DR. EVERTON FERNANDES (Orcid ID : 0000-0001-7062-3295)

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Riboflavin induces *Metarhizium* spp. to produce conidia with elevated tolerance to UV-B, and upregulates photolyases, laccases and polyketide synthases genes

Abbreviated running headline:

Riboflavin increases UV-B tolerance

Ronaldo A. Pereira-Junior[1,2], Carla Huarte-Bonnet[1,2], Flávia R. S. Paixão[1,2], Donald W. Roberts[3], Christian Luz[1], Nicolás Pedrini[2], Éverton K. K. Fernandes[1*]

[1] Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, GO, Brazil.

[2] Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Universidad Nacional de La Plata - CONICET, La Plata, Buenos Aires, Argentina.

[3] Department of Biology, Utah State University, Logan, UT, USA.

* To whom correspondence should be addressed at Universidade Federal de Goiás, Instituto de Patologia Tropical e Saúde Pública, Avenida Esperança s/n, Campus Samambaia, Goiânia, GO, Brazil, 74690-900.

Phone: +55 (62) 3209-6118; e-mail: evertonkort@ufg.br

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Abstract

Aims: The effect of nutritional supplementation of two *Metarhizium* species with riboflavin (Rb) during production of conidia was (a) evaluated on conidial tolerance (based on germination) to UV-B radiation and (b) on conidial expression following UV-B irradiation, of enzymes known to be active in photoreactivation, viz., photolyase (*Phr*), laccase (*Lcc*) and polyketide synthase (*Pks*).

Methods and Results: *Metarhizium acridum* (ARSEF 324) and *Metarhizium robertsii* (ARSEF 2575) were grown either on (a) potato dextrose agar medium (PDA), (b) PDA supplemented with 1% yeast extract (PDAY), (c) PDA supplemented with Rb (PDA+Rb), or (d) PDAY supplemented with Rb (PDAY+Rb). Resulting conidia were exposed to 866.7 mW m^{-2} of UV-B Quaite-weighted irradiance to total doses of 3.9 kJ m^{-2} or 6.24 kJ m^{-2}. Some conidia also were exposed to 16 klux of white light after being irradiated, or not, with UV-B to investigate the role of possible photoreactivation. Relative germination of conidia produced on PDA+Rb (regardless Rb concentration) or on PDAY and exposed to UV-B was higher compared to conidia cultivated on PDA without Rb supplement, or to conidia suspended in Rb solution immediately prior to UV-B exposure. The expression of *MaLac3* and *MaPks2* for *M. acridum*, as well as *MrPhr2*, *MrLac1*, *MrLac2* and *MrLac3* for *M. robertsii* was higher when the isolates were cultivated on PDA+Rb and exposed to UV-B followed by exposure to white light, or exposed to white light only.

Conclusions: Rb in culture medium increase the UV-B tolerance of *M. robertsii* and *M. acridum* conidia, and which may be related to increased expression of photolyase, laccase and pks genes in these conidia.

Significance and Impact of the Study: The enhanced UV-B tolerance of *Metarhizium* spp. conidia produced on Rb-enriched media may improve the effectiveness of these fungi in biological control programs.

Keywords: Laccase, *Metarhizium acridum*, *Metarhizium robertsii*, photolyase, photoreactivation, polyketide synthase, riboflavin, tolerance to UV-B.

1. Introduction

Entomopathogenic fungi (EF) are environmentally friendly alternatives to exclusive use of chemical pesticides. Among these fungi, *Metarhizium* is the most investigated genus for microbial control of arthropod pests and vertebrate-disease vectors (Zimmermann 1993; Bahiense *et al.* 2007; Fernandes and Bittencourt 2008; Gindin *et al.* 2009; Sousa *et al.* 2013; Rodrigues *et al.* 2015; Gutierrez *et al.* 2016). The use of EF as biological control agents may be limited, however, due to the extended time the fungus requires to initiate infection and kill their arthropod hosts. Additionally, their success may be limited by their vulnerability to abiotic stresses, particularly high temperatures (Fernandes *et al.* 2010; Rangel *et al.* 2010; Barreto *et al.* 2016) and ultraviolet radiation (UV), UV-A and UV-B (Braga *et al.* 2001a,b,c,d; Rangel *et al.* 2004; 2005; Fernandes *et al.* 2007; Fang *et al.* 2010; Fernandes *et al.* 2015).

Negative effects of UV-B radiation on several EF have been reported, i.e., *M. anisopliae* s.l. Metchnikoff (Alves *et al.* 1998; Braga *et al.* 2001a,b,c; Falvo *et al.* 2016), *M. acridum* Driver & Milner (Moore *et al.* 1993; Braga *et al.* 2001a,b,c; Rangel *et al.* 2005), *Beauveria bassiana* Balsamo-Crivelli (Fernandes *et al.* 2007), *Lecanicillium lecanii* Zare & Gams (=*Verticillium lecanii*) and *Lecanicillium aphanocladii* Zare & Gams (=*Aphanocladium album*) (Braga *et al.* 2002). UV radiation may reduce conidial viability of

EF and cause delayed germination of surviving conidia (Rangel *et al.* 2008; Fernandes *et al.* 2015). Amongst these EF, *M. acridum* is well known for its notable tolerance to UV-B radiation (Morley-Davies *et al.* 1995; Fargues *et al.* 1996), suggesting that this species may be important in investigating mechanisms involved in fungal protection against UV-B radiation (Braga *et al.* 2001a,b,c).

