UNIVERSITY OF SÃO PAULO
SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO

The photobiology of *Metarhizium acridum*: light quality, stress tolerance, and gene regulation

A fotobiologia de *Metarhizium acridum*: qualidade de luz, tolerância ao estresse e regulação gênica

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Doctoral Thesis presented to the Graduate Program of the School of Pharmaceutical Sciences of Ribeirão Preto for the degree of Doctor in Sciences

Concentration area: Bioagents and Biotechnology Applied to Pharmacy

Candidate: Guilherme Thomaz Pereira Brancini
Supervisor: Prof. Dr. Gilberto Úbida Leite Braga

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“Any road followed precisely to its end leads precisely nowhere. Climb the mountain just a little bit to test that it's a mountain. From the top of the mountain, you cannot see the mountain.”

Frank Herbert

RESUMO

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Metarhizium acridum é um importante fungo entomopatogênico utilizado no controle biológico de insetos praga. O sucesso do controle biológico depende majoritariamente da habilidade do fungo em tolerar fatores ambientais geradores de estresse tais como o calor e a radiação ultravioleta. Um dos fatores ambientais geradores de estresse é a radiação solar ultravioleta-B (UV-B, 280-315 nm), que é capaz de atrasar a germinação dos conídios e até causar inativação do fungo, reduzindo assim a eficiência do controle de insetos. Foi anteriormente observado que o crescimento de *M. acridum* na presença de luz visível induz o fungo a produzir conídios com elevada tolerância à radiação UV-B. A luz visível é um importante estímulo para muitos fungos, pois ela regula diversos processos biológicos e serve ainda como sinal espacial e temporal. A resposta à luz em fungos varia de acordo com a qualidade de luz e pode ser dividida em respostas à luz azul, verde e vermelha. Na presente tese, três importantes questões são abordadas: (1) qual qualidade de luz (azul ou vermelha) é responsável pelo aumento da tolerância à radiação UV-B após exposição à luz? (2) Como a luz aumenta a tolerância à radiação UV-B? (3) Como a luz regula a expressão gênica tanto no nível transcricional como no pós-transcricional? Aqui é mostrado que a luz azul, e não a luz vermelha, aumenta a tolerância do fungo à radiação UV-B. Além disso, a luz induz a expressão de um gene que codifica uma fotoliase e foi observado que a fotorreativação, e não o reparo no escuro, é o principal mecanismo envolvido na tolerância à radiação UV-B. O uso da transcriptômica via sequenciamento de mRNA revelou que a luz regula a transcrição de aproximadamente 11% dos genes no genoma. Apesar disso, o uso de proteômica quantitativa mostrou que a luz alterou a abundância de apenas 57 proteínas, ou seja, poucas mudanças no nível de mRNA foram traduzidas em mudanças no nível proteico. A proteômica também revelou que a exposição à luz causou uma redução na abundância de proteínas envolvidas com o processo de tradução, tais como subunidades do fator de iniciação de tradução 3 e proteínas ribossomais. Essa redução na atividade traducional é consistente com um modelo em que a luz atua como sinal e como estresse para a célula. Além disso, a redução na atividade traducional é uma possível explicação para o número reduzido de proteínas reguladas pela luz. Finalmente, os resultados apresentados aqui enfatizam a importância de se medir os níveis proteicos para um entendimento completo da resposta à luz em fungos.

Palavras-chave: *Metarhizium*, fotobiologia, tolerância ao estresse, proteômica, transcriptômica.

ABSTRACT

BRANCINI, G. T. P. **The photobiology of *Metarhizium acridum*: light quality, stress tolerance, and gene regulation.** 2019. 112f. Thesis (Doctorate). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2019.

Metarhizium acridum is an important entomopathogenic fungus currently used for the biological control of insect pests. The success of biological control is heavily dependent on the fungus ability to tolerate environmental stressors such as heat and ultraviolet radiation. One of such stressor is solar ultraviolet-B radiation (UV-B, 280-315 nm), which is capable of delaying conidia germination and even inactivate the fungus, thus reducing insect control efficiency. It was previously shown that growing *Metarhizium* in the presence of visible light induces the fungus to produce conidia with increased tolerance to UV-B radiation. Visible light is an important stimulus for many fungi as it regulates a wide variety of biological processes and serves additionally as a signal for space and time. Responses to light in fungi vary according to light quality and can be divided in responses to blue, green, and red light. In the present thesis, three major questions are addressed: (1) what radiation color (blue or red) is responsible for the increased tolerance to UV-B radiation after light exposure? (2) How does light exposure increase tolerance to UV-B radiation? (3) How does light globally regulate gene expression both transcriptionally and post-transcriptionally? Here it is shown that blue light, and not red light, increases tolerance to UV-B radiation. Also, light induces the expression of a photolyase-coding gene and it was observed that photoreactivation, and not dark repair, is the major component behind UV-B radiation tolerance. Transcriptomics via mRNA-Sequencing revealed that light regulates the transcription of approximately 11% of the genome. However, quantitative proteomics showed that light changed the abundance of only 57 proteins, thus showing that few changes at the mRNA level were translated to the protein level. Proteomics also revealed that light exposure caused a reduction in the abundance of translation-related proteins such as subunits of the eukaryotic translation initiation factor 3 and ribosomal proteins. This reduction in translational activity is consistent with a model in which light is both a signal and a stress to the cell. Furthermore, decreased translational activity is a potential explanation for the reduced number of light-regulated proteins. Finally, the results presented here highlight the importance of measuring protein levels in order to fully understand light responses in fungi.

Keywords: *Metarhizium*, photobiology, stress tolerance, proteomics, transcriptomics.

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1 – INTRODUCTION

1.1 – Light responses in fungi

Visible light (400-700 nm) is perhaps the most important environmental signal for many fungi as it regulates a variety of biological processes such as the balance between sexual and asexual development, spore germination, vegetative growth, secondary metabolism, circadian time keeping, phototropism, pathogenicity, nutrient uptake, and stress tolerance (Yu and Fischer, 2019). Visible radiation is made of photons of different wavelengths and fungi possess a range of photoreceptors that sense distinct spectral colors (Fig. 1).

Fungi responses to different radiation colors will vary based on the presence or absence of the necessary photoreceptor as well as the pathways activated or inhibited after light exposure (Yu and Fischer, 2019). In the following sections, we provide an overview of light regulation in fungi focusing on blue, red, and green light responses.

