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Metarhizium robertsii and *M. acridum* conidia produced on riboflavin-supplemented medium have increased UV-A tolerance and upregulated photoprotection and photoreactivation genes

Carla Huarte-Bonnet · Ronaldo A. Pereira-Junior · Flávia R. A. Paixão · Gilberto U. L. Braga · Donald W. Roberts · Christian Luz · Nicolás Pedrini · Éverton K. K. Fernandes

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Abstract The aim of this study was to evaluate the effect of riboflavin supplementation of culture medium on conidial UV-A tolerance of *M. acridum* (Driver & Milner) (Hypocreales: Clavicipitaceae) and *M. robertsii* (Bischoff, Rehner & Humber) (Hypocreales: Clavicipitaceae). These fungi were produced on culture medium supplemented, or not supplemented, with riboflavin. Relative germination and expression patterns of some photoprotection-related genes were evaluated after irradiating with artificial UV-A, or with filtered solar radiation (> 320 nm; UV-A and visible radiation). *M. acridum* conidia harvested from riboflavin-supplemented culture medium demonstrated enhanced UV-A tolerance when irradiated with artificial UV-A. Nevertheless, relative germination of conidia of both species produced on riboflavin-supplemented medium and exposed to filtered solar radiation was significantly higher than those produced on medium not supplemented with riboflavin. Riboflavin increased the transcription of photolyases, laccases and polyketide synthase genes. However, each fungal species induced different genes patterns involved in DNA repair and photoprotection. The addition of riboflavin to the substrate used for mass production of *Metarhizium* spp. and the resulting enhancement of conidial tolerance to solar radiation may improve the effectiveness of these fungi in biological control programs.

Carla Huarte-Bonnet and Ronaldo A. Pereira-Junior have contributed equally to this work.

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C. Huarte-Bonnet · R. A. Pereira-Junior · F. R. A. Paixão · C. Luz · É. K. K. Fernandes (✉)
Instituto de Patologia Tropical E Saúde Pública, Universidade Federal de Goiás, Avenida Esperança s/n, Campus Samambaia, Goiânia, GO 74690-900, Brazil
e-mail: evertonkort@ufg.br

C. Huarte-Bonnet · R. A. Pereira-Junior · F. R. A. Paixão · N. Pedrini (✉)
Instituto de Investigaciones Bioquímicas de La Plata – INIBIOLP, CONICET - Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 1900 La Plata, Argentina
e-mail: npedrini@med.unlp.edu.ar

R. A. Pereira-Junior
Centro Universitário de Goiás – Uni-ANHANGUERA, Goiânia, GO, Brazil

G. U. L. Braga
Departamento de Análises Clínicas, Toxicológicas E Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil

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Introduction

Entomopathogenic fungi such as *Metarhizium* spp. Sorokin, 1883 (Hypocreales: Clavicipitaceae) offer an environmentally friendly alternative to chemical pesticides. Their use as biological pesticides has been limited, however, by their relatively slow killing speed compared to chemical insecticides as well as their low tolerance to abiotic stresses, such as solar ultraviolet (UV) radiation (Braga et al. 2001a, b, c, d; Fernandes et al. 2007; Santos et al. 2011). UV radiation is part of the spectrum of electromagnetic radiation emitted by the sun and is conventionally divided in UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (100–280 nm). Only UV-A and UV-B radiation reach the Earth's surface, because atmospheric ozone drastically reduces the penetration of radiation with wavelengths shorter than 320 nm and completely excludes those below 290 nm (Braga et al. 2015; Caldwell and Flint 1997). UV-A is responsible for approximately 95% of the total energy of the UV spectrum that reaches the planet surface and UV-B is responsible for the remaining 5% (Braga et al. 2015; Schuch et al. 2017). Both UV-A and UV-B radiation have deleterious effects on *Metarhizium* spp. conidia, and although the effects of UV-B irradiation on conidia of entomopathogenic fungi have been studied extensively (e.g., Braga et al. 2001a, c, d, 2006; Falvo et al. 2016; Fang et al. 2010; Fernandes et al. 2007, 2015; Hunt et al. 1994; Miller et al. 2004; Nascimento et al. 2010; Pereira-Junior et al. 2018; Posadas et al. 2012; Rangel et al. 2005, 2006, 2011), information on the effects of UV-A irradiation is scarce (Braga et al. 2001c; Cadet et al. 2015; Fargues et al. 1997; Rueda Páramo et al. 2015; Yao et al. 2010).

DNA is the main target of solar UV radiation in living cells (Schuch et al. 2017). The type of DNA damage caused by the radiation depends on the wavelength of the incident UV photon (Braga et al. 2015; Douki et al. 2003; Kuluncsics et al. 1999). Damage can occur either from a direct photoreaction triggered by the absorption of UV-B or UV-A photons or by photosensitization mediated by endogenous photosensitizers (Cadet et al. 2012). UV-B is strongly absorbed by DNA while UV-A is only weakly absorbed. Thus, the direct lesion caused by the former is much more frequent than by the latter (Kuluncsics et al. 1999). Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4)-pyrimidone photoproducts [(6–4) photoproducts] are the two main bipyrimidine photoproducts induced directly by UV-B radiation (Fang and St. Leger 2012; Kuluncsics et al. 1999; Nascimento et al. 2010). Although UV-A constitutes a large part of UV radiation it is far less efficient in producing direct DNA photo-lesions than UV-B (Douki et al. 2003; Kuluncsics et al. 1999). Bipyrimidine photoproducts are also the main type of DNA damage induced by UV-A radiation, although produced a thousand times less efficiently than UV-B and through a mechanism different from that triggered by UV-B (Douki et al. 2003; Kuluncsics et al. 1999). UV-A radiation also excites endogenous chromophores, which causes oxidative DNA damage, such as single-strand breaks, oxidized pyrimidines and oxidized purines (essentially 8-oxo-7,8-dihydroguanine) through reactive oxygen species (Douki et al. 2003; Kuluncsics et al. 1999; Schuch et al. 2009; Yagura et al. 2017).

