


## ORIGINAL ARTICLE

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

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## Keywords

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

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## Abstract

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

**Methods and Results:** *Metarhizium acridum* (ARSEF 324) and *Metarhizium robertsii* (ARSEF 2575) were grown either on (i) potato dextrose agar medium (PDA), (ii) PDA supplemented with 1% yeast extract (PDAY), (iii) PDA supplemented with Rb (PDA+Rb), or (iv) PDAY supplemented with Rb (PDAY+Rb). Resulting conidia were exposed to 866.7 mW m<sup>-2</sup> of UV-B Quante-weighted irradiance to total doses of 3.9 or 6.24 kJ m<sup>-2</sup>. Some conidia also were exposed to 16 klux of white light (WL) 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 WL, or exposed to WL only.

**Conclusions:** Rb in culture medium increases the UV-B tolerance of *M. robertsii* and *M. acridum* conidia, and which may be related to increased expression of *Phr*, *Lac* 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.

## 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, 2001b, 2001c, 2001d; 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. *Metarhizium anisopliae* s.l. (Metch.) Sorok. (Alves *et al.* 1998; Braga *et al.* 2001a, 2001b, 2001c; Falvo *et al.* 2016), *Metarhizium acridum* Driver & Milner (Moore *et al.* 1993; Braga *et al.* 2001a, 2001b, 2001c; Rangel *et al.* 2005), *Beauveria bassiana* (Bals.) Vuill. (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, 2001b, 2001c).

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 (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 monomerize 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., *M. 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 noncovalently linked chromophores. One chromophore, flavin-adenine dinucleotide (FADH), is completely reduced by a catalytic cofactor. This cofactor 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; Halaoui *et al.* 2006; Avalos and Limón 2014; Chen *et al.* 2015). The production of melanin, for example, is a defence 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 defence mechanisms and virulence (Soler-Rivas *et al.* 1997; Jacobson 2000; Halaoui *et al.* 2006). Melanin biosynthesis has two well-elucidated pathways in fungi, i.e. the dihydroxynaphthalene (DHN) and the L-3,4-dihydroxyphenylalanine 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 *Metarhizium robertsii* Bischoff, Rehner & Humber, and that nonmelanin 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 catalyse a one-electron oxidation concomitantly 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 blastospore 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 B<sub>2</sub>, 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 cofactor, 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 gene expression of some enzymes possibly involved in both fungal photoprotection and DNA-damage repair subsequent to UV-B exposure were investigated.

## Materials and methods

### Fungal cultures

The isolates *M. acridum* ARSEF 324 and *M. robertsii* 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). 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 (i) potato dextrose agar medium (PDA) (Difco Laboratories, Sparks, MD), (ii) PDA supplemented with 1 g l<sup>-1</sup> yeast extract (Difco Laboratories) (PDAY), (iii) PDA supplemented with 0.00125, 0.0025, 0.005, 0.01 or 0.02% (w/v) Rb (Alamar Tecnocientífica Ltda., São Paulo, Brazil) (PDA+Rb) or (iv) PDAY supplemented with 0.01% Rb (w/v) (PDAY+Rb). All cultures were grown in polystyrene Petri dishes (90 × 15 mm, Cralplast<sup>®</sup>; Cral Produtos para Laboratórios Ltda., Cotia, Brazil) incubated at 27 ± 1°C and relative humidity (RH) > 80%, in the dark, for 15 days.