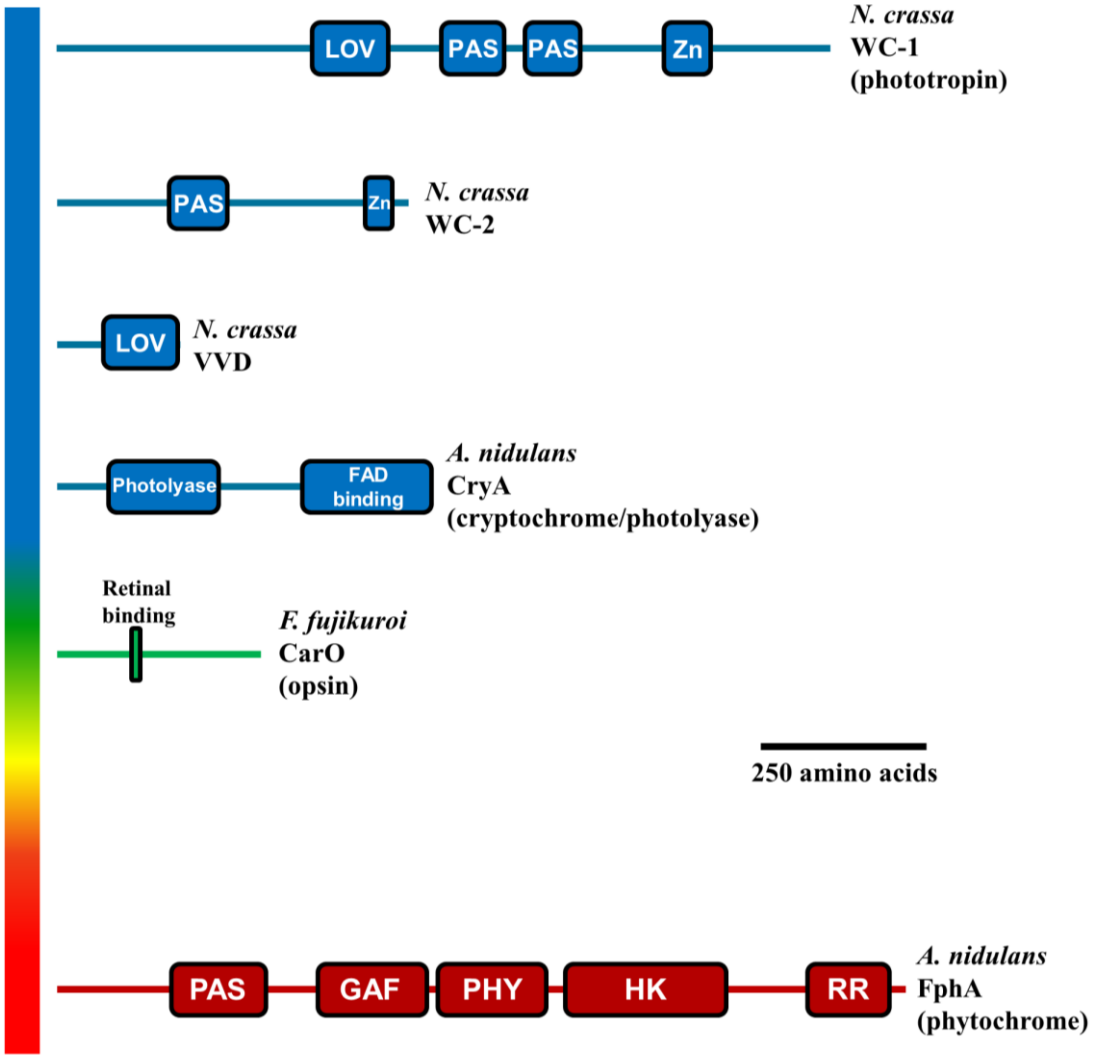

Fig. 1 – Classical fungal photoreceptors from *Neurospora crassa*, *Aspergillus nidulans*, and *Fusarium fujikuroi* responsible for sensing blue, green, and red light. LOV: *L*ight, *O*xygen, *V*oltage; PAS: *P*er-*A*rnt-*S*im; Zn: zinc finger DNA binding; GAF: c*G*MP-specific phosphodiesterase, *A*denylyl cyclase, *F*ormate hydrogen lyase; PHY: phytochrome; HK: histidine kinase; RR: response regulator. Source: adapted from Yu and Fischer (2019)

1.1.1 – Blue light responses

Responses to blue light were first characterized in the model ascomycete *Neurospora crassa*. Light regulates many aspects of *N. crassa* biology including mycelial carotenoid biosynthesis, development, conidiation, formation of protoperithecia, and the circadian clock (Linden; Rodriguez-Franco; Macino, 1997). When growing in slants in the presence of blue light (~450 nm), *N. crassa* will appear

completely orange as both conidia and mycelium accumulate carotenoids. However, mutants for the *wc-1* (*w*hite *c*ollar-1) do not accumulate carotenoids in the mycelium as a response to light. This results in the formation of a white mycelial matt underneath the orange conidia resembling a white collar (Harding and Turner, 1981). Later, it was unveiled that the *wc-1* gene encodes a zinc finger transcription factor binding to GATA-like sequences and is itself light-regulated (Ballario et al., 1996). A second gene, *wc-2*, is also essential for light responses in *N. crassa*. WC-1 and WC-2 interact via their PAS (Per-Arnt-Sim) domains (Fig. 1) and form the heterodimer White Collar Complex (WCC) that then drives the expression of light-regulated genes after illumination (Talora et al., 1999; Yu and Fischer, 2019). Despite the amount of evidence at the time, confirmation that WC-1 was indeed a photoreceptor came only in 2002 after the publication of two studies. In the first, Froehlich and co-workers reported that, in the presence of light, WC-1 binds to specific regions named Light-Responsive Elements (LRE) on light-regulated genes (Froehlich et al., 2002). Simultaneously, He and coworkers reported that WC-1 possesses a specific type of PAS domain named LOV (Light, Oxygen, Voltage) that binds a FAD molecule with peak absorption at 450 nm (He et al., 2002). When taken together, these studies showed that WC-1 is both a bona fide photoreceptor and a transcription factor binding to the promoter regions of light-regulated genes.