The two major lesions induced in DNA by UV radiation (CPDs and 6–4 photoproducts) are repaired by photolyases, which are flavoproteins that use a near-UV/blue-light photon (300–500 nm) as a co-substrate (Sancar 2008). Enzymes that repair CPDs are referred to as CPD photolyase, and enzymes that repair (6–4) photoproduct are called (6–4) photolyase (Sancar 2008). All photolyases contain flavin adenine dinucleotide (FAD) group as the catalytic cofactor. Riboflavin [7,8-dimethyl-10-(1′-D-ribityl) isoalloxazine, vitamin B_2] serves as the precursor of the flavins coenzymes such as FAD (Abbas and Sibirny 2011).

Conidia of some species can accumulate melanin or melanin-like pigments that might act as sunscreens (Braga et al. 2015). Melanins are dark pigmented multifunctional polymers formed by phenolic or indolic monomers, usually complexed with proteins and carbohydrates (Butler and Day 1998). Fungal melanins, among other functions, protect conidia

D. W. Roberts
Department of Biology, Utah State University, Logan, UT, USA

against abiotic stresses. They are mostly synthesized via the dihydroxynaphthalene (DHN) pathway, where a polyketide synthase (PKS) catalyzes the first step in this metabolic route (Braga et al. 2015). Laccases also can be responsible for conidial pigmentation and pathogenesis. A laccase, *MrLac1*, was fully characterized in *M. robertsii* (= *M. anisopliae s.l.*) and reportedly contributes to conidial pigmentation (Fang et al. 2010). The expression of *MrLac1*, however, was present also in non-pigmented cells, suggesting additional roles for this enzymes.

Our recent studies were initiated in the hope to discover a simple way to improve UV-A and UV-B tolerance of fungal products destined for use in insolated field environments. We recently demonstrated that supplementing culture medium with riboflavin increased the tolerance of resulting *M. robertsii* and *M. acridum* (*M. anisopliae* var. *acridum*) conidia to UV-B, and this was related to increased expression of photolyase, laccase and PKS genes in these conidia (Pereira-Junior et al. 2018). The responses of *Metarhizium* spp. conidia produced on riboflavin-supplemented media to the tolerance to UV-A radiation or to full solar spectrum have not yet been investigated.

The current study examines the effects of riboflavin supplementation on conidia of *M. acridum* and *M. robertsii*. We evaluated if riboflavin could increase conidia tolerance to UV-A radiation emitted by UV-A fluorescent lamps and to filtered solar radiation. To study whether riboflavin could influence gene expression, we quantified transcription of genes involved in photorepair and photoprotection.

Materials and methods

Fungal isolates and culture conditions

Two isolates of *Metarhizium* spp. were studied: *Metarhizium acridum* (= *M. anisopliae* var. *acridum*) (ARSEF 324) and *Metarhizium robertsii* (= *M. anisopliae s.l.*) (ARSEF 2575), both from the USDA—Agricultural Research Service Collection of Entomopathogenic Fungal Cultures of the USA (ARSEF; Robert W. Holley Center for Agriculture & Health, USDA, Ithaca, NY, USA). Selection of these isolates was based on their known UV tolerance reported in the literature (Braga et al. 2001a, b, c, 2006; Hunt et al. 1994; Miller et al. 2004; Rangel et al. 2004, 2005). Both isolates were grown either on potato-dextrose-agar medium (PDA) (Difco Laboratories, Sparks, USA) or on PDA supplemented with 0.01% (w/v) riboflavin (PDAR) (Alamar Tecno-Científica Ltda., São Paulo, Brazil), in polystyrene Petri plates (90 × 15 mm, CRAL Artigos para Laboratórios Ltda., Cotia, Brazil) at 27 ± 1 °C and RH > 90%, in the dark, for 15 days. PDA or PDAR plates were completely covered with a disc of 90 g m^{-2} sterile tracing paper (Edispel Distribuidora, Goiânia, Brazil) before fungal inoculation to prevent contamination of conidia with culture medium during their harvesting.

Conidial tolerance to UV-A radiation

Exposure to radiation from UV-A lamps in a photostability chamber

Conidial suspensions on plates, with lids removed, were exposed to 16,000 mW m^{-2} of UV-A radiation for 30, 60, 90 and 105 min at 27 ± 1 °C in a photostability chamber (Series 6540, Caron Products & Services Inc., Marietta, OH, USA), which is equivalent to 28.8, 57.6, 86.4 and 100.8 kJ m^{-2} of UV-A radiation, respectively. Control plates were held in the chamber during exposure but covered with aluminum foil to block all UV radiation. The spectral irradiance (Supplemented Fig. S1a) was measured with an USB 2000 + Rad spectroradiometer (Ocean Optics, Dunedin, FL, USA). After irradiation, plates were incubated for either 24 h or 48 h at 27 ± 1 °C and RH > 90% in the dark and then stained with Amann lactophenol with cotton blue solution.