UV radiation may cause mutagenesis and cell death from damage to cellular components, either by inducing the formation of cyclobutane pyrimidine dimers (CDPs) and pyrimidine photoproducts in DNA or by producing reactive-oxygen species (ROS) (Franklin and Haseltine 1986; Mitchell *et al.* 1990; Griffiths *et al.* 1998; Nascimento *et al.* 2010; Fang and St. Leger 2012). DNA is a major target of UV radiation and may cause severe DNA damage, but the CPDs can be restored to their monomeric form by photoreactivation by the action of two specific photoactive enzymes that repair the DNA damage: CPD photolyase and 6-4 photolyase, known collectively as DNA photolyases (Schleicher *et al.* 2007). Photoreactivation is a light-dependent DNA repair mechanism, which uses visible light to monomerizes UV-induced CPDs by activating photolyases (Yasui and Eker 1998; Friedberg *et al.* 2006; Fang and St. Leger 2012). In fact, photoreactivation has been reported in many EF, e.g., *Beauveria* spp., *Metarhizium anisopliae* s.l., *Isaria farinosa* Holmsk. (=*Paecilomyces farinosus*) and *L. lecanii*, which increased the ability of UV-irradiated conidia to germinate (Chelico *et al.* 2006). Photolyases are flavoproteins that contain two non-covalently linked chromophores. One chromophore, flavin-adenine dinucleotide (FADH), is completely reduced by a catalytic co-factor. This co-factor performs repair functions after excitation by either direct absorption of photons or energy-transfer resonance of the second chromophore, which is a pigment that collects sunlight and increases repair efficiency (Kao *et al.* 2005).

Cellular pigments also protect the cell by blocking UV radiation (Butler and Day 1998; Halaouli *et al.* 2006; Avalos and Limón 2014; Chen *et al.* 2015). The production of melanin, for example, is a defense mechanism of some organisms stressed by UV radiation, free radicals, gamma rays, dehydration, or extreme temperatures. Melanin contributes to resistance against cell-wall hydrolytic enzymes by preventing cell lysis. Melanin and other pigments are also involved in the formation and stability of spores (Mayer and Harel 1979), as well as in defense mechanisms and virulence (Soler-Rivas *et al.* 1997; Jacobson, 2000; Halaouli *et al.* 2006). Melanin biosynthesis has two well elucidated pathways in fungi, i.e, the dihydroxynaphthalene (DHN) and the L-3,4-dihydroxyphenylalanine (L-DOPA) routes, both involved in the biosynthesis of pigments and mycotoxins (Langfelder *et al.* 2003; Chiang *et al.* 2010; Eisenman and Casadevall 2012; Chen *et al.* 2015). The DHN melanin biosynthesis is initiated by the polyketide synthase (PKS) to form 1,8 DHN intermediate, which is polymerized into melanin by catalase phenoloxidase, peroxidase, laccase, and catalase enzymes (Butler and Day, 1998). Rangel *et al.* (2006a) and Chen *et al.* (2015) documented that melanin is not produced by *M. robertsii*, and that non-melanin pigments produced are not associated with stress resistance. In addition, Gonzales *et al.* (2010) reported that *M. anisopliae* s.l. does not produce carotenoids, but suggested that this condition may vary between species and isolates, and that other pigments also may be involved in UV protection.

Cellular pigmentation is also regulated by laccases (Fang *et al.* 2010). Not all laccase functions are known, but in addition to production of pigments, they are associated with the detoxification of xenobiotics and synthesis and degradation of lignin (Morozova *et al.* 2007). Laccases are enzymes that catalyze a one-electron oxidation con-comitantly with the four-electron reduction of molecular oxygen to water (Baldrian 2006), preventing the formation of hydrogen peroxide. In filamentous fungi such as *M. anisopliae* s.l., laccase class 1 (*MLac1*) is expressed during conidia and blastospores production or appressoria formation. The

polyphenol oxidase, like MLAC1, is involved in pigmentation, pathogenicity, virulence, and tolerance to abiotic stresses (Fang *et al.* 2010).

Many other mechanisms may be involved in tolerance of EF to UV radiation; for example, a simple change in composition of the substrate used during fungal growth may cause fluctuation of conidial tolerance (Rangel *et al.* 2006b; 2008; 2015). Riboflavin (Rb), ordinarily known as vitamin B2, is a potential enhancer of UV-radiation tolerance in fungal spores, as demonstrated for *Aspergillus nidulans* Winter and *Ashbya gossypii* Guilliermond (Stahmann *et al.* 2001). Rb is an essential metabolic precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are coenzymes involved in oxidative metabolism (Abbas and Sibirny 2011). Although it is known that the biochemical pathway of Rb produces FAD co-factor, no correlation between Rb and DNA repair of CDPs or pigment production has been documented. The current study evaluates the effect of Rb supplementation during culture on *Metarhizium* spp. conidial tolerance to UV-B radiation. Additionally, changes in levels of some enzymes possibly involved in both fungal photoprotection and DNA-damage repair subsequent to UV-B exposure were investigated.

2. Materials and methods

2.1 Fungal Cultures

The isolates *Metarhizium acridum* ARSEF 324 and *Metarhizium robertsii* Bischoff, Rehner & Humber ARSEF 2575 were obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Emerging Pests and Pathogens Research, Robert W. Holley Center for Agriculture and Health, at Cornell University, Ithaca, NY, 14853-2901, USA). These isolates were selected because their tolerances to UV-B radiation are well known (Hunt *et al.* 1994; Braga *et al.* 2001a,b,c; Miller *et al.* 2004; Rangel *et al.* 2004; 2005; Braga *et al.* 2006). ARSEF 324 was isolated from *Austracris guttulosa* Walker

(Orthoptera: Acrididae) in Queensland, Australia, and ARSEF 2575 was isolated from *Curculio caryae* Horn (Coleoptera: Curculionidae) in South Carolina, USA.

Fungi were grown either on a) potato dextrose agar medium (PDA) (Difco Laboratories, Sparks, USA), b) PDA supplemented with 1g L^{-1} yeast extract (Difco Laboratories) (PDAY), c) PDA supplemented with 0.00125%, 0.0025%, 0.005%, 0.01%, or 0.02% (w/v) Rb (Alamar Tecno-Científica Ltda., São Paulo, Brazil) (PDA+Rb), or d) PDAY supplemented with 0.01% Rb (w/v) (PDAY+Rb). All cultures were grown in polystyrene Petri dishes (90 × 15 mm, Cralplast®, Cral produtos para laboratórios LTDA., Cotia, Brazil) incubated at 27 ± 1 ºC and relative humidity (RH) > 80%, in the dark, for 15 days.