More recently, the WC-1-driven blue light response in *N. crassa* was found to be dependent on histone acetylation. Residue K14 of histone H3 associated with *albino-3* (*al-3*) is acetylated after illumination in a WC-1-dependent manner (Grimaldi et al., 2006). A deletion mutant for the histone H3 gene has the same blind phenotype observed for *wc-1* mutants and does not display light induction of *al-3*. Loss of photoinduction also occurs after deletion of *ngf-1* coding for an acetyltransferase

involved in histone acetylation (Grimaldi et al., 2006). Later, it was observed that WC-1 and NGF-1 interact and the resulting complex assembles in the dark on the promoters of light-regulated genes (Brenna et al., 2012). Upon light exposure, NGF-1 acetylates histone H3 and activates gene transcription (Brenna et al., 2012).

In terms of genome-wide gene regulation after illumination, transcriptomic analyses by micro-array revealed that approximately 5.6% of the genome is regulated by light in *N. crassa* (Chen et al., 2009). Light-regulated genes were divided in Early Light Response Genes (ELRG) and Late Light Response Genes (LLRG) with the former peaking within 15-45 min of light exposure and the latter after 45-90 min. The main difference between the two classes is that ELRG are regulated by the WCC itself whereas LLRG are regulated by different transcription factors. However, because LLRG are light-regulated, their transcriptional regulators must also be upregulated after light exposure and some are indeed direct targets of the WCC. Therefore, the authors unveiled that light response in *N. crassa* is hierarchical in nature (Chen et al., 2009). It was also reported that co-regulated genes normally share biological functions. For example, four genes involved in carotenoid biosynthesis present almost identical regulation. Genes involved in DNA repair were classified as ELRG while genes coding for antioxidant enzymes were placed among the LLRG (Chen et al., 2009).

In 2014, a more thorough analysis, performed with RNA-Seq, revealed that as much as 31% of all expressed genes in *N. crassa* are light-regulated (Wu et al., 2014). More importantly, the authors showed that light exposure also results in gene downregulation, which was a then-undescribed phenomenon in *N. crassa* (Wu et al., 2014). In this regard, light exposure resulted in downregulation of genes involved in ribosome biogenesis, a known cellular response to stress (Spriggs; Bushell; Willis,

2010). This is consistent with previous work showing that light exposure represents a stress to the fungal cell (Wang; Yoshida; Hasunuma, 2007; Canessa et al., 2013).

One important protein in the regulation of blue light responses in *N. crassa* is the LOV domain protein VIVID (VVD) (Fig. 1). VVD binds WC-1 and thus interferes with the formation of the WCC (Hunt et al., 2010). Because *vvd* transcription is under the control of the WCC (and is therefore light-induced) VVD plays a role in photoadaptation. Mutants for *vvd* lack the ability to photoadapt and display increased expression of light-regulated genes when grown in constant light (Shrode et al., 2001).

Cryptochromes and photolyases are also relevant blue light photoreceptors in fungi (Fig. 1). Photolyases use blue light to repair UV-damaged DNA whereas cryptochromes are defined as proteins with sequence homology to photolyases but that lack DNA repair activity, normally fulfilling regulatory roles (Sancar, 2003). In *N. crassa*, the gene *cry* encodes a cyptochrome that has been shown to bind both single- and double-stranded DNA (Froehlich et al., 2010). However, CRY does not seem to regulate gene expression for either early or late light-responsive genes (Froehlich et al., 2010). Conversely, in *Aspergillus nidulans* the cryptochrome CryA is a regulator of development. CryA represses sexual development under UV-A light (350-370 nm) and *cryA* deletion mutants undergo sexual development under inappropriate conditions (Bayram et al., 2008). Interestingly, CryA has been shown to possess DNA repair activity in repair-deficient *Escherichia coli* and also when overexpressed in UV-sensitive (*uvsB*Δ) *A. nidulans* (Bayram et al., 2008). In *Fusarium fujikuroi*, the cryptochrome CryD cooperates with the WC-1 homologue, WcoA, to modulate light-induced carotenoid biosynthesis. Furthermore, VVD homologue VvdA regulates WcoA activity and therefore also plays a role in photocarotenogenesis (Castrillo and Avalos, 2015).

1.1.2 – Red light responses

Blue light responses normally dominate and fungi like *N. crassa* respond exclusively to this color. However, some fungi will also respond to red light. It is the case for *A. nidulans*. In addition to homologues for WC-1 and WC-2, named LreA and LreB (*L*ight *R*esponse), *A. nidulans* possesses a fungal phytochrome (FphA) responsible for sensing red light (650~705 nm).

Molecular studies have shown that FphA is a two-component regulatory protein made of photoreceptor and regulatory domains. The photoreceptor domain contains the PAS, GAF (c*G*MP-specific phosphodiesterase; *A*denylyl cyclase; *F*ormate hydrogen lyase), and PHY (*Phy*tochrome) domains and the regulatory domain contains a histidine kinase and a response regulator domain (Fig. 1) (Brandt et al., 2008).

The roles of red light in *A. nidulans* development were reported long ago when Mooney and Yager observed that red light (680 nm) was essential for conidiogenesis and that the fungus reproduced sexually by forming cleistothecia in the absence of light (Mooney and Yager, 1990). Later, it was shown that FphA is responsible for repressing sexual development in the presence of red light (Blumenstein et al., 2005).

In *A. nidulans*, unlike observed for *N. crassa*, both blue and red light regulate development. The blue photoreceptors LreA and LreB (homologues of WC-1 and WC-2) interact with FphA both genetically and physically (Purschwitz et al., 2008). At the genetic level, LreA and LreB are activators of sexual development and are inhibited by FphA under light, leading to asexual development and conidiogenesis. At the molecular level, there is a direct interaction between LreB and FphA (Purschwitz et al., 2008). Therefore, LreA and LreB interact to form a complex similar to the WCC and LreB also interacts with FphA which, in turn, interacts with the master regulator of development velvet A (VeA) (Purschwitz et al., 2008). Furthermore, because development and

secondary metabolism are closely linked in fungi (Calvo et al., 2002), the authors evaluated the effects of light exposure on sterigmatocystin (ST) production. In *A. nidulans*, secondary metabolite production is favored in the dark. Both white and blue light inhibited ST production. However, red light increased ST biosynthesis to levels above those of the dark control with a peak production at 700 nm (Purschwitz et al., 2008).