Conidial UV-A tolerance was evaluated as previously described (Fernandes et al. 2007; Pereira-Junior et al. 2018). Conidia produced either on PDA or PDAR were harvested and suspended in 0.01% (v/v) polyoxyethylene sorbitan monooleate (Tween 80®, Sigma Chemical Co., Saint Louis, MO, USA) solution. Suspensions were agitated, filtered through 8-µm filters to remove clumps of conidia, and the concentration adjusted to 2 × 10^6 conidia ml^{-1}. A 20 µl aliquot was inoculated, without spreading, onto the center of 35 mm Petri plates with PDA medium supplemented with yeast extract 1 g l^{-1} (PDAY), 0.002% (w/v) benomyl (50% active ingredient; Benlate®, DuPont, São Paulo, SP, Brazil) and 0.05% (w/v) chloramphenicol. Benomyl allows germination but

kills germ tubes, and thereby prevents mycelial overgrowth that may obviate viewing conidial germination. Chloramphenicol is bactericidal. At least 300 conidia were evaluated per plate at × 400 magnification, and relative percent germination was calculated according to Braga et al. (2001b). Four independent experiments were performed.

Exposure to filtered solar radiation (UV-A and visible radiation)

Additional fungal-inoculated plates were exposed to 19,126 mW m^{-2} filtered solar radiation for 30, 60, 90 or 120 min in an out-door trial, which was equivalent to 34.5, 68.9, 103.35 or 137.8 kJ m^{-2} of UV-A radiation, respectively (supplementary Fig. S1b). During exposure, the plates were floated on water in plastic trays, with ice added as needed to avoid temperature increase, and the temperature inside one non-inoculated plate was monitored using a data logger (HOBO® H8, Onset Computer Corporation, Bourne, MA, USA). The plates were covered with a 0.1 mm-thick clear polyester film (JSC Industries, La Mirada, CA, USA) that blocks the transmission of UV wavelengths shorter than 320 nm (Braga et al. 2001c). Thus, conidia were exposed to solar UV-A and visible radiation. Control plates were covered with aluminum foil to block all UV radiation. After irradiation, the plates were incubated for 48 h at 27 ± 1 °C and RH > 90% in the dark, and assessment of relative germination was conducted as described above. Three experiments were performed on three different sunny days in Goiânia, Goiás, Brazil [Center-West Brazil (16° 40′ 30″ S and 49°14′ 42″ W, 749 m elevation)], from June 16th to July 9th, 2017, with all exposures starting at noon.

Gene transcription in *Metarhizium* spp. conidia exposed to UV-A and/or white light

A layer of mycelia often grew over the conidial layer on both PDA and PDAR plates. Most of this mycelial layer was removed from the culture plates with a spatula to allow irradiation of conidia, and for RNA extractions to be mostly from conidia. The culture plates were then exposed immediately to either (1) UV-A radiation in a photostability chamber at its 'UV-A' setting for 105 min (100.8 kJ m^{-2}), (2) white light (WL) at its 'white light' setting for 2 h at 16 Klux (supplementary Fig. S1c), or (3) to UV-A followed by white light at the same doses. The WL treatment was conducted to investigate the role of possible photoreactivation. Control plates were covered with aluminum foil to block all UV-A radiation and WL. After irradiation, conidia were harvested from culture plates with a spatula and immediately macerated in liquid nitrogen with mortar and pestle. Total RNA was extracted using the RNeasy Plant Mini Kit® (Qiagen, Hilden, Germany) with an on-column DNA digestion step (DNase I, Qiagen, Hilden, Germany), following the protocol recommended by the manufacturer. Total RNA was quantified using a Nanodrop® spectrophotometer (Thermo Scientific, Wilmington, USA), and its integrity analyzed in 1% (w/v) agarose gel. Synthesis of the first chain of cDNA was performed with the GoScript Reverse Transcription System® (Promega Corporation, Madison, USA), using 360 ng of total extracted RNA. The real-time polymerase chain reaction (q-PCR) was carried out with iQ SYBR Green Supermix (BioRad, Hercules, USA). The amplification was performed in an Mx3000P qPCR System (Stratagene, La Jolla, USA) at the following thermal conditions: denaturation at 95 °C for 10 min, followed by 40 cycles with three-segment amplification (30 s at 95 °C for denaturation, 1 min at 55 °C for annealing, and 30 s at 72 °C for DNA-chain elongation). The genes investigated and the primers used are listed in Table 1. The efficiency of qPCR reaction was optimized between 85 and 115% for all primers tested. Each sample was amplified in duplicate, and three independent assays were conducted. The relative expression ratio (RER) of each target gene was calculated according to Nordgard et al. (2006) as follows:

$$RER = \frac{{E_T}^{\Delta C_t T(\text{control}-\text{treatment})}}{\sqrt{{E_{R1}}^{\Delta C_t R_1(\text{control}-\text{treatment})} \times {E_{R2}}^{\Delta C_t R_2(\text{control}-\text{treatment})}}}$$

where E is the PCR efficiency, C_t is the cycle threshold, $R1$ and $R2$ are reference genes [Glyceraldehyde 3-phosphate dehydrogenase and γ-actin (Fang and Bidochka 2006)], T is target gene, ΔC_t is the C_t difference between the control (not irradiated) and treatments (irradiated with UV-A, WL or UV-A + WL).