### Evaluation of conidial tolerance to UV-B radiation

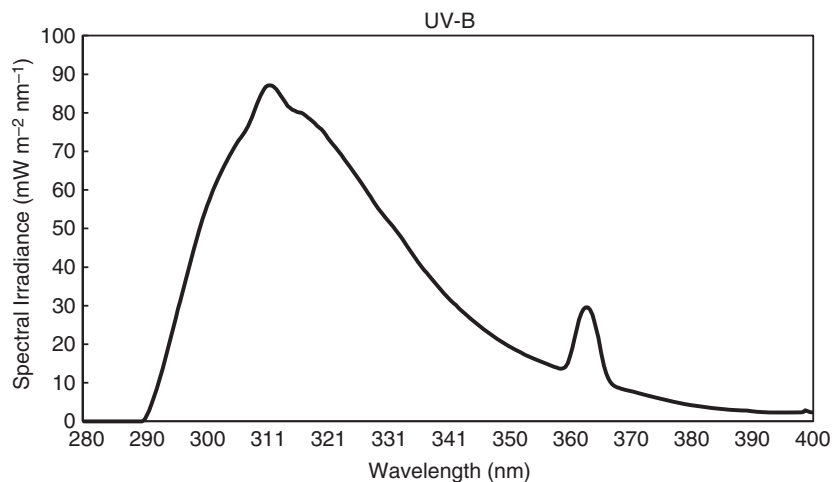
Conidial tolerance to UV-B was evaluated as described by Fernandes *et al.* (2007). Conidia were suspended in 0.01% polyoxyethylene sorbitan monooleate (Tween 80<sup>®</sup>; Sigma Chemical Co., St Louis, MO) solution (v/v). In addition, conidia of fungi grown on PDA were also suspended in aqueous 0.01% Tween 80 solution containing 0.01% Rb. The concentration of suspensions was adjusted to 1 × 10<sup>6</sup> conidia per ml, and 20 µl were inoculated on the centre

(without spreading) of a Petri plate (60 × 15 mm, Cralplast) with PDAY medium plus 0.002% (w/v) benomyl (Hi-Yield Chemical Company, Bonham, TX) (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<sup>-2</sup> of Quait-weighted irradiance (Quait *et al.* 1992a,b) for 2 h in a chamber containing four ultraviolet lamps (UVB-313 EL/40W; Q-Lab Corporation, Westlake, OH), which corresponded to a dose of 6.24 kJ m<sup>-2</sup> for both fungal isolates tested. During irradiation, plates were covered with a 0.13 mm thick cellulose diacetate film (JCS Industries, Le Mirada, CA), 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<sup>®</sup> (Onset Computer Corporation, Bourne, MA) 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, FL). After irradiation, the plates were incubated for 48 h at 27 ± 1°C 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 per cent 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.

### Evaluation of conidial tolerance to UV-B radiation followed by white light exposure (photoreactivation)

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 Evaluation of conidial tolerance to UV-B radiation and exposed to UV-B for 1 : 15 h (3.9 kJ m<sup>-2</sup>) or 2 h (6.24 kJ m<sup>-2</sup>). A second batch of plates, after being irradiated with UV-B, was exposed to 16 klux of white light (WL: 400–700 nm; see Fig. S1) in a photostability chamber (Caron, Marietta, GA) for 2 h to allow photoreactivation. A third group of plates was exposed to only WL for 2 h. Temperature and humidity in the



**Figure 1** Irradiance spectrum of the cellulose diacetate-filtered lamps of the UV-B irradiation chamber. Based on the Quate-weighted irradiance (Quate *et al.* 1992a,b), the lamps provided 866.7 mW m<sup>-2</sup> of UV-B radiation at the exposure-shelf level in the chamber.

photostability chamber were monitored and held at  $27 \pm 1^\circ\text{C}$  and  $\text{RH} > 80\%$ . Control plates were covered with aluminium foil to block all UV radiation and WL. Plates were incubated for 48 h in the dark, stained, and the relative per cent germination calculated as described above. All tests were performed three independent times with a new culture batch for each repetition.

#### 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  $90 \text{ g m}^{-2}$  sterile tracing paper (Edispel Distribuidora, Goiânia, Brazil) during culture 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  $27 \pm 1^\circ\text{C}$  in the dark, after which aerial mycelium was mostly removed from the cultures with a spatula, which allowed UV-B and WL exposures as well as RNA extractions to be primarily from conidia. Then, the plates (with lids removed) were immediately exposed to either (i) UV-B at  $3.9 \text{ kJ m}^{-2}$ , (ii) WL at 16 klux or (iii) UV-B followed by WL 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<sup>®</sup> (Qiagen, Hilden, Germany) plus an on-column DNA digestion step (DNase I<sup>®</sup>; Qiagen) following the protocol recommended by the manufacturer. Total RNA was quantified using a Nanodrop<sup>®</sup> spectrophotometer (Thermo Scientific, Wilmington, DE), and its integrity analysed on a 1% (w/v) agarose gel. Synthesis of cDNA was performed with the GoScript Reverse Transcription System<sup>®</sup>

(Promega Corporation, Madison, WI), 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, CA). The amplification was performed in an Mx3000P qPCR system (Stratagene, La Jolla, CA), 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  $95^\circ\text{C}$  for 10 min, followed by 40 cycles with three-segment amplification (30 s at  $95^\circ\text{C}$  for denaturation, 1 min at  $55^\circ\text{C}$  for annealing, and 30 s at  $72^\circ\text{C}$  for DNA chain elongation).