The phytochrome FphA was also recently found to regulate histone modification in response to light. Photoinduction of the light-regulated gene *ccgA* correlates with acetylation of lysine 9 in histone H3 (H3K9) (Hedtke et al., 2015). Because H3K9 was acetylated after illumination, the authors studied the interactions of VeA, LreA, and FphA with the acetyltransferase GcnE and the deacetylase HdaA. All three proteins interact with GcnE and HdaA. Based on the findings, the following model is proposed: in the dark, VeA and LreA are bound to the *ccgA* promoter and interact with HdaA to prevent transcription of *ccgA*. Upon illumination, LreA is released from the promoter and FphA, together with VeA, induce acetylation of H3K9 via the SAGA/AdaB/GcnE complex, thus activating *ccgA* transcription (Hedtke et al., 2015).

Histone modification is one of the functions that FphA exerts in nuclei. However, a cytoplasmic regulatory role for this photoreceptor was also unveiled. Yu and coworkers performed an *A. nidulans* screening looking for blind mutants and isolated one blind strain that presented a point mutation in the *sakA* gene involved in the high osmolarity glycerol (HOG) pathway (Yu; Armant; Fischer, 2016). Photoinduction of the conidiation-related gene *conJ* and of *ccgA* is abolished in both *fphA* and *sakA* deletion mutants, indicating that light sensing and the HOG pathway were intertwined. Indeed, *conJ* and *ccgA* expression was induced by 0.5 M NaCl. For the HOG pathway to be activated by light, illumination should result in SakA shuttling into nuclei and also

in SakA phosphorylation. A SakA-GFP protein shifted to nuclei after illumination with either white or red light in an FphA-dependent manner. Shuttling into nuclei was also observed with 0.5 M NaCl, but this was FphA-independent. Illumination with white or red light also resulted in FphA-dependent SakA phosphorylation. At the molecular level, FphA was found to directly interact with the phosphotransfer protein YpdA which is part of the two-component system involved in HOG pathway activation. Therefore, light triggers the stress-activated HOG pathway in *A. nidulans* during signaling (Yu; Armant; Fischer, 2016).

So far we have seen that whereas blue-light responses dominate in *N. crassa*, red light is more important to *A. nidulans*. Some fungi display more complex interplay between blue- and red-light responses. It is the case of the plant pathogen *Alternaria alternata*. This fungus possesses blue, red, and green light photoreceptors that were named exactly the same as their *A. nidulans* orthologues. Light induction of the conidiation-related gene *ccgA* is reduced in a Δ*fphA* background and lost in Δ*lreA* strains (Igbalajobi; Yu; Fischer, 2019). This is markedly different from *N. crassa* and *A. nidulans*. In the former, *ccg-1* induction is strictly dependent on WC-1 whereas in the latter *ccgA* photoinduction depends on FphA. There is also interplay between light and HOG signaling pathways in *A. alternata*. Both blue and red light cause HogA phosphorylation and this response is lost in both Δ*fphA* and Δ*lreA* strains together with *ccgA* photoinduction (Igbalajobi; Yu; Fischer, 2019). Interestingly, Δ*fphA* and Δ*lreA* strains display higher tolerance to oxidative stress (H_2O_2 and menadione) and increased expression of genes coding for catalases and superoxide dismutases (Igbalajobi; Yu; Fischer, 2019). This is in contrast to *Botrytis cinerea* in which deletion of WC-1 led to reduced tolerance to oxidative stress (Canessa et al., 2013) and highlights the variability between fungi in terms of light response and photoreceptor roles.

1.1.3 – Green light responses

Green light sensing in fungi is achieved via opsins. The *N. crassa* NOP-1 is a G protein coupled receptor with the classical seven helix transmembrane domain (Bieszke et al., 1999a). A conserved Lys residue in the seventh helix binds all-*trans*-retinal via a protonated Schiff base, thus forming a bacterial rhodopsin-like protein with peak absorption at 534 nm (Bieszke et al., 1999a). The mechanism through which NOP-1 operates is not known, but light exposure causes isomerization of retinal followed by deprotonation of the Schiff base. These are followed by protein conformational changes that likely trigger signal transduction (Bieszke et al., 1999a).

nop-1 mRNA levels increase during *N. crassa* asexual development and after light exposure, indicating it could perform a role in conidiation and/or light response, despite the fact that Δ*nop-1* mutants present no overt conidiation or photoresponse phenotype (Bieszke et al., 1999b). Interestingly, *nop-1* levels were higher in a Δ*wc-2* background, indicating WC-2 can negatively regulate *nop-1* expression (Bieszke; Li; Borkovich, 2007). Furthermore, deleting *nop-1* resulted in increased expression of conidiation-related genes *con-10* and *con-13* irrespective of light exposure (Bieszke; Li; Borkovich, 2007). More recently, photoinduction of *con-6* and *con-10* was found to be higher in a Δ*nop-1* background, indicating that NOP-1 can negatively regulate WCC activity at certain loci, presumably by regulating a WCC repressor (Olmedo et al., 2010).

The plant pathogen ascomycete *F. fujikuroi* has two genes coding for opsins, namely *opsA* and *carO,* and both have been shown to be light regulated (Estrada and Avalos 2009). Strains lacking the *N. crassa wc-1* homologue *wcoA* have increased expression of *opsA* and *carO*, indicating that WcoA is a negative regulator of their expression. However, photoinduction of *opsA* and *carO* is lost in the *wcoA* mutant

(Estrada and Avalos, 2009). *opsA* is homologous to *N. crassa nop-1* and its deletion in *F. fujikuroi* leads to no overt phenotype be it growth, development, virulence or secondary metabolite production. However, there is reduced expression of some structural genes involved in carotenoid biosynthesis, such as *carB* and *carRA* (Estrada and Avalos, 2009).