Table 1 *Metarhizium robertsii* and *Metarhizium acridum* genes tested and the real-time quantitative primers sequences used

Gene	Forward (3′–5′)	Reverse (5′–3′)	Genebank accession number/references
Metarhizium robertsii			
CDP photolyase (*MrPhr1*)	CATGCCGATTTGGACTTGCTTG	TGGAACAGCGCAATCAAACAGG	JN694762.1
6–4 photolyase (*MrPhr2*)	TCGCCGTTTCTTCACTTTGGTG	TCTGCGTAAACTTGGCACCGAC	JN694761.1
Laccase 1 (*MrLac1*)	CGCCTGGCTTACGTGATAC	GCTCAGGCCATGCATCAAC	XM_007825745.1
Laccase 2 (*MrLac2*)	TCCCTGGGTCAACGAAAGCC	CGCCGCGATAAAGTTCATGC	XM_007826363.1
Laccase 3 (*MrLac3*)	CTATACTTTCCGAGCCACGCAG	CCCTTTCCATTCCCTCGATAGC	XM_007828431.1
Polyketide synthase 1 (*MrPks1*)	CATTCCGCCTCTCTCATTGCC	TGTGCGGCGCATGATATGG	XM_007825743.2
Polyketide synthase 2 (*MrPks2*)	CATCAGCGCCATCGGTTTAGAC	CGGGATAGGGATTGGTTTGTGG	XM_007821237.2
Metarhizium acridum			
6–4 Photolyase (*MaPhr2*)	TTCATGCCGATTTGGAATTGC	TGCTTGTTTGATGGTGCCTCTG	XM_007813640.1
Laccase 1 (*MaLac1*)	AATGCGCGTACCCAACTCTG	CCACGGCGACATGTGATCTG	XM_007813533.1
Laccase 2 (*MaLac2*)	CGGCCAATCCACAGCTAATG	TGGCATCGGCTGTGTTTGAC	XM_007809667.1
Laccase 3 (*MaLac3*)	CCGCATCCCATCCACAAAC	GCTGTGGTTGGCATGGAGAC	XM_007812616.1
Polyketide synthase 1 (*MaPks1*)	CCGTTCCCGACTCACAATTACC	GCACAGCAAGCGGCGTTAATC	XM_007813534.1
Polyketide synthase 2 (*MaPks2*)	CGGCTTATCCTCCAACGAATCC	TCTGCTGCCTCTGGAAATCTCC	XM_007817459.1
Reference genes (for both)			
Gamma actin	TCCTGACGGTCAGGTCATC	CACCAGACATGACGATGTTG	Partial sequence (Fang and Bidochka 2006)
Glyceraldehyde 3-phosphate dehydrogenase	GACTGCCCGCATTGAGAAG	AGATGGAGGAGTTGGTGTTG	AY461523

Statistical analysis

All data sets were previously checked for normality and homoscedasticity with Shapiro–Wilk and Barlett tests, respectively. Differences among the mean relative percent germination of conidia, and differences among the relative-gene expression ratios were determined by two-way ANOVA, which examined the effect of two independent factors: the radiation dose/time and the culture medium, followed by the Sidak's multiple comparisons test. No data transformation was required prior to ANOVA.

P-values < 0.05 were considered as significant. ANOVA followed by Sidak's multiple comparisons test were fit using GraphPad Prism® version 7.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Conidial tolerance to UV-A radiation

Exposure to UV-A from lamps

Conidia produced by *M. robertsii* (ARSEF 2575) and *M. acridum* (ARSEF 324) on PDA medium supplemented, or not, with riboflavin, exhibited a dose-dependent delay in germination following UV-A exposure (Fig. 1). No significant difference in tolerance to UV-A was found for *M. robertsii* conidia produced on medium supplemented, or not, with riboflavin and exposed to radiation emitted by UV-A lamps in a photostability chamber (Fig. 1a) ($P > 0.05$ for 24 h and 48 h incubation). In contrast, *M. acridum* conidia produced on riboflavin-supplemented medium were more tolerant (24 h incubation: $F_{3,24} = 7.93$ and $P = 0.0008$; 48 h incubation: $F_{3,24} = 5.12$ and $P = 0.007$) to radiation showing an increased germination rate compared to conidia produced on medium without riboflavin and irradiated for 60 min (followed by 24 h and 48 h incubation, $P < 0.01$) or for 105 min (48 h incubation, $P < 0.001$) (Fig. 1b). Although deleterious effects were observed after 105 min irradiation, *M. acridum* conidia produced on riboflavin-PDA supplemented medium reached similar relative germination levels as *M. robertsii* grown on either medium (Fig. 1).