2.2 Evaluation of conidial tolerance to UV-B radiation

Conidial tolerance to UV-B was evaluated as described by Fernandes *et al.* (2007). Conidia were suspended in 0.01% polyoxyethylene sorbitan monooleate (Tween 80®, Sigma Chemical Co., Saint Louis, USA) solution (v/v). In addition, conidia of fungi grown on PDA were also suspended in aqueous 0.01% Tween 80® solution containing 0.01% Rb. The concentration of suspensions was adjusted to 1×10^6 conidia mL^{-1}, and 20 µL were inoculated on the center (without spreading) of a Petri plate (60 × 15 mm, Cralplast®) with PDAY medium plus 0.002% (w/v) benomyl (Hi-Yield Chemical Company, Bonham, USA) (Milner *et al.* 1991; Braga *et al.* 2001a) and 0.05% (w/v) chloramphenicol. Benomyl has very little effect on conidial germination; but is lethal to germ tubes immediately after germination. It was used in treatment and control groups to allow monitoring of germination for 48 h by preventing mycelial overgrowth (Milner *et al.* 1991). Chloramphenicol was used to prevent bacterial contamination.

The plates (with lids removed) were exposed to 866.7 mW m^{-2} of Quaite-weighted irradiance (Quaite *et al.* 1992a,b) for 2 h in a chamber containing four ultraviolet lamps (UVB-313 EL/40W, Q-Lab Corporation, Westlake, USA), which corresponded to a dose of 6.24 kJ m^{-2} for both fungal isolates tested. During irradiation, plates were covered with a 0.13 mm thick cellulose diacetate film (JCS Industries, Le Mirada, USA), which blocks UV-C radiation (below 280 nm) and the UV-B short-wavelength (280–290 nm), but permits the passage of most UV-B (290–320 nm) and the minimal UV-A (320–400 nm) radiation emitted by the lamps (Fig. 1). Control plates were covered with aluminium foil to block all UV radiation. The temperature inside the irradiation chamber was monitored with a data logger HOBO® (Onset Computer Corporation, Bourne, USA) during the tests, and in all experiments temperature was maintained at 27 ± 1 °C. The spectral irradiance was measured with a USB 2000 + Rad Spectroradiometer (Ocean Optics, Dunedin, USA). After irradiation, the plates were incubated for 48 h at 27 ± 1 ºC and RH > 80% in the dark. The spots where conidia were inoculated were stained with Amann lactophenol and cotton blue solution to facilitate evaluation of conidial germination. A minimum of 300 conidia were evaluated per plate, and relative percent germination of treated conidia in relation to the controls was calculated as described by Braga *et al.* (2001b). All tests were performed three independent times.

2.3 Evaluation of conidial tolerance to UV-B radiation followed by white light exposure

Conidia produced on PDA or PDA supplemented with 0.01% Rb were suspended in 0.01% Tween 80® and inoculated on PDAY + benomyl 0.002% (w/v) plates as mentioned in section 2.2 and exposed to UV-B for 1:15 h (3.9 kJ m^{-2}) or 2 h (6.24 kJ m^{-2}). A second batch of plates, after being irradiated with UV-B, was exposed to 16 klux of white light in a photostability chamber (Caron, Marietta, USA) for 2 h to allow photoreactivation. A third group of plates was exposed to only white light for 2 h. Temperature and humidity in the

photostability chamber was monitored and held at 27 ± 1 °C and RH > 80%. Control plates were covered with aluminium foil to block all UV radiation and white light. Plates were incubated for 48 h in the dark, stained, and the relative percent germination calculated as described above. All tests were performed three independent times with a new culture batch for each repetition.

2.4 Gene expression of *Metarhizium* spp. conidia irradiated with UV-B and/or white light

Conidia were produced on PDA medium or PDA supplemented with 0.01% Rb. The medium was completely covered with a disc of 90 $g.m^{-2}$ sterile tracing paper (Edispel Distribuidora, Goiânia, Brazil) during cultures with the inoculation conidia being placed on the top of the paper. The tracing paper sheet was used to avoid contamination of the samples with culture medium during harvesting of conidia. The plates were incubated for 15 days at 27 ± 1 °C in the dark, after which aerial mycelium was mostly removed from the cultures with a spatula, which allowed UV-B and white light exposures as well as RNA extractions to be primarily from conidia. Then, the plates (with lids removed) were immediately exposed to either (a) UV-B at 3.9 kJ m^{-2}, (b) white light (WL) at 16 klux, or (c) UV-B followed by white light at the same doses.

After irradiation, conidia were harvested from cultures with a spatula and immediately macerated with mortar and pestle in liquid nitrogen, and total RNA was extracted using the RNeasy Plant Mini Kit® (Qiagen, Hilden, Germany) plus 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 on a 1% (w/v) agarose gel. Synthesis of cDNA was performed with the GoScript Reverse Transcription System®

(Promega Corporation, Madison, USA), using 360 ng of the total extracted RNA. The real-time polymerase chain reaction (qPCR) was carried out with iQ SYBR Green Supermix (BioRad, Hercules, USA). The amplification was performed in an Mx3000P qPCR system (Stratagene, La Jolla, USA), and the primers used are listed in Table 1. Fungal sequences used for primer design were obtained from Genbank. The following qPCR thermal program was used: denaturation at 95 ºC for 10 min, followed by 40 cycles with three-segment amplification (30 s at 95 ºC for denaturation, 1 min at 55 ºC for annealing, and 30 s at 72 ºC for DNA chain elongation).