Unlike *opsA*, *carO* encodes an opsin displaying green light-dependent proton pump activity and it mainly localizes to conidia plasma membrane (Garcia-Martinez et al., 2015). Conidia from $CarO^-$ strains germinate faster than those from $CarO^+$ strains under illumination, indicating that CarO slows down germination under light (Garcia-Martinez et al., 2015). It is speculated that this is achieved by regulating a local proton gradient as ambient pH can affect conidia germination. Furthermore, CarO could also be playing a role in nutrient uptake by maintaining a proton gradient across the membrane. This could prove energetically advantageous to the fungus as maintaining proton gradients are normally a function of H^+ ATPases (Garcia-Martinez et al., 2015). Finally, green light sensing opsins are much more prevalent in phytopathogenic and endophytic fungi, presumably because green light is abundant in the phyllosphere as a result of chlorophyll-filtered sunlight (Garcia-Martinez et al., 2015).

1.2 – *Metarhizium*

1.2.1 – *Metarhizium* spp. as biological control agents

Metarhizium (Ascomycota; Sordariomycetes) is a genus of filamentous entomopathogenic fungi that can be found in both cultivated and undisturbed soil worldwide. Owing to their ability of infecting and killing insects, *Metarhizium* spp. have been used for the biological control of insect pests and disease vectors in an attempt to replace – either totally or partially – the use of chemical insecticides (Lacey

et al., 2015). Biological control is usually performed by applying formulations containing fungal conidia in the field. Upon contact with the host cuticle, conidia germinate and invade the insect, eventually causing its death by a combination of hyphal growth, nutrient consumption, and production of toxic metabolites (Kershaw et al., 1999; Scholte et al., 2004).

In 2007, the number of commercial products containing *Metarhizium* spp. worldwide was 47 (Faria and Wraight, 2007). In Brazil, the annual production of *Metarhizium* by ten sugarcane mills and private companies in the states of Alagoas and São Paulo is estimated at 1,882 tons (Li et al., 2010). In the states of Alagoas, Pernambuco, and Sergipe, *Metarhizium anisopliae* is used control the sugarcane spittlebug *Mahanarva posticata* and the root spittlebug *Mahanarva fimbriolata* with an estimated 300,000 ha of sugarcane being treated each year (Li et al., 2010). In China, *Metarhizium acridum* is used to control grasshoppers in grasslands in the northern part of the country. The fungus is also used to control grasshopper populations on rice and bamboo plantations in southern China (Li et al., 2010).

The fungus *M. acridum* is considered a specialist entomopathogen, capable of infecting exclusively orthopteran insects such as locusts and grasshoppers (Hu et al., 2014). Compared to other *Metarhizium* species, *M. acridum* has an increased number of genes related to genomic stability and the fungus is indeed the most tolerant to abiotic stresses such as UV radiation and heat, which is advantageous to biological control (Hu et al., 2014; Braga et al., 2015).

The success of biological control employing mycoinsecticides depends on maintaining fungal viability under stressful field conditions. Environmental factors such as UV radiation and high temperatures, both resulting from solar exposure, can delay conidia germination and even inactivate the fungus (Braga et al., 2001; Rangel et al.,

2005b). Ultraviolet-B radiation (UV-B, 280-315 nm) is known to interact directly with and damage DNA by inducing the formation of cyclobutane pyridimide dimers (CPD) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PP) which hinders germination and growth until the damages are repaired (Nascimento et al., 2010). *Metarhizium* inactivation by heat and high temperatures is also a concern (Keyser et al., 2014) and locusts infected with *M. acridum* bask in the sun to increase their body temperature in a phenomenon termed behavioral fever (Clancy et al., 2018).

Many studies have focused on increasing *Metarhizium* tolerance to environmental stressors, some of which by the genetic engineering of strains (Ortiz-Urquiza and Keyhani, 2015). Tseng and coworkers cloned an *A. alternata* gene for melanin biosynthesis and inserted it in the *M. anisopliae* genome. Because melanin can serve a photoprotecting pigment, there was increased tolerance to UV-B radiation in the mutant strain. Also, increased mortality of *Plutella xylostella* larvae due to a reduction in UV-induced germination delay was also observed (Tseng; Chung; Tzean, 2011). In a similar way, inserting a photolyase coding gene from *Halobacterium salinarum* into the fungus genome resulted in increased survival of the mutant strain after UV-B exposure and increased virulence against *Anopheles gambiae* (a malaria vector) when compared to the wild type (Fang and St Leger, 2012).

While the success of genetic engineering is evident, there are other ways of increasing *Metarhizium* tolerance to stressors without having to resort to transgenic organisms. Both chemical and physical environmental factors are known to regulate *Metarhizium* tolerance to a variety of stresses. For instance, cultivating *M. acridum* on rich medium results in conidia with higher tolerance to UV-B radiation when compared to conidia obtained by infecting the grasshopper *Melanoplus sanguinipes* (Rangel et al., 2005a). Similarly, *Metarhizium robertsii* conidia produced in a medium with

supplemented salicylic acid are more thermotolerant when compared to conidia obtained from non-supplemented medium (Rangel et al., 2012). One environmental stimulus that can regulate *Metarhizium* tolerance to stress is light.

1.2.2 – The photobiology of *M. acridum*

As previously shown, light is an essential environmental signal that regulates many biological processes in fungi. Exposure to visible light also modulates stress tolerance in *Metarhizium*. It was previously shown that growing *M. robertsii* under a 12:12h light:dark photoperiod induces the fungus to produce conidia with increased tolerance to UV-B radiation when compared to conidia obtained from cultures that grew in the dark (Rangel et al., 2011). The genome of *M. acridum* bears genes encoding for all classical photoreceptors except green light-sensing opsins (Table 1).

Table 1 – The *Metarhizium acridum* genome bears genes coding for the classical photoreceptors with the exception of opsins

	Metarhizium acridum	
Photoreceptor	**Name**	**Gene ID**
WC-1	White collar 1	MAC_01685
WC-2	Cutinase gene palindrome-binding protein	MAC_09544
VVD	Cellulose signaling associated protein ENVOY	MAC_03457
Cryptochromes/Photolyases	Putative cryptochrome DASH	MAC_07571
	Cryptochrome-2	MAC_03703
	Photolyase	MAC_05491
Opsins	-	-
Phytochromes	Putative phytochrome-like histidine kinase	MAC_04734

Knowing that light regulates UV (particularly UV-B) radiation tolerance and that *M. acridum* possesses genes encoding for different photoreceptors, three immediate questions rise to mind: (1) what are the effects of different light colors on the acquisition of UV-B tolerance? (2) What is the molecular mechanism behind such

increase in tolerance? (3) How does light globally regulate gene expression in *M. acridum*? These questions are addressed in the following three chapters. In Chapter 1, we investigate the effects of blue and red light on the acquisition of UV-B tolerance and also examine the kinetics of tolerance acquisition after light exposure. In Chapter 2, we study the photoinduction of photolyase- and UV endonuclease-coding genes and the roles of photoreactivation in determining UV-B tolerance. Finally, in Chapter 3 we combine RNA-sequencing and mass spectrometry-based high-throughput quantitative proteomics to study how light regulates gene expression at both the mRNA and the protein levels.