Each sample was analysed in duplicate, and three independent repetitions were conducted. The relative expression ratio (RER) was calculated using the  $C_t$  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:

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

where  $E$  is the estimated PCR efficiency (100%),  $C_t$  is the cycle threshold,  $T$  is the target gene,  $R_1$  is *gamma actin*,  $R_2$  is *glyceraldehyde 3-phosphate dehydrogenase*, and  $\Delta C_t$  is the difference between the control (not exposed) and the treatment (exposed to UV-B, UV-B+WL, or WL).

#### Statistical analysis

Differences among the mean relative per cent germination of conidia, and among the RERs 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, CA).

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

Gene	Forward (3'–5')	Reverse (5'–3')	Genebank accession number/References
<i>Metarhizium robertsii</i>			
CDP photolyase ( <i>MrPhr1</i> )	CATGCCGATTGGACTTGCTTG	TGGAACAGCGCAATCAACAGG	JN694762.1
6-4 photolyase ( <i>MrPhr2</i> )	TCGCCGTTTCTTCACTTTGGTG	TCTGCGTAAACTTGGCACCGAC	JN694761.1
Laccase 1 ( <i>MrLac1</i> )	CGCCTGGCTTACGTGATAC	GCTCAGGCCATGCATCAAC	XM_007825745.1
Laccase 2 ( <i>MrLac2</i> )	TCCTGGGTCAACGAAAGCC	CGCCGCGATAAAGTTCATGC	XM_007826363.1
Laccase 3 ( <i>MrLac3</i> )	CTATACTTCCGAGCCACGCAG	CCCTTCCATTCCCTCGATAGC	XM_007828431.1
Polyketide synthase 1 ( <i>MrPks1</i> )	CATTCCGCCTCTCTATTGCC	TGTGCGGGCGCATGATATGG	XM_007825743.2
Polyketide synthase 2 ( <i>MrPks2</i> )	CATCAGCGCCATCGGTTTAGAC	CGGGATAGGGATTGGTTGTGG	XM_007821237.2
<i>Metarhizium acridum</i>			
6-4 Photolyase ( <i>MaPhr</i> )	TTCATGCCGATTGGAATTGC	TGCTTGTTGATGGTGCCTCTG	XM_007813640.1
Laccase 1 ( <i>MaLac1</i> )	AATGCGCGTACCCAACCTCTG	CCACGGCGACATGTGATCTG	XM_007813533.1
Laccase 3 ( <i>MaLac3</i> )	CCGCATCCCATCCACAAC	GCTGTGGTTGGCATGGAGAC	XM_007812616.1
Polyketide synthase 1 ( <i>MaPks1</i> )	CCGTTCCCGACTCACAATTACC	GCACAGCAAGCGGCGTTAATC	XM_007813534.1
Polyketide synthase 2 ( <i>MaPks2</i> )	CGGTTATCTCTCAACGAATCC	TCTGCTGCCTCTGGAAATCTCC	XM_007817459.1
<i>Reference genes (for both)</i>			
Gamma actin	TCCTGACGGTCAGTTCATC	CACCAGACATGACGATGTTG	Partial sequence (Fang and Bidochka 2006)
Glyceraldehyde 3-phosphate dehydrogenase	GACTGCCCGCATTGAGAAG	AGATGGAGGAGTTGGTGTG	AY461523

## Results

### 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<sup>-2</sup>), 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).