Each sample was analyzed in duplicate, and three independent repetitions were conducted. The relative expression ratio (RER) was calculated using the Ct values of each target gene and the two reference genes (gamma actin and glyceraldehyde 3-phosphate dehydrogenase) in control (not irradiated) and treated (UV-B; UV-B + WL; WL) samples, according to Nordgard *et al.* (2006), as follows:

$$RER = \frac{{E_T}^{\Delta C_t T(control-treatment)}}{\sqrt{{E_{R1}}^{\Delta C_t R_1(control-treatment)} \times {E_{R2}}^{\Delta C_t R_2(control-treatment)}}} \quad (1)$$

where E is the estimated PCR efficiency (100%), C_t is the cycle threshold, T is the target gene, $R1$ is *gamma actin*, $R2$ is *glyceraldehyde 3-phosphate dehydrogenase*, and ΔC_t is the difference between the control (not exposed) and the treatment (exposed to UV-B, UV-B + WL, or WL).

2.5. Statistical analysis

Differences among the mean relative percent germination of conidia, and among the relative expression ratios of genes were determined by analysis of variance (ANOVA)

followed by the Student-Newman-Keuls (SNK) test. P values < 0.05 were considered significant. ANOVA followed by SNK were fit using Instat 3.05 (GraphPad Software Inc., San Diego, USA).

3. Results

3.1. Conidial tolerance to UV-B radiation

Relative germination of conidia of both fungal isolates, ARSEF 2575 and ARSEF 324, produced on PDAY or PDA supplemented with 0.01% Rb, and exposed to UV-B radiation (6.24 kJ m^{-2}), was considerably higher (for ARSEF 324: $F_{4,10} = 103.87$; $P < 0.001$; for ARSEF 2575: $F_{4,10} = 29.1$; $P < 0.001$) in comparison to the relative germination of conidia of the same isolates produced on PDA medium without supplementation (Fig. 2). Relative germination of conidia produced on PDA and suspended in Rb solution (PDA + Rb Sol) immediately prior to irradiation was similar to the relative germination of irradiated conidia produced on PDA and suspended in 0.01% Tween 80® solution without Rb (see Fig. 2). Additionally, conidia of ARSEF 324 ($F_{5,12} = 47.8$; $P < 0.001$) and ARSEF 2575 ($F_{5,12} = 5.7$; $P = 0.006$) were approximately 25% more tolerant to UV-B radiation when produced on PDA medium supplemented with Rb, regardless its concentration (0.00125% – 0.02%) in comparison to conidia produced on PDA without Rb (data not shown).

3.2. Conidial tolerance to UV-B radiation followed by white light exposure (photoreactivation)

Conidia of both isolates (*M. acridum* ARSEF 324 and *M. robertsii* ARSEF 2575) produced on PDA medium supplemented with Rb were more tolerant to UV-B at 3.9 kJ m^{-2} or 6.24 kJ m^{-2} than conidia produced on pure PDA medium without Rb, whether followed or not by exposure to 16 klux white light (Fig. 3). In fact, *M. acridum* conidia produced on PDA

supplemented or not with Rb and then exposed to UV-B at 6.24 kJ m^{-2} followed by exposure to white light were more tolerant to UV-B than conidia that were not exposed to white light ($F_{5,12}$ = 100.1; P < 0.0001). Conidia of *M. robertsii* had improved tolerance to UV-B radiation in response to white light exposure only when they were produced on medium containing Rb and exposed to 6.24 kJ m^{-2} ($F_{5,12}$ = 598.8; P < 0.0001). No significant effect on relative germination of *M. acridum* or *M. robertsii* was detected when conidia were exposed to white light only (Fig. 3).

3.3. Gene expression of *Metarhizium* spp. irradiated conidia

The expression of *MaLac3* gene of *M. acridum* conidia produced on PDA supplemented with Rb was higher than the expression of this gene of *M. acridum* cultured on PDA when exposed to a) UV-B radiation followed by exposure to white light (UV-B + WL), or b) exposed to white light only (WL) ($F_{5,12}$ = 11.2; P = 0.0002). The expression of *MaPks2* gene also increased when Rb was added to the culture medium and then the produced conidia exposed to UV-B + WL ($F_{5,12}$ = 4.1 and P = 0.02). The expression of *MaPhr, MaLac1 and MaPks1* genes of conidia produced on PDA medium supplemented with Rb did not differ from conidia produced on PDA without Rb in any treatment groups (P > 0.05) (Fig. 4).

The expression of *MrPhr2*, *MrLac2* and *MrLac3* genes of *M. robertsii* increased when the fungus was grown on PDA medium with Rb in comparison to conidia produced on PDA without Rb, but only when their conidia were exposed to UV-B + WL or WL alone (Fig. 5). Conidia exposed to UV-B + WL showed increased expression of *MrLac1* gene only when it was grown on PDA medium supplemented with Rb. The expression of *MrPhr1, MrPks1* and *MrPks2* genes did not differ among the treatment groups (P > 0.05).

Conidia of both isolates produced on PDA supplemented with Rb did not express the genes tested in significantly greater amounts than conidia produced on pure PDA medium in any irradiation treatment.

4. Discussion

In this study, conidia of *M. robertsii* (ARSEF 2575) and *M. acridum* (ARSEF 324) when produced on Rb-supplemented PDA culture medium exhibited enhanced tolerance to UV-B radiation. PDAY medium also increased the tolerance of conidia, likely because yeast extract contains B-complex vitamins, including Rb. In an earlier study (Rangel *et al.* 2004), on the other hand, there were no differences in conidial tolerance to UV-B radiation when *M. robertsii* (ARSEF 23 and ARSEF 2575) conidia were produced on PDA or PDAY. In the current study, *M. acridum* ARSEF 324 was less tolerant to UV-B than in our previous studies. Some of our earlier studies utilized colony-forming units (CFU) to assess viability rather than percent conidial relative germination; which makes comparisons difficult (Braga *et al.* 2001a,c; Rangel *et al.* 2005; 2006). The viability results reported in the current study were acquired in four experiments (two in the USA, two in Brazil) with three to four repetitions each.