2 – OBJECTIVES

1) To study how light quality influences the photoinduction of tolerance to UV-B radiation;

2) To investigate the mechanism through which light exposure increases tolerance to UV-B radiation;

3) To elucidate how light exposure affects gene expression both transcriptionally and post-transcriptionally.

Chapter 1

Exposure of *Metarhizium acridum* mycelium to light induces tolerance to UV-B radiation

Running title: Light induction of UV-B tolerance in *Metarhizium acridum*

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ABSTRACT

Metarhizium acridum is an entomopathogenic fungus commonly used as a bioinsecticide. The conidium is the fungal stage normally employed as field inoculum in biological control programs and must survive under field conditions such as high ultraviolet-B exposure. Light, which is an important stimulus for many fungi, has been shown to induce the production of *Metarhizium robertsii* conidia with increased stress tolerance. Here we show that a two-hour exposure to white or blue/UV-A light of fast-growing mycelium induces tolerance to subsequent UV-B irradiation. Red light, however, does not have the same effect. In addition, we established that this induction can take place with as little as 1 min of white-light exposure. This brief illumination scheme could be relevant in future studies of *M. acridum* photobiology and for the production of UV-B resistant mycelium used in mycelium-based formulations for biological control.

Keywords: *Metarhizium*, light, stress tolerance, ultraviolet radiation, blue light, fungal photobiology

Introduction

Metarhizium (Ascomycota, Sordariomycetes) comprises a wide variety of entomopathogenic fungal species, which have been employed for biological control of insects, mainly in agriculture (Faria and Wraight, 2007; Lacey et al., 2015). For the efficiency of this biological control, conidia must survive under field conditions, including, for instance, exposure to solar ultraviolet radiation. Ultraviolet-A (UV-A) (320-400 nm) and ultraviolet-B (UV-B) (280-320 nm) inactivate conidia and delay their germination, resulting in reduced killing of insect hosts (Braga et al., 2001; Nascimento et al., 2010; Tseng; Chung; Tzean, 2011; Fang and St Leger, 2012; Braga et al., 2015).

Therefore, increasing the tolerance of *Metarhizium* spp. to UV irradiation is of great interest for the use of *Metarhizium* as a biological control agent (Fernandes et al., 2015). Conidia with increased tolerance to UV-B radiation can be produced by varying chemical and physical parameters present during mycelial growth (Rangel et al., 2015). For example, growth of *Metarhizium robertsii* on a rich medium induces the production of conidia with higher tolerance to UV-B radiation as compared with those produced by infecting the insects *Galleria mellonella* and *Zophobas morio* (Rangel et al., 2004). Furthermore, growth of *Metarhizium robertsii* in the presence of visible light also induces the production of conidia with increased tolerance to UV-B radiation (Rangel et al., 2011).

Light is a major stimulus for many fungi and regulates a wide range of biological processes, including phototropism, sexual/asexual development and production of secondary metabolites (Rodriguez-Romero et al., 2010; Fuller; Loros; Dunlap, 2015). Most fungi respond to blue light using photoreceptors homologous to the White Collar Complex first described in *Neurospora crassa*, with some responding also to red light through phytochromes (Rodriguez-Romero et al., 2010; Fuller; Loros; Dunlap, 2015). In the ascomycete *Neurospora crassa*, blue light regulates as much as 31% of the expressed genes in a vegetative growing mycelium, including genes related to overall metabolism, carotenoid production and ribosome biogenesis (Wu et al., 2014). Blue light is also responsible for entraining *N. crassa* to the circadian clock (Froehlich et al., 2002). Although *N. crassa* has been shown to possess phytochromes, no red light responses have so far been demonstrated for this fungus (Froehlich et al., 2005).

For *Aspergillus nidulans*, however, both blue and red light responses are known (Bayram et al., 2010). Initially, it was established that red light is necessary for

conidiogenesis (Mooney and Yager, 1990). Later, it was shown that red light represses sexual development through the phytochrome FphA (Blumenstein et al., 2005). Purschwitz et al. (2008) characterized responses of *A. nidulans* to both blue and red light and identified physical interactions between blue (LreA, LreB) and red (FphA) photoreceptors (Purschwitz et al., 2008).

Regardless of wavelength, response to light in fungi is fast – being achieved within minutes – and transient (Chen et al., 2009; Ruger-Herreros et al., 2011; Wu et al., 2014). For *M. robertsii*, as previously stated, white light during mycelial growth induces the production of conidia with increased tolerance to UV-B stress (Rangel et al., 2011). However, this response was reported for colonies exposed to visible light throughout their development. To the best of our knowledge, there is no information in the literature as to whether a brief light exposure of vegetative growing mycelium could induce higher tolerance of its colonies to a subsequent exposure to UV-B radiation in *Metarhizium*. Therefore, we investigated whether a short exposure to white light could induce tolerance to UV-B radiation in *M. acridum* mycelium. Because analysis of *M. acridum* genome (Gao et al., 2011) shows the presence of genes encoding for blue- and red-light photoreceptors (similar to *N. crassa white collar 1* and *A. nidulans fphA*, respectively), we further verified if exposures to blue/UV-A or red light alone could induce tolerance to UV-B radiation.

Materials and methods

Strain and conidia production

Metarhizium acridum strain ARSEF 324 was obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Robert W. Holley Center for Agriculture & Health, Ithaca, NY, USA). Conidia were produced by growing the fungus

on Potato Dextrose Agar (PDA) (Difco) supplemented with 5 g L^{-1} yeast extract (Difco) (PDAY) in complete darkness at 28°C for 10-14 days.