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

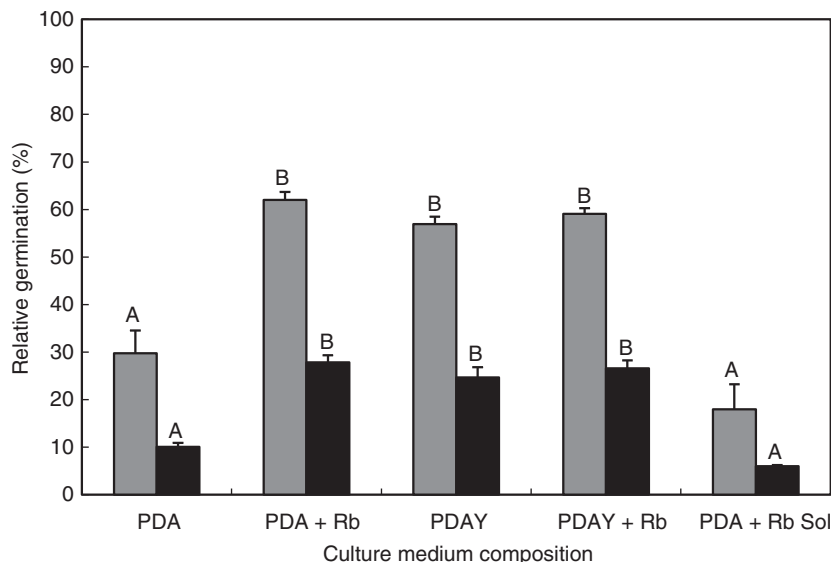
Conidia of both isolates (*M. acridum* ARSEF 324 and *M. robertsii* ARSEF 2575) produced on PDA medium supplemented with Rb were more tolerant to UV-B at 3.9 or 6.24 kJ m<sup>-2</sup> than conidia produced on pure PDA

medium without Rb, whether followed or not by exposure to 16 klux WL (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<sup>-2</sup> followed by exposure to WL were more tolerant to UV-B than conidia that were not exposed to WL ( $F_{5,12} = 100.1$ ;  $P < 0.0001$ ). Conidia of *M. robertsii* had improved tolerance to UV-B radiation in response to WL exposure only when they were produced on medium containing Rb and exposed to 6.24 kJ m<sup>-2</sup> ( $F_{5,12} = 598.8$ ;  $P < 0.0001$ ). No significant effect on relative germination of *M. acridum* or *M. robertsii* was detected when conidia were exposed to WL only (Fig. 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 (i) UV-B radiation followed by exposure to WL (UV-B+WL), or (ii) exposed to WL 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



**Figure 2** Relative germination of *Metarhizium acridum* (ARSEF 324, grey bars) and *M. robertsii* (ARSEF 2575, black bars) conidia after exposure to UV-B irradiance of  $866.7 \text{ mW m}^{-2}$  for 2 h (total dosage =  $6.24 \text{ kJ m}^{-2}$ ). Conidia were produced on (i) PDA medium without supplement (PDA); (ii) PDA supplemented with 0.01% riboflavin (PDA+Rb); (iii) PDA supplemented with 1% yeast extract (PDAY); or (iv) 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+Rb Sol). Relative germination was calculated in relation to nonirradiated 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.

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 ( $P > 0.05$ ).