The conidial tolerance of *M. acridum* and *M. robertsii* to UV-B did not change when conidia were suspended in 0.01% Rb solution (rather than produced on PDA+Rb), suggesting that these fungi need to metabolize and accumulate the Rb to acquire photoprotection. Conversely, asco- and conidiospores of *Ashybia gossypii* and *Aspergillus nidulans*, respectively, were more tolerant to UV radiation (254 nm) when suspended in saline solution with 0.8 mM Rb compared to conidia suspended in aqueous solution (Stahmann *et al.* 2001). Therefore, reducing susceptibility of *Metarhizium* spp. conidia to solar UV-B radiation in the field, will require supplementing the media used for conidial production with Rb. According

to Jackson *et al.* (2010), the production of EF can be nutritionally manipulated to increase the efficacy of fungal propagules in biological control programs. Additionally, efficacy of EF may be improved by formulating fungal propagules with appropriate adjuvants (Kim *et al.* 2013; Barreto *et al.* 2016).

Riboflavin supplementation, regardless the concentrations tested, increased conidial tolerance to UV-B for both of the *Metarhizium* isolates we investigated. The low concentration of Rb required to enrich the culture medium, allied to the low cost of this vitamin, make it a promising compound for enhancing the stress tolerance of commercially produced *Metarhizium* spp. conidia. Since solar UV-B radiation represents a serious challenge to fungal biological control agents, enhancing the persistence of fungal propagules in the field by mitigating the effects of such irradiation is crucially important.

Photoreactivation is a process that repairs DNA damage from UV radiation using enzymes that require activation by visible light (Friedberg *et al.* 2006). Similar to the results of Chelico *et al.* (2006) with *M. anisopliae* s.l. exposed to UV-C, in the current study *M. robertsii* conidia irradiated with UV-B demonstrated photoreactivation, viz., exposure to white light after UV-B resulted in high relative germination; this response was associated with high expression levels of photolyase genes stimulated by exposure to the white light (see Fig. 5A). Fang and St. Leger (2012), also demonstrated that CDP photolyases are important mechanisms to repair DNA damage in *M. robertsii* and *B. bassiana* exposed to UV radiation. In fact, visible light, especially the blue spectrum, enhances the expression and activation of photolyases in mycelium and conidiophores of *Trichoderma harzianum* Rifai (Berrocal-Tito *et al.* 2000). Activated photolyases eliminate cyclobutane pyrimidine dimers (CPDs) in damaged DNA, allowing irradiated cells to recover. Also, flavin-adenine dinucleotide (FADH) is a chromophore of photolyases that has a repair function upon excitation by either direct photon absorption or resonance energy transfer from a second chromophore (Kao *et al.*

2005). Therefore, chromophores derived from Rb in *Metarhizium* spp. possibly improve the efficiency of DNA-damage repair in irradiated cells.

Conidia of *M. acridum* ARSEF 324 irradiated with UV-B followed by exposure to white light had higher relative germination, even when conidia were produced in culture medium without Rb supplementation (see Fig. 3); however, no over-expression of photolyases was detected, even by conidia produced on PDA supplemented with Rb were exposed to UV-B or white light, or the combination of UV-B and white light (see Fig. 4A). Accordingly, photolyase augmentation apparently is not the major mechanism for protecting *M. acridum* ARSEF 324 conidia from UV-B radiation, or if it is important, it is not influenced by Rb supplementation. The production of CPDs is variable in fungi and is particularly low in *M. acridum* ARSEF 324 and *A. nidulans* in comparison to *Aspergillus fumigatus* Fresenius (Nascimento *et al.* 2010). The low expression rates of photolyases in irradiated *M. acridum* might be the result of low CPD production in this isolate, but this was not measured in our study.

The expression of laccase and PKS genes, however, was higher when conidia were exposed to UV-B radiation followed by exposure to white light. Among other metabolic pathways, these enzymes are involved in cell pigmentation, which may confer photoprotection to fungi by blocking UV-B radiation. *M. acridum* ARSEF 324 is highly pigmented due to dark-green conidia. The origin of this isolate, Queensland, Australia, suggests that it might need to adapt to high doses of UV radiation by developing mechanisms for photoprotection, such as cellular pigmentation. In fact, pigmentation in EF is linked to significant protection against UV radiation; for example, wild-type dark-green-pigmented *M. robertsii* (ARSEF 23) conidia were more tolerant to simulated solar UV radiation than three groups of mutants with yellow, purple, or white conidia (Braga *et al.* 2006). The current study suggests that cell pigmentation by high expression of *MaLac* or *MaPks* genes in

ARSEF 324 might protect their conidia against UV radiation by expressing genes potentially involved in pigmentation.

With *M. robertsii* ARSEF 2575, the over-expression of the three *MrLac* genes suggests a relationship to increased pigmentation and the improved UV-B tolerance of conidia. These results agree with Fang *et al.* (2010), where laccase MLAC1 contributed to conidial tolerance of ARSEF 2575 to UV-B radiation and heat, and is also involved in conidial pigmentation and pathogenicity. Therefore, both DNA repair and cell pigmentation are potentially responsible for photoprotection in *M. robertsii* ARSEF 2575.

In general, the genes studied here were upregulated when conidia of both *Metarhizium* species were exposed to white light combined, or not, with UV-B radiation exposure. However, different patterns of relative expression were observed between the two isolates, indicating that certain genes are more relevant for one isolate than the other. The great genotypic and phenotypic intraspecific variability in EF (Braga *et al.* 2001a,c; Fernandes *et al.* 2007), including their differing mechanisms of photoprotection and DNA repair, can be pointed to as possible causes for differences in tolerance against UV-B radiation among isolates. Variation in natural tolerance of *M. anisopliae* s.l. and *B. bassiana* s.l. isolates to UV-B radiation has been associated with their latitude of origin and with the microenvironment of their original sites (Bidochka *et al.* 2001; Braga *et al.* 2001c; Bidochka *et al.* 2002; Fernandes *et al.* 2007).