Exposure to solar UV-A and visible radiation

In the outdoor irradiation tests, the germination of conidia produced on PDA medium supplemented with riboflavin and exposed to solar UV-A and visible radiation (wavelength > 320 nm; see supplementary Fig. S1) was significantly higher for both *M. robertsii* and *M. acridum* isolates in comparison to conidia produced on PDA not supplemented with riboflavin [ARSEF 2575: ($F_{3,23} = 38.00$; $P < 0.0001$); ARSEF 324: ($F_{3,23} = 20.73$; $P < 0.0001$)] (Fig. 2).

Gene transcription

With *M. robertsii*, transcription of CDP- and 6–4-photolyase genes (*MrPhr1* and *MrPhr2*) were higher in conidia exposed to UV-A and/or white light than the controls (*MrPhr1*: $F_{2,12} = 7.96$ and $P = 0.01$; *MrPhr2*: $F_{2,12} = 25.42$ and $P < 0.0001$). The transcript level of *MrPhr1* was significantly higher ($P < 0.05$) after exposure to UV-A + WL in conidia produced on PDAR than in conidia produced on PDA only (14.2- and 2.2-fold expression, respectively) (Fig. 3a). In medium supplemented with riboflavin, *MrPhr2* transcript levels in conidia after exposure to UV-A and UV-A + WL (20.7- and 21.2-fold expression, respectively) were significantly higher ($P < 0.001$) than in conidia produced on PDA without riboflavin (5.1- and 1.6-fold increase, respectively). For laccases, *MrLac1* expression was notably higher ($F_{2,12} = 40.41$ and $P < 0.0001$) in conidia produced on medium with riboflavin and exposed to UV-A or UV-A + WL (14.2- and 58.7-fold expression, respectively). No significant differences were observed for transcript level of *MrLac1* in conidia produced on PDA or PDAR and then exposed to white light alone, nor for *MrLac2* and *MrLac3* genes ($P > 0.05$ for both *MrLac2* and *MrLac3*) in all conditions tested (Fig. 3b). For *M. robertsii* polyketide synthases, the expression of *MrLac2* and *MrLac3* genes was increased by riboflavin supplementation after UVA + WL exposure (*MrPks1*: $F_{2,12} = 20.64$ and $P < 0.0001$; *MrPks2*: $F_{2,12} = 5.47$ and $P = 0.02$).

M. acridum photolyase gene (*MaPhr2*) was transcriptionally induced after UV-A exposure, regardless of the media tested ($F_{2,12} = 10.75$ and $P = 0.002$). However, the transcript level of photolyase in conidia produced on PDA was significantly higher (32.1-fold increase) than those found in conidia produced on PDAR (10.9-fold increase) (Fig. 4a). The transcript level of *MaLac1* was significantly higher ($F_{2,12} = 11.47$ and $P = 0.001$) in conidia produced on PDAR than in conidia produced on PDA, after exposure to UV-A (8.7- and 1.5-fold expression, respectively) (Fig. 4b). The expression of *MaLac2* and *MaLac3*, however, did not differ significantly ($P > 0.05$ for both genes). For all irradiation treatments, the expression of polyketide synthase genes in conidia produced on PDAR was not significantly different than those detected in conidia produced on