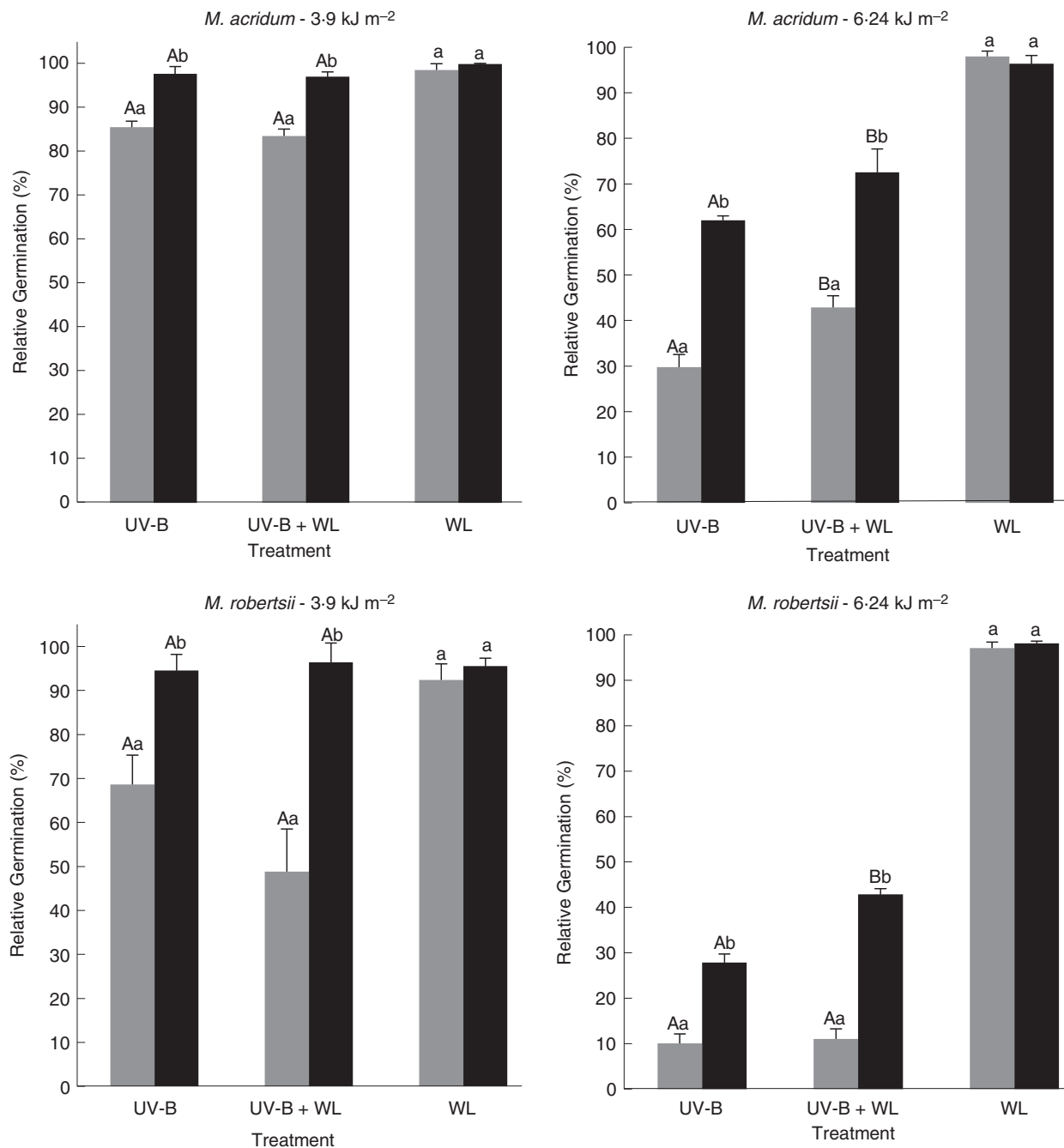
Conidia of both isolates produced on pure PDA medium (without supplement) did not express the genes tested in significantly greater amounts than conidia produced on PDA medium supplemented with Rb in any irradiation treatment.

## Discussion

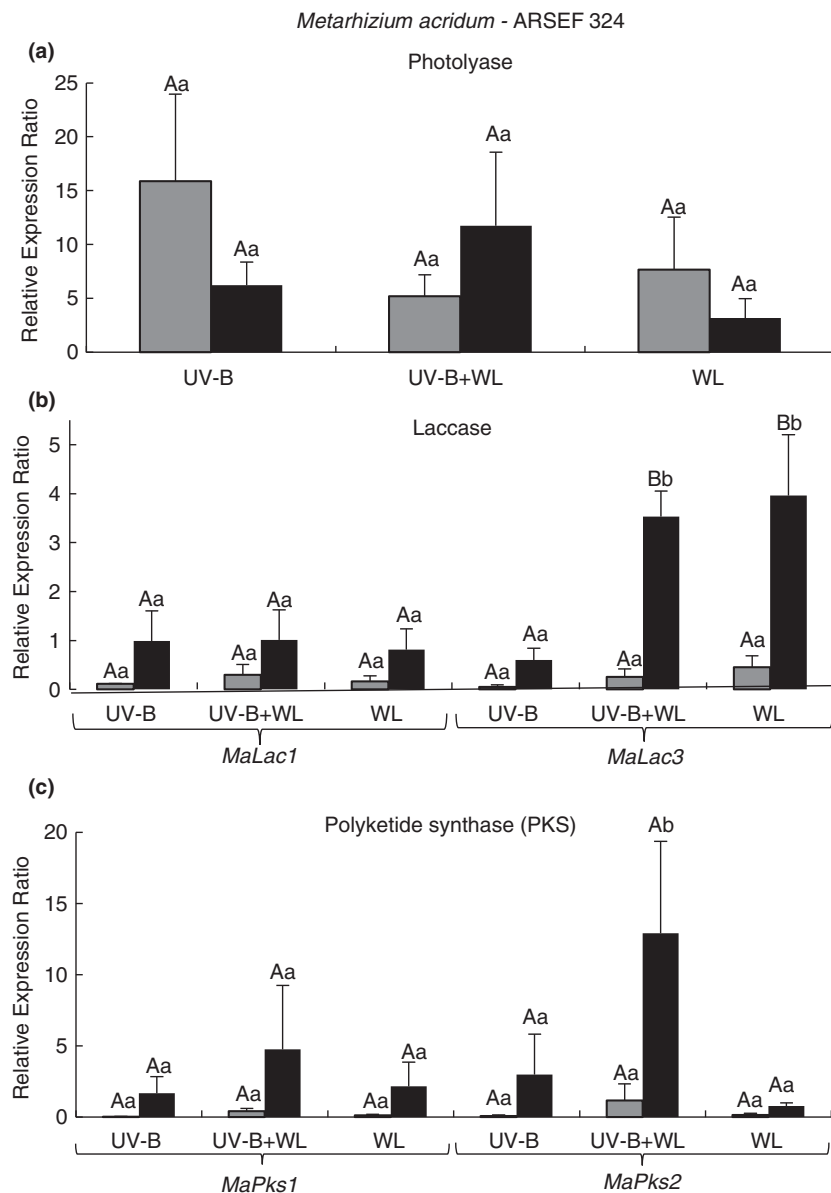
In this study, conidia of *M. robertsii* (ARSEF 2575) and *M. acridum* (ARSEF 324) when produced on Rb-supplemented PDA culture medium exhibited enhanced tolerance to UV-B radiation. PDAY medium also increased the tolerance of conidia, likely because yeast extract contains B-complex vitamins, including 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 per cent conidial relative germination; which makes comparisons difficult (Braga et al. 2001a, 2001c). 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 *A. nidulans*, respectively, were more tolerant to UV radiation (254 nm) when suspended in saline solution with  $0.8 \text{ mmol l}^{-1}$  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).