Conidia are known to be resistant, dormant fungal structures that remain dormant until external environmental conditions are favorable for germination (Hagiwara *et al.* 2016). However, in this study, conidia of both *M. robertsii* and *M. acridum* revealed expression of photolyase, laccase, or polyketide synthase genes. In fact, enzymatic metabolic activity in dormant conidia has been demonstrated by revealing an induced activity of α-amylase, invertase, and glucose dehydrogenase in *Aspergillus oryzae* Ahlburg (Sinohara 1970). Also,

induction of dormant conidia of *Fusarium solani* f. sp. *pisi* Snyder & Hansen by contact with plant cuticles triggers expression of the cutinase gene (Woloshuk and Kolattukudy 1986), and conidia suspended in water demonstrated an important respiratory metabolism normally initiated during fermentation (Novodvorska *et al.* 2016). Dormant conidia of *M. robertsii* and *M. acridum*, therefore, may respond to environmental stimuli by activating mechanisms for cell maintenance and cell damage repair.

Although Rb is a precursor of FMN and FAD, known oxidation-reduction cofactors in many organisms (Mironov *et al.* 1994), the role of this compound in mechanisms that enhance conidial tolerance against UV-B currently is not fully understood. The expression of genes involved in at least two important pathways for photoprotection was influenced by addition of Rb to culture media; however, it is not clear how Rb participates in both molecular and physiological responses. Further biochemical and molecular studies will be needed to fully determine the role of Rb in UV-tolerance pathways of EF. In addition, future studies should consider the consequences of Rb supplementation on conidial yield, pathogenesis and virulence against arthropod hosts, etc. In fact, Shah *et al.* (2005) reported that conidia of *M. anisopliae* s.l. produced on culture medium supplemented with 1% yeast extract were more virulent to *Tenebrio molitor* Linnaeus than conidia produced on other artificial media. We indicate here that the addition of Rb as a supplementation compound to commonly used substrates in mass production of *Metarhizium* spp. could be a useful approach for enhancing conidial UV-B tolerance, improving the efficacy of current fungal biological control programs.

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Conflict of interest

The authors have declared no conflict of interests.

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8. Captions for figures

Fig. 1. Irradiance spectrum of the cellulose-diacetate-filtered lamps of the UV-B irradiation chamber. Based on the Quaite-weighted irradiance (Quaite *et al.,* 1992a,b), the lamps provided 866.7 mW m^{-2} of UV-B radiation at the exposure-shelf level in the chamber.

Fig. 2. Relative germination of *Metarhizium acridum* (ARSEF 324, gray bars) and *M. robertsii* (ARSEF 2575, black bars) conidia after exposure to UV-B irradiance of 866.7 mW m^{-2} for 2 h (total dosage = 6.24 kJ m^{-2}). Conidia were produced on 1) PDA medium without supplement (PDA); 2) PDA supplemented with 0.01% riboflavin (PDA + Rb); 3) PDA supplemented with 1% yeast extract (PDAY); or 4) PDA supplemented with 1% yeast extract and 0.01% Rb (PDAY + Rb). Conidia were suspended in Tween 80 0.01% solution and applied onto the surface PDAY medium immediately before exposure to UV-B radiation. Conidia were also produced on PDA and suspended in 0.01% Rb solution, applied to the surface of PDAY medium, and immediately irradiated (PDA + Rib Sol). Relative germination was calculated in relation to non-irradiated controls. Bars with the same letter within the same isolate do not differ significantly (ARSEF 324: $F_{4,10} = 103.9$, $P < 0.0001$; ARSEF 2575: $F_{4,10} = 29.1$, $P < 0.0001$). Error bars are standard errors of three independent trials.

Fig. 3. Relative germination of *Metarhizium acridum* (ARSEF 324) and *M. robertsii* (ARSEF 2575) conidia exposed to 1) UV-B irradiance of 866.7 mW m^{-2} for 1.5 h (total dosage = 3.9 kJ m^{-2}) or 2 h (total dosage = 6.24 kJ m^{-2}) (UV-B), or 2) to UV-B (6.24 kJ m^{-2}) followed by exposure to white light (16 klux) (UV-B + WL), or 3) to white light only (WL). Conidia were produced on PDA medium with (black bars) or without (gray bars) 0.01% riboflavin. Relative germination was calculated in relation to non-irradiated controls. Bars with the same capital letter within the same culture medium (PDA, or PDA + Rb) and different treatments (UV-B,

or UV-B + WL) do not differ significantly. Bars with the same small letters within the same treatment (UV-B, or UV-B + WL, or WL) and different culture medium (PDA or PDA + Rb) do not differ significantly. ARSEF 324 exposed to 3.9 kJ m^{-2} ($F_{5,12}$ = 28.5 and $P < 0.0001$) or 6.24 kJ m^{-2} ($F_{5,12}$ = 100.0 and $P < 0.0001$); ARSEF 2575 exposed to 3.9 kJ m^{-2} ($F_{5,12}$ = 11.6 and $P = 0.003$) or 6.24 kJ m^{-2} ($F_{5,12}$ = 598.8 and $P < 0.0001$). Error bars are standard errors of three independent trials.

Fig. 4. Relative expression ratio of photolyase (A), laccase (B) and polyketide synthase (C) in *Metarhizium acridum* (ARSEF 324) conidia produced on PDA medium with (black bars) or without (gray bars) riboflavin and exposed to: 1) UV-B irradiance of 866.7 mW m^{-2} for 1:30 h (total dosage = 3.9 kJ m^{-2}) (UV-B), or 2) to UV-B (3.9 kJ m^{-2}) followed by exposure to white light (16 klux) (UV-B + WL), or 3) to white light only (WL). Bars with the same capital letter within the same gene and culture medium do not differ significantly: A = photolyase ($P > 0.05$); B = laccase (laccase 1: $P > 0.05$; laccase 3.1: $F_{5,12}$ = 11.2 and P = 0.0002); C = PKS (PKS 1: $P > 0.05$; PKS 2: $F_{5,12}$ = 4.1 and $P = 0.02$). Bars with the same small letter within the gene expression of ARSEF 324 grown on PDA with or without Rb (PDA + Rb or PDA) and exposed to UV-B, UV-B + WL, or WL, do not differ significantly. Error bars are standard error of three independent trials. Gamma actin and glyceraldehyde 3-phosphate dehydrogenase were the reference genes.