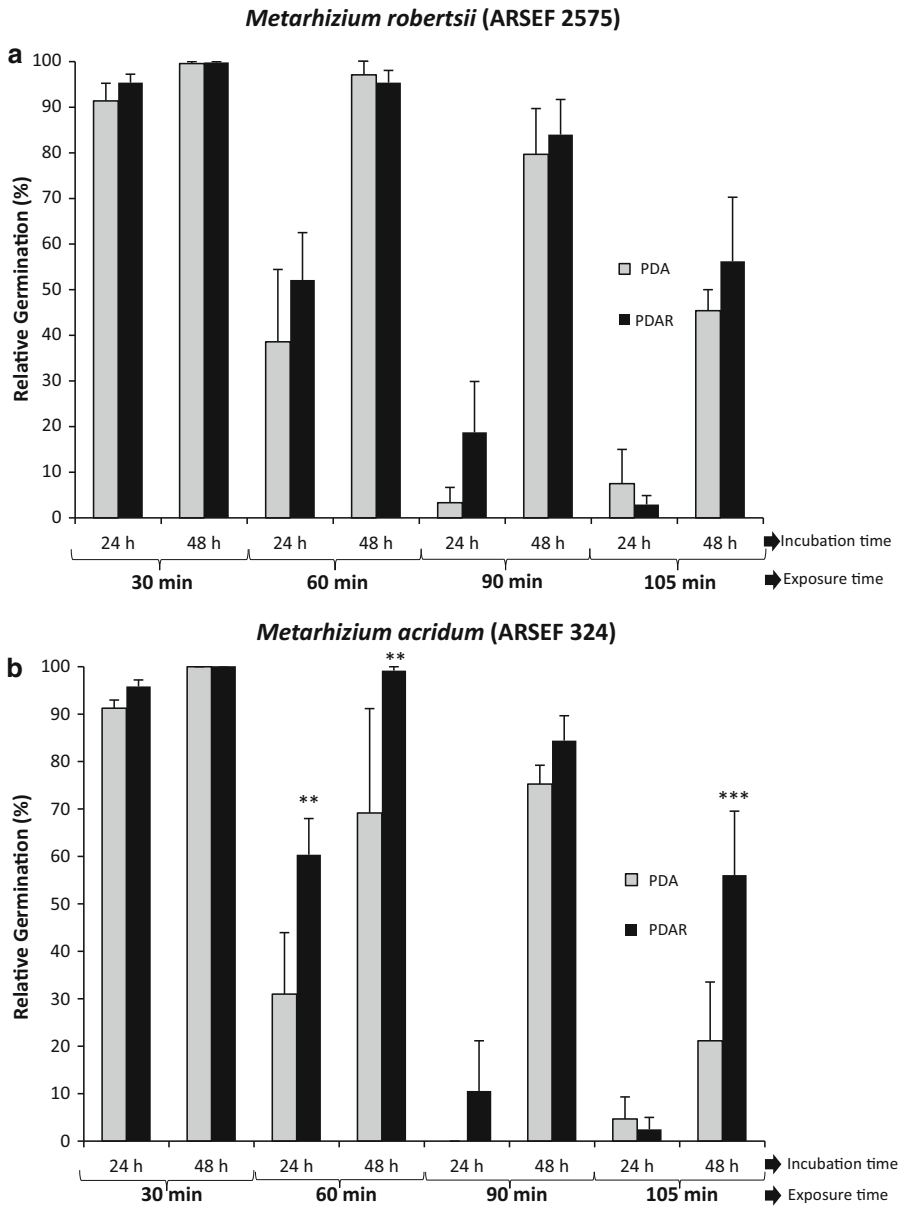

Fig. 1 Relative germination of conidia of *Metarhizium robertsii* ARSEF 2575 (**a**) and *M. acridum* ARSEF 324 (**b**) produced on PDA medium (gray) or on PDA supplemented with 0.01% riboflavin (= PDAR) (black), and incubated for 24 h and 48 h after being exposed to 16,000 mW m^{-2} of UV-A radiation for 30, 60, 90 or 105 min (2.8, 57.6, 86.4 and 100.8 kJ m^{-2}, respectively). Relative germination was calculated in relation to non-irradiated controls. Error bars are SE of four independent trials. Asterisks indicate significant difference between the relative germination of conidia produced on PDA or PDAR in the same incubation period: **($P < 0.01$), ***($P < 0.001$)

PDA ($P > 0.05$ for both *MaPks1* and *MaPks2*) (Fig. 4c).

Discussion

Riboflavin supplementation of culture medium induced *M. acridum* to produce conidia with increased tolerance to UV-A radiation, whether from UV-A lamps within a photostability chamber or under polyester-filtered solar radiation (wavelengths > 320 nm), which include UV-A and visible radiation. However, supplementing culture media with riboflavin increased tolerance of *M. robertsii* conidia only to filtered solar radiation, but not to UV-A radiation emitted by UV-A lamps. In a recent study, we also demonstrated that growing *M. robertsii* and *M. acridum* on riboflavin-supplemented medium produced conidia with increased tolerance to UV-B radiation (Pereira-Junior et al. 2018). The effect of UV-A (present study) or UV-B (Pereira-Junior et al. 2018)

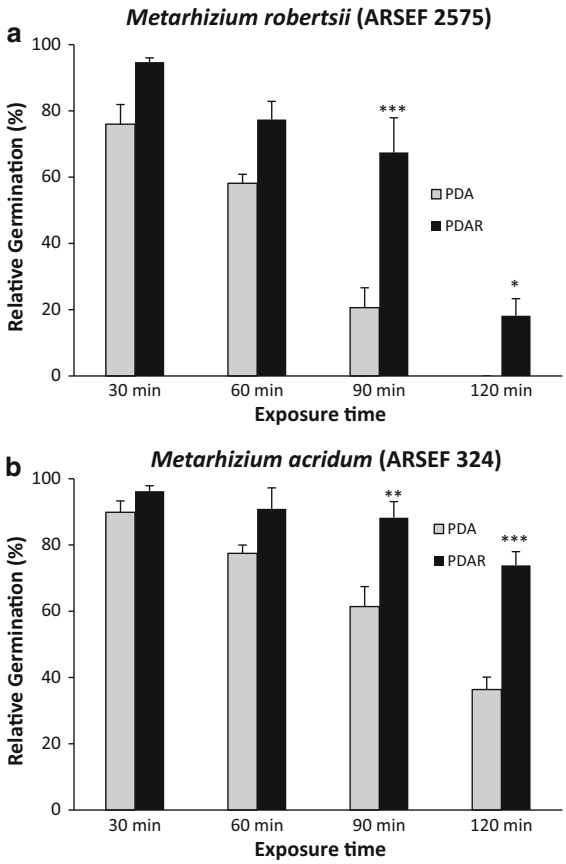

Fig. 2 Relative germination of conidia of *Metarhizium robertsii* ARSEF 2575 (**a**) and *M. acridum* ARSEF 324 (**b**) produced on PDA medium (gray), or PDA supplemented with 0.01% riboflavin (= PDAR) (black), and exposed to filtered solar UV-A radiation for 30, 60, 90 or 105 min. Relative germination was calculated in relation to non-irradiated controls. Error bars are SE of four independent trials. Asterisks indicate significant difference between the relative germination of conidia produced on PDA or PDAR in the same incubation period: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$)