**Figure 3** Relative germination of *Metarhizium acridum* (ARSEF 324) and *M. robertsii* (ARSEF 2575) conidia exposed to (i) UV-B irradiance of 866.7 mW m<sup>-2</sup> for 1 : 15 h (total dosage = 3.9 kJ m<sup>-2</sup>) or 2 h (total dosage = 6.24 kJ m<sup>-2</sup>) (UV-B), or (ii) to UV-B (6.24 kJ m<sup>-2</sup>) followed by exposure to white light (16 klux) (UV-B+WL), or (iii) to white light only (WL). Conidia were produced on PDA medium with (black bars) or without (grey bars) 0.01% riboflavin. Relative germination was calculated in relation to nonirradiated 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<sup>-2</sup> ( $F_{5,12} = 28.5$  and  $P < 0.0001$ ) or 6.24 kJ m<sup>-2</sup> ( $F_{5,12} = 100.0$  and  $P < 0.0001$ ); ARSEF 2575 exposed to 3.9 kJ m<sup>-2</sup> ( $F_{5,12} = 11.6$  and  $P = 0.003$ ) or 6.24 kJ m<sup>-2</sup> ( $F_{5,12} = 598.8$  and  $P < 0.0001$ ). Error bars are standard errors of three independent trials.



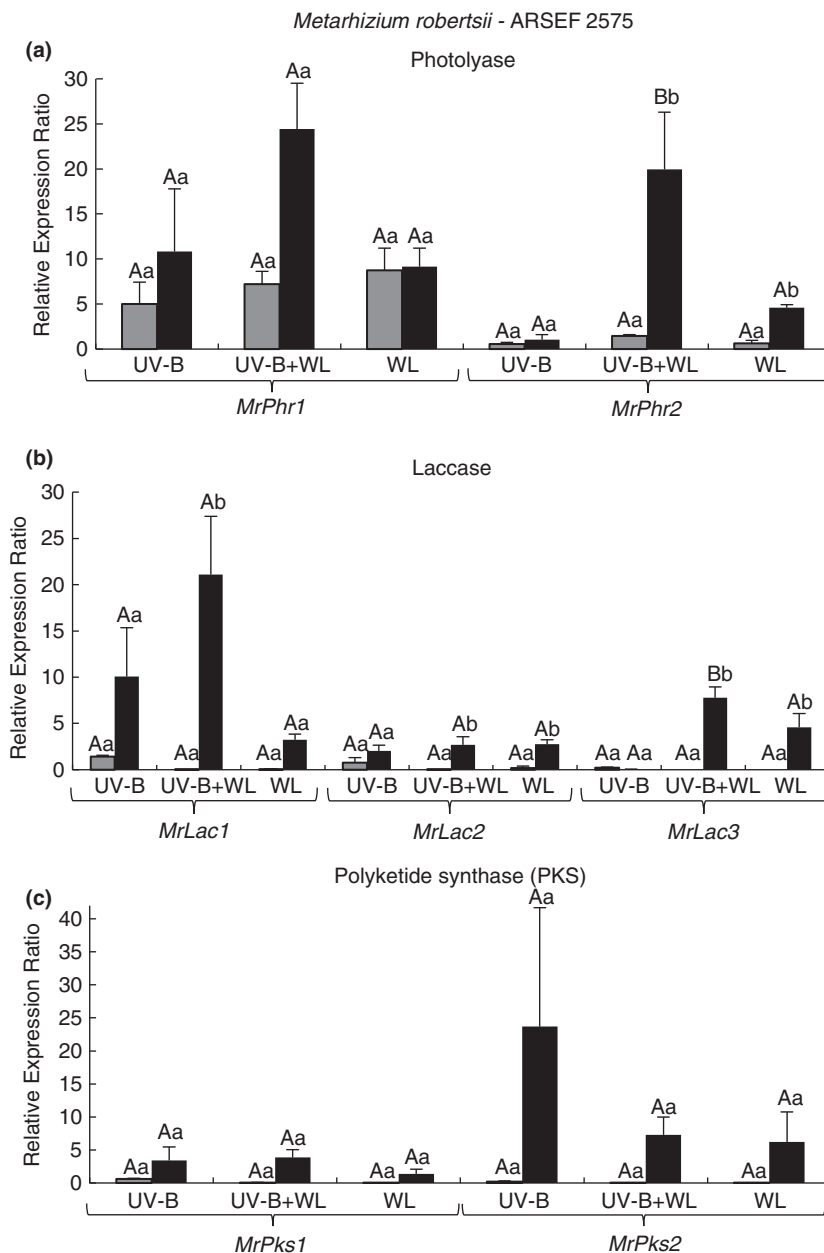
**Figure 4** Relative expression ratio of photolyase (a), laccase (b) and polyketide synthase (c) in *Metarhizium acridum* (ARSEF 324) conidia produced on PDA medium with (black bars) or without (grey bars) riboflavin and exposed to: (i) UV-B irradiance of 866.7 mW m<sup>-2</sup> for 1 : 15 h (total dosage = 3.9 kJ m<sup>-2</sup>) (UV-B), or (ii) to UV-B (3.9 kJ m<sup>-2</sup>) followed by exposure to white light (16 klux) (UV-B+WL) or (iii) 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.

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, makes 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 WL after UV-B resulted in high relative germination; this response was associated with high expression levels of *MrPhr2* stimulated by exposure to the WL (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 CPDs in damaged

**Figure 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 (grey bars) riboflavin and exposed to (i) UV-B irradiance of  $866.7 \text{ mW m}^{-2}$  for 1 : 15 h (total dosage =  $3.9 \text{ kJ m}^{-2}$ ) (UV-B), or (ii) to UV-B ( $3.9 \text{ kJ m}^{-2}$ ) followed by exposure to white light (16 klux) (UV-B+WL) or (iii) 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.



DNA, allowing irradiated cells to recover. Also, 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 WL had higher relative germination, even when conidia were produced in culture medium without Rb supplementation, indicating photoreactivation (see Fig. 3); however, no over-expression of *MaPhr* was detected,

even by conidia produced on PDA supplemented with Rb and exposed to UV-B or WL, or the combination of UV-B and WL (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 *MaPhr* 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 *Lac* and *Pks* genes, however, was higher when conidia were exposed to UV-B radiation followed by exposure to WL. 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 WL 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 favourable for germination (Hagiwara et al. 2016). However, in this study, conidia of both *M. robertsii* and *M. acridum* revealed expression of photolyase, laccase, or PKS genes. In fact, enzymatic metabolic activity in dormant conidia has been demonstrated by revealing an induced activity of  $\alpha$ -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 (a source of Rb) 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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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Irradiance spectrum of white light emitted by the photostability chamber lamps, at 16 klux.