Fig. 5. Relative expression ratio of photolyase (A), laccase (B) and polyketide synthase (C) in *Metarhizium robertsii* (ARSEF 2575) conidia produced on PDA medium with (black bars) or without (gray bars) riboflavin and exposed to 1) UV-B irradiance of 866.7 mW m^{-2} for 1:30 h (total dosage = 3.9 kJ m^{-2}) (UV-B), or 2) to UV-B (3.9 kJ m^{-2}) followed by exposure to white

light (16 klux) (UV-B + WL), or 3) to white light only (WL). Bars with the same capital letter within the same gene and culture medium do not differ significantly: A = photolyase (CDP photolyase: $P > 0.05$; 6-4 photolyase: $F_{5,12} = 11.72$ and $P = 0.0016$); B = laccase (laccase 1: $F_{5,12} = 7.53$ and $P = 0.027$; laccase 2: $F_{5,12} = 7.18$ and $P = 0.0013$; laccase 3: $F_{5,12} = 31.65$ and $P < 0.0001$); C = PKS (PKS 1 and PKS 2: $P > 0.05$). Bars with the same small letter within the gene expression of ARSEF 2575 grown on PDA with or without Rb (PDA + Rb or PDA) and exposed to UV-B, UV-B + WL, or WL, do not differ significantly. Error bars are standard error of three independent trials. Gamma actin and glyceraldehyde 3-phosphate dehydrogenase were the reference genes.

Table 1 *Metarhizium robertsii* and *M. acridum* real-time quantitative primer sequences.

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 (*MaPhr*)	TTCATGCCGATTTGGAATTGC	TGCTTGTTTGATGGTGCCTCTG	XM_007813640.1
Laccase 1 (*MaLac1*)	AATGCGCGTACCCAACTCTG	CCACGGCGACATGTGATCTG	XM_007813533.1
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

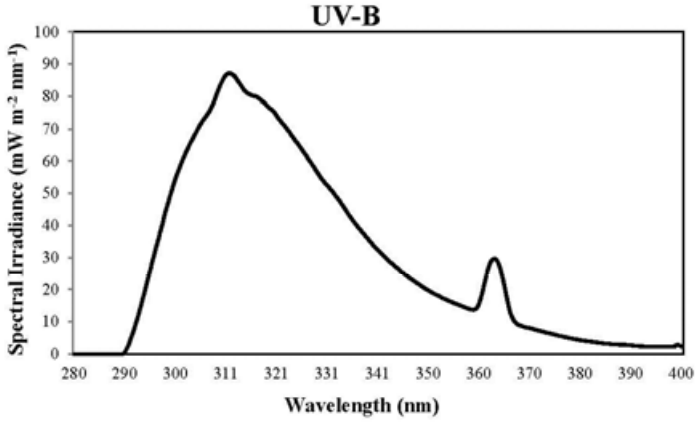

Fig. 1.

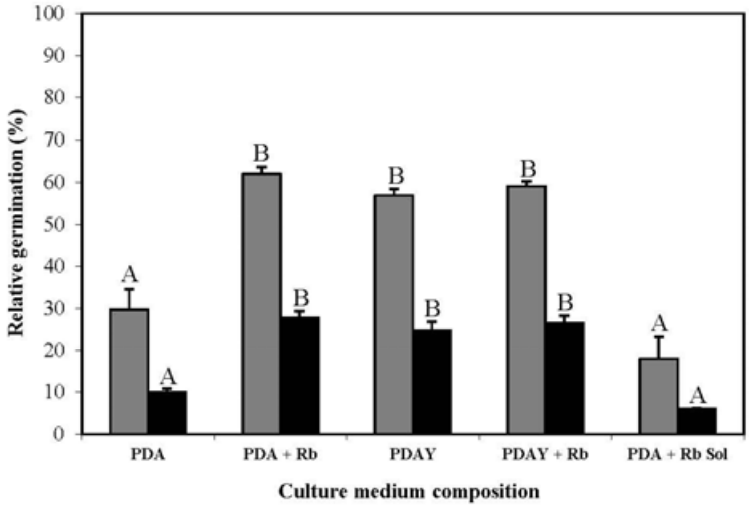

Fig. 2.