Mycelial growth

Conidia obtained from the plates were used to prepare a 10^4 conidia mL^{-1} suspension in 0.01% (v/v) Tween® 80 (Sigma). This suspension (80 µL) was used to inoculate 60-mm PDAY plates. The plates were incubated in complete darkness at 28°C for 26 h to obtain colonies.

Light induction

Twenty-six-hour-old colonies were exposed for 2 h under one of the following light sources: two white light fluorescent lamps (Osram, Germany) (5.3 W m^{-2}), red-light LED (54 W m^{-2}, peak emission = 630 nm, full width at half maximum = 16.5 nm) or blue/UV-A-light LED (60 W m^{-2}, peak emission = 400 nm, full width at half maximum = 15.0 nm). A dark treatment was also prepared in which no illumination was applied. Immediately after light exposure, both illuminated and dark plates were exposed to UV-B radiation.

UV-B exposures

UV-B exposures were conducted in a temperature-controlled growth chamber (410 7NDR, Nova Ética, São Paulo, Brazil) at 28 ± 1 °C for 3 h. The UV-B irradiance was provided by two fluorescent lamps (TL 20W/12 RS; Philips, Eindhoven, Holland). Samples were covered with a 0.13 mm thick cellulose diacetate film (JCS Industries), which blocked radiation below 290 nm. This allowed the passage of most UV-B and UV-A, but prevented samples from being exposed to UV-C (< 280 nm) and short

wavelength UV-B. The spectral irradiance of the filtered UV lamps is shown in (De Menezes et al., 2015). The DNA damage action spectrum developed by Quaite et al. (1992) was used to calculate the biologically effective weighted UV irradiance (UV_{BE})(Quaite; Sutherland; Sutherland, 1992). Musil (1995) developed a formulation for this action spectrum which we normalized to unit at 300 nm and weighted the measured spectral irradiance from 290 to 365 nm (Musil, 1995). The UV_{BE} at the sample level was 1.0 W m^{-2}. Control groups were prepared by inducing the colonies but not exposing them to UV-B radiation. After the exposure, all the plates were kept at 28 °C in complete darkness. Four independent experiments were performed.

Minimum time of visible light exposure required for induction of UV-B tolerance

Twenty-six-hour-old colonies were exposed to white light for 0 (dark), 5, 15, 30, 60 and 120 min. After the light induction was over, plates were dark-incubated until the longest exposure (120 min) was done. For instance, the 15 min plates were light-illuminated for 15 min and then dark-incubated for 1 h 45 min. Then, plates were exposed to UV-B as described in the previous section. Control plates were light-induced for 120 min and were not exposed to UV-B. We chose 120 min as the induction time for control plates because any light effect would most likely be better visualized after the longest induction period used. Three independent experiments were performed.

Minimum time of dark incubation after light induction necessary to achieve increased UV-B tolerance

Twenty-six-hour-old colonies were light-induced for 5 minutes and then incubated in the dark for 0, 10, 25, 55 and 115 minutes before UV-B exposure. Dark treatment received no light before UV-B stress. Control plates were light-induced for 5

minutes but were not exposed to UV-B radiation. Three independent experiments were performed.

Effect of colony age on the light-induced increase in UV-B tolerance

Colonies were allowed to develop for 26, 18 or 12 h before being induced under white light for 2 h. Dark treatments for each growth time were also prepared. After light induction, both light and dark treatments were exposed to UV-B radiation. Three independent experiments were performed.

Relative survival after UV-B exposure

At the time of light induction and subsequent UV-B exposure, colonies are barely visible to the naked eye. Therefore, colonies that continued to grow after UV-B exposure and achieved good naked eye visibility were considered as surviving the UV-B stress (Supplementary Figure S1). Colony counting was performed 48 and 72 h after UV-B irradiation. Relative survival was obtained by calculating the ratio of surviving colonies of each treatment to control groups (not exposed to UV-B). Plates were photographed with a Panasonic Lumix FZ40 digital camera 72 h after UV-B exposure.

Spectroradiometry

All radiation (emitted by LEDs, white and UV-B fluorescent lamps) measurements were performed using a cosine-corrected irradiance probe (CC-3-UV, Ocean Optics, Dunedin, FL, USA) screwed onto the end of an optical fiber coupled to a USB4000 spectroradiometer (Ocean Optics, Dunedin, FL). Total irradiance of the white fluorescent lamps as well as the red and blue/UVA LEDs was calculated by integrating irradiance in the visible (400-700 nm), red (570-680 nm) and blue/UVA (360-450 nm)

regions of the spectrum. For emission spectra of the light sources used, see Supplementary Figure S2.

Statistical analysis

Analysis of variance (ANOVA) was performed with GraphPad Prism 5.01 (GraphPad Software). Means were considered statistically different if $P < 0.05$ in Tukey's post-test.

Results and Discussion

The colonies of *M. acridum* that have been exposed to 2 h of white or blue/UV-A light show an increased tolerance to UV-B radiation unlike colonies kept in the dark or exposed to red light (Fig. 1A).

Furthermore, colonies previously induced with white or blue/UV-A light showed a reduced growth delay after UV-B exposure as compared to either colonies exposed to red light or those kept in the dark (Fig. 1B).