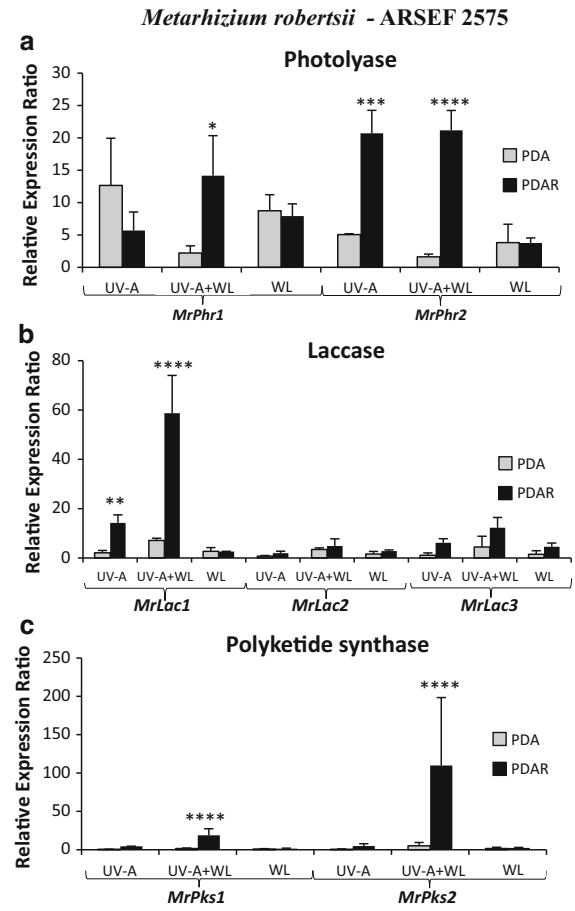

Fig. 3 Relative expression ratio of photolyase (**a**), laccase (**b**) and polyketide synthase (**c**) in *Metarhizium robertsii* (ARSEF 2575) grown on PDA medium with (PDAR) or without riboflavin (PDA) and exposed to: (1) 100.8 kJ m^{-2} of UV-A irradiance (UV-A), (2) UV-A (100.8 kJ m^{-2}) followed by exposure to 16 Klux of white light (UV-A + WL) for 2 h, or (3) white light only (WL) for 2 h. Error bars are SE of three independent trials. Asterisks indicate significant difference between the gene expression of conidia produced on PDA or PDAR: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$), ****($P < 0.0001$)

exposure on germination and gene expression of *M. acridum* and *M. robertsii* conidia produced on medium supplemented or not supplemented with riboflavin is summarized in supplementary Table S1.

Supplementation with riboflavin resulted in increased tolerance to filtered solar radiation for both *M. robertsii* and *M. acridum* conidia (Fig. 2). Results showed that the protective effect of the riboflavin supplementation against UV-A depends on the fungal strain and on the radiation used. In the case of *M. robertsii*, the difference between the tolerances of the conidia produced with or without riboflavin supplementation was much higher when filtered solar radiation was used. In this situation, besides the UV-A radiation there is an intense visible (white) light background that improves the photoreactivation of the pyrimidines dimers by photolyases, as documented by (Chelico et al. 2005, 2006). As riboflavin is the precursor of the FAD which is the catalytic center of these enzymes, we are tempted to speculate that conidia produced on riboflavin-supplemented medium have improved photoreactivation. However, this hypothesis should be confirmed in future studies. Photoreactivation is well documented in

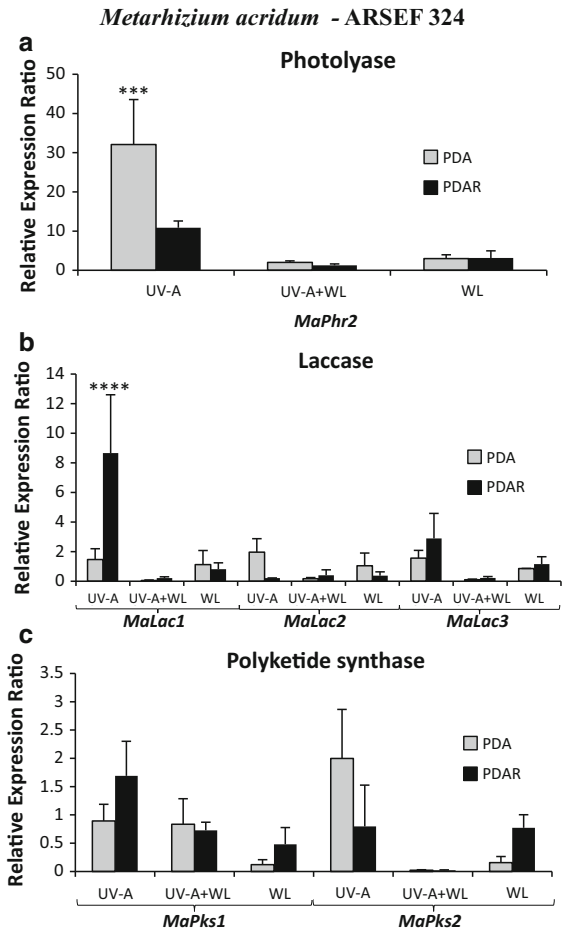

Fig. 4 Relative expression ratio of photolyase (**a**), laccase (**b**) and polyketide synthase (**c**) in *Metarhizium acridum* (ARSEF 324) grown on PDA medium with (PDAR) or without riboflavin (PDA) and exposed to: (1) 100.8 kJ m^{-2} of UV-A irradiance (UV-A), (2) UV-A (100.8 kJ m^{-2}) followed by exposure to 16 Klux of white light (UV-A + WL) for 2 h, or (3) white light only (WL) for 2 h. Error bars are SE of three independent trials. Asterisks indicate significant difference between the gene expression of conidia produced on PDA or PDAR: ***($P < 0.001$), ****($P < 0.0001$)