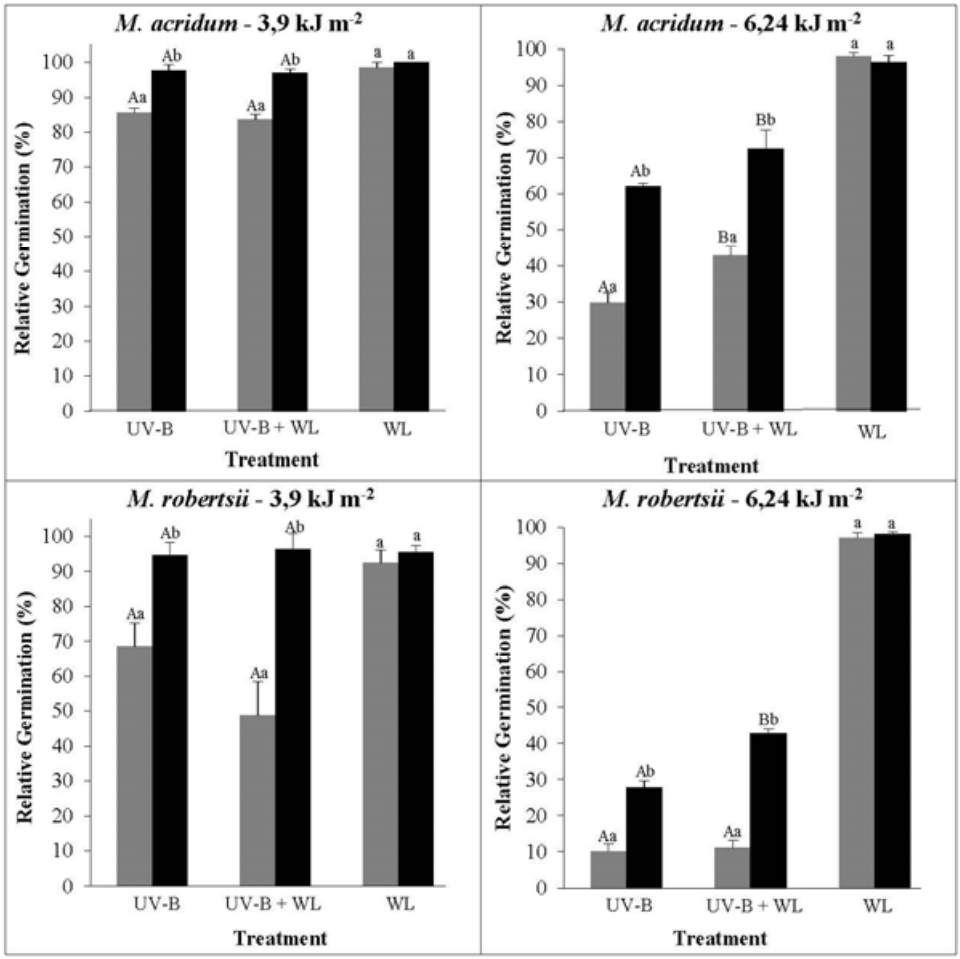

Fig. 3.

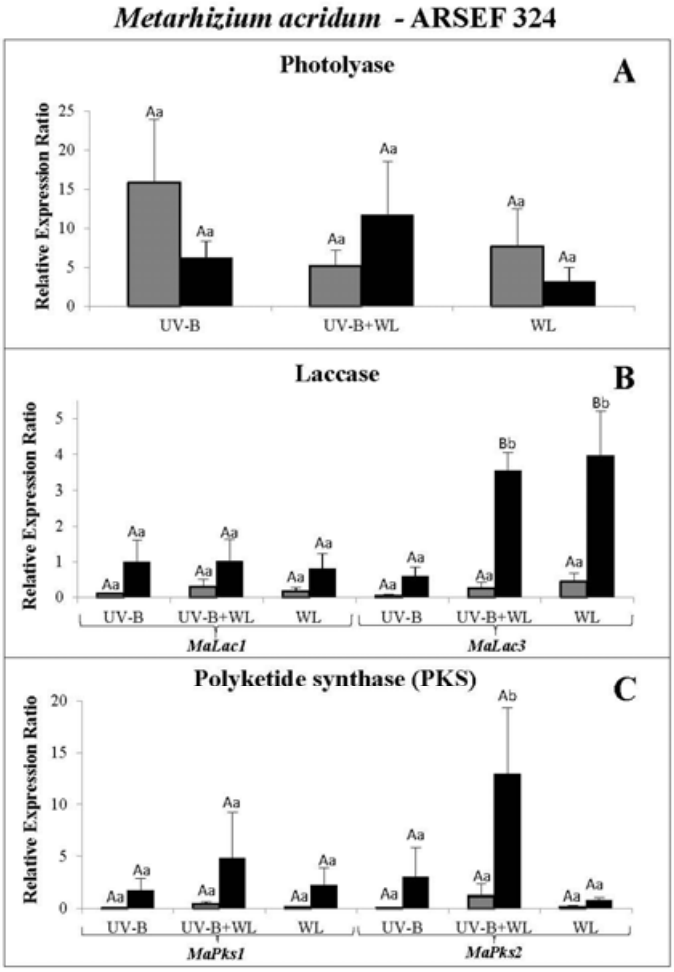

Fig. 4.

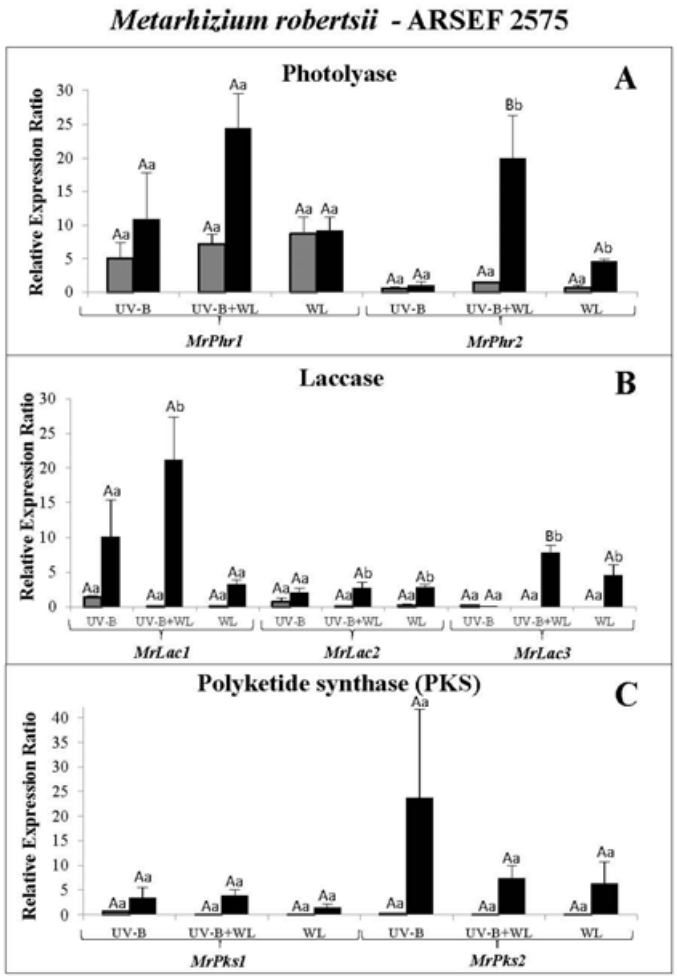

Fig. 5.