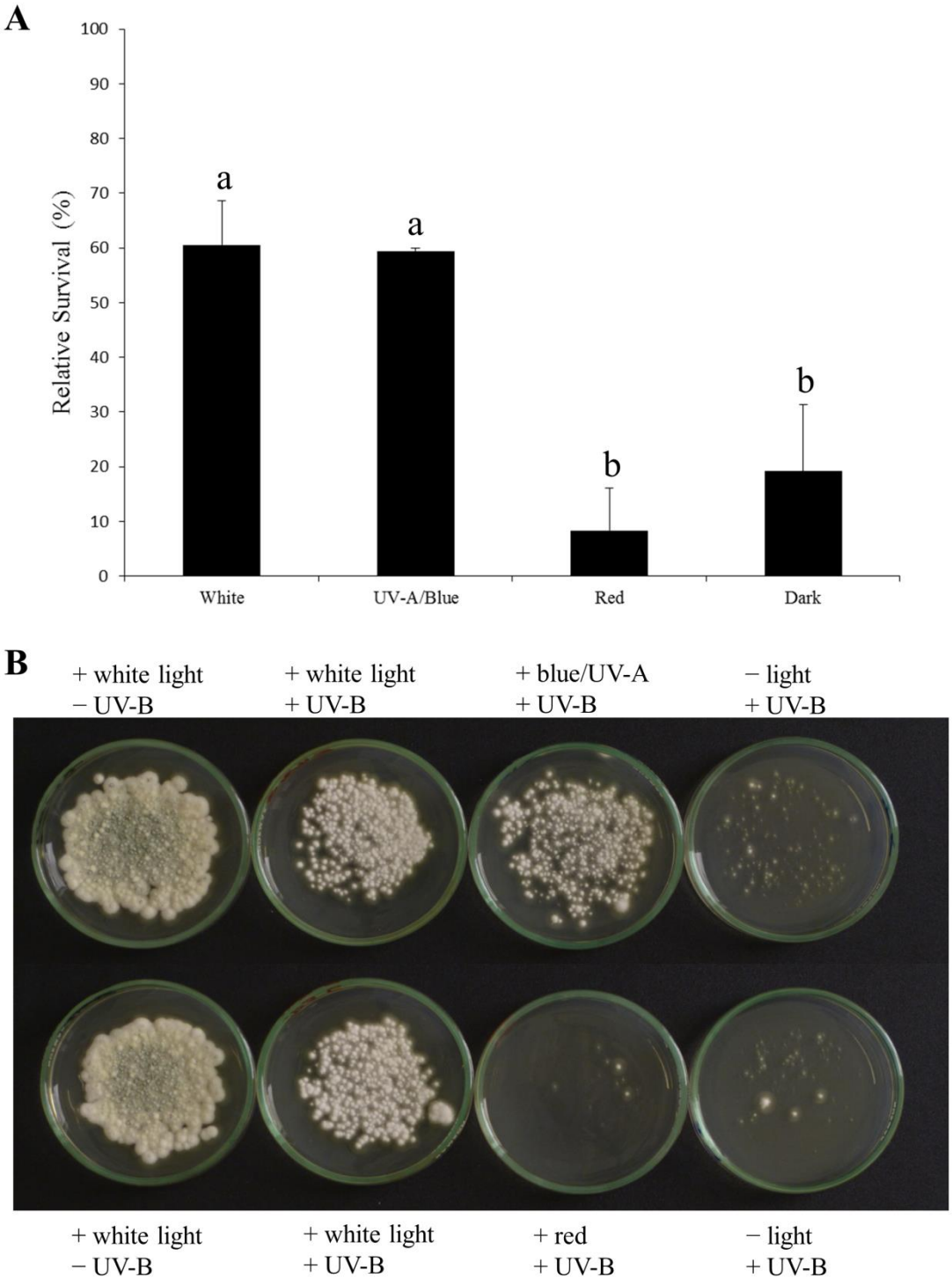

Fig. 1 – Light induction of UV-B tolerance by different spectral regions. (A) Effect of a 2-h induction with white, blue/UV-A or red light versus no induction (dark) on *M. acridum* relative survival to subsequent UV-B radiation. Colonies were counted 48 and 72 h after UV-B treatment. Error bars represent standard deviation from four independent experiments. Different lower-case letters indicate that means are statistically different ($P < 0.01$). (B) Effect of a 2-h induction with white, blue/UV-A or red light versus no induction (dark) on *M. acridum* colony development after UV-B radiation. Images were obtained 72 h after UV-B exposure

In order to characterize the duration of the exposure to light that was required for the increase in the survival under UV-B radiation we exposed colonies of *M. acridum* to white light for a varying amount of time, from 5 to 120 min (in the case of light exposure times below 120 minutes, induction was followed by dark incubation to totalize 120 minutes). We observed that the time of light illumination necessary to induce UV-B tolerance was as little as 5 minutes (Fig. 2).

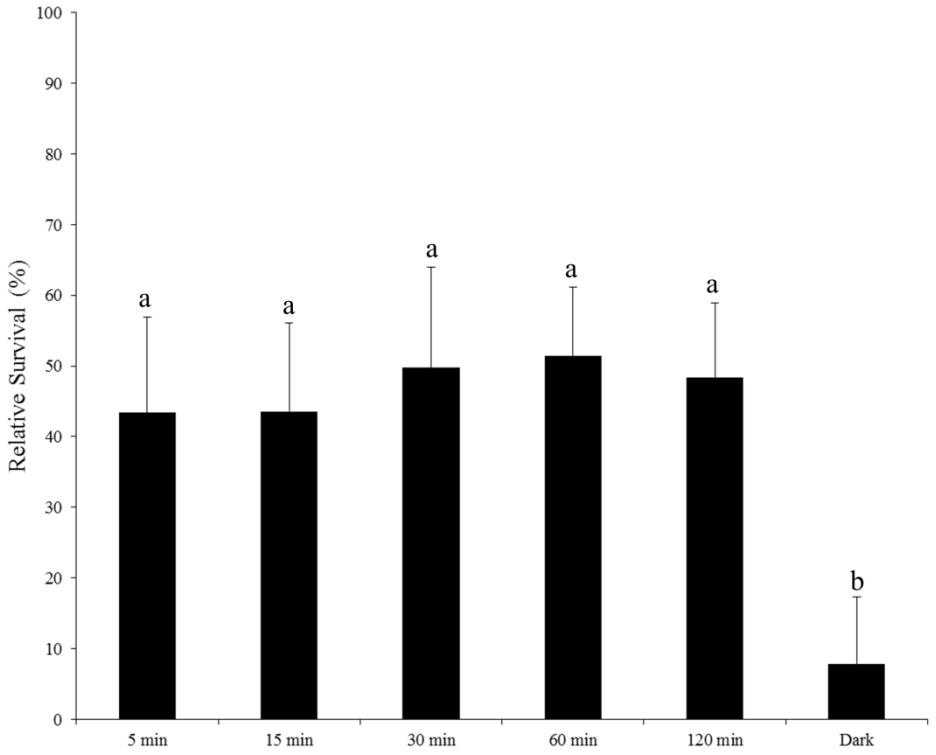

Fig. 2 – Effect of induction with white light for 0 (dark), 5, 15, 30, 60 or 120 minutes on *M. acridum* relative survival to subsequent UV-B radiation. After light induction and before UV-B exposure, colonies were incubated in the dark for the remaining time to complete 2 h (see Materials and Methods). Colonies were counted 48 and 72 h after UV-B treatment. Error bars represent