entomopathogenic fungi, including *Metarhizium* spp. (Braga et al. 2002, 2015; Chelico et al. 2005, 2006), and they are important to repair of DNA damaged by UV radiation. In the current study, transcripts of both photolyase genes (*MrPhr1* and *MrPhr2*) increased in conidia produced on PDAR when exposed to UV-A followed by exposure to white light in the laboratory, suggesting an increase in fungal photoreactivation that correlates well with the increase in tolerance to solar radiation. Increased photoreactivation following up-regulation of a photolyase gene was also demonstrated in *M. acridum* (Brancini et al. 2018). In fact, although UV radiation has been reported to cause DNA damage, it also contributes to the photoreactivation process (Brancini et al. 2018; Sancar 1990).

Transcription of laccases (*MrLac1* and *MaLac1*) in both fungal species as well as polyketide synthase genes (*MrPks1* and *MrPks2*) in *M. robertsii* were increased in conidia exposed to either UV-A alone or followed by white light. Both laccase and polyketide synthase enzymes contribute to conidial pigmentation (Chen et al. 2015; Fang et al. 2010; Schümann and Hertweck 2006). Thus, these genes may be involved in UV protection by blocking radiation that causes cellular damage. There was significant correlation between relative expression of these genes and germination rates of *M. robertsii* conidia produced on PDAR and then exposed to UV-B radiation (Pereira-Junior et al. 2018).

Reduced viability of *M. robertsii* conidia exposed to solar UV-A at 3 kJ m^{-2} was reported previously, whereas there was no reduction in viability of *M. acridum* (Braga et al. 2001c). In the present study, viability (germination) of *M. acridum* conidia produced on medium enriched with riboflavin was reduced to approximately 75% after exposure to solar UV-A for 120 min (Fig. 2), whereas the viability was reduced to ca. 55% when exposed to artificial UV-A for 105 min. The literature reports that *M. acridum* is more tolerant to solar UV-A radiation (Braga et al. 2001c) [and also to UV-B (Braga et al. 2001a; Pereira-Junior et al. 2018)] than *M. robertsii*. In these studies the fungal isolates were cultivated on PDAY, i.e., PDA supplemented with yeast extract (1 g l^{-1}), a component which contains B-complex vitamins, including riboflavin. In the current study, the tolerance to artificial UV-A of *M. acridum* conidia produced on PDAR medium was, at most, as high as that of *M. robertsii*. *M. acridum*, however, had higher relative mean germination than *M. robertsii* when exposed to solar UV-A radiation. These results indicate a distinctive dose–response pattern of fungal isolates to UV-A radiation, i.e., conidia that tolerate low doses might not be as tolerant to higher doses. The variation of response among fungal isolates to different UV wavelengths and irradiation doses suggest that several mechanisms might be involved in DNA damage reparation and UV photoprotection, and that different mechanisms are triggered in each isolate to mitigate UV-caused stress with different UV-A doses. In fact, it

is well established that, in mammalian cells, UV-B induces DNA damage mainly by a direct photoreaction (thymine dimerization), whereas UV-A also damage DNA indirectly via photosensitizing reactions (Cadet et al. 2015).

The results presented in the current study, as well as our results reported previously (Pereira-Junior et al. 2018), cannot fully explain the protective effects of riboflavin to *Metarhizium* spp. against UV radiation. Although we measured the expression pattern of some genes potentially involved in this tolerance, other genes are likely to be involved in these pathways as well. Moreover, cross-talk between enzymes may be possible, but currently is not known. Transcriptomic and proteomics analyses might be necessary to clarify the identity and role of other genes involved in UV photoprotection and repair, and thereby provide a better understanding of UV tolerance of fungi to different UV wavelengths and doses. Hopefully, the use of riboflavin in culture media, to some degree, may produce conidia which overcome the frequently noted negative effects of environmental UV radiation on entomopathogenic fungi when used as biological control agents in field applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Carla Huarte-Bonnet is currently involved in developing and characterizing fungal microsclerotia-like structures.

Ronaldo A. Pereira-Junior studies formulations to improve the tolerance of fungi to UV radiation.

Flávia R. A. Paixão is dedicated to the investigation of microsclerotia formation, its tolerance to stresses, and its virulence against *Aedes aegypti*.

Gilberto U. L. Braga is professor of genetics and his main research interest lies on fungal photobiology and antimicrobial photodynamic treatment.

Donald W. Roberts is insect pathologist who dedicated many decades to the biological control of arthropod pests.

Christian Luz research lies on invertebrate pathology, biodiversity and formulation of entomopathogenic fungi.

Nicolás Pedrini is devoted to the study of entomopathogenic fungi and insect-host interactions.

Éverton K. K. Fernandes is professor of parasitology, with main research interest on biological control of ticks and responses of fungi to stresses.