that 53 isolates of *B. bassiana* presented a high variability in tolerance to UV-B, ranging from 16 % to almost 80 %. These authors also reported that, in general, *B. bassiana* conidia survival was inversely related to UV-B exposure time. Our results showed the same tendency. It is important to highlight that isolate Bb 259 presented the highest natural tolerance to UV-B radiation, reaching 78.5 % of relative germination after 4 h of exposure and 48 h of incubation. This result may explain the lack of significant differences in the percentage of egg mortality between the formulated and the unformulated fungi.

Since the speed of germination has been pointed out as an important factor for fungal virulence [22, 23, 24], it is logical to assume that the virulence of conidia damaged by radiation will be reduced. Formulation of *B. bassiana* to provide protection to UV-B light will be necessary to increase its survival and efficacy. In our study, addition of 5 % (v/v) of oil enhanced the UV-B tolerance of *B. bassiana* conidia. We found that sunflower, corn and soybean oils provided the best degree of protection from artificial UV-B radiation after 4 h of exposure. Similarly, Alves *et al*. (1998) [7] found that peanut oil efficiently protected conidia of the related fungus *Metarhizium anisopliae* after 4 h and 6 h of exposure to UV-B radiation.

Moore *et al.* (1993) [25] found that oxybenzone resulted in a good protection of conidia from the entomopathogenic fungus *Metarhizium flavoviridae* exposed to UV-B. In contrast, our studies revealed that the protectant oxybenzone was ineffective to protect *B. bassiana* conidia from UV-B radiation.

The virulence of the fungal isolates against *R. microplus* eggs was similar after 4 h of UV-B exposure when the isolates were formulated with 0.5 % (v/v) of sunflower oil. However, the virulence of the unformulated fungal suspension decreased drastically when they were exposed to UV-B radiation for 4 h. Similarly, Inglis *et al*. (1996) [26] found that *B. bassiana* conidia formulated in sunflower oil were more efficient against grasshopper nymphs than those formulated in water when they were exposed to UV light. Leemon and Jonsson (2008) [27] observed that spores of entomopathogenic fungi in an canola oil emulsion exposed to UV-B caused higher mortality rates in ticks than comparative aqueous suspensions. Ramle *et al*. (2004) [28] suggested that oil formulation enhances adhesion of the conidia to the insect cuticle. These authors also reported that other advantages of oil over water formulation include the ready suspension of the lipophilic conidia of *B. bassiana* in oil. In addition, *Beauveria* spp. conidia maintain their viability in oils for longer than those in aqueous formulation [29]. Prior *et al*. (1988) [30] pointed out that an oil drift evaporates more slowly than water, thus giving the conidia more time to germinate and infect. Oil emulsifiable fungal formulations allow the use of conventional hydraulic sprayers and water, the cheapest and most readily available carrier liquid for pesticides.

Both natural tolerance and an efficient UV-B protectant are important qualities for a mycoacaricide to be an adequate tool in an integrated pest management program. However, the possible high virulence of the isolate also needs to be considered. In our research, Bb 259 ($LC_{50} = 6.58 \times 10^7$) was the most naturally tolerant isolate and with the addition of 5 % (v/v) of sunflower oil it reached values of 69.93 % of egg mortality, whereas isolate Bb 98 ($LC_{50} = 1.15 \times 10^7$) presented a low tolerance to UV-B radiation, but with the addition of sunflower oil, it reached values of 89.6 % of egg mortality.

In conclusion, UV-B is undoubtedly one of the most important factors affecting the survival of fungal conidia in the field. This work demonstrates that the formulation of the six *B. bassiana* isolates selected with 0.5 % (v/v) of sunflower oil was efficient to control tick eggs in the laboratory. Moreover, the selection of the most tolerant fungal isolate (Bb 259) as well as the selection of the most virulent fungal isolate (Bb 98) formulated with sunflower oil will provide valuable tools for their use in an integrated management program of *R. microplus* ticks. Particularly to protect ticks eggs, those are exposed to UV-B in the grass.

In this research, we only investigated the effect of UV-B, the most harmful fraction of UV radiation. However, since solar radiation in the field presents a combination of wavelengths, it is necessary to study the effectiveness of this oil formulation in this complex system. Thus, field assays are needed to complete the evaluation of the performance of this formulation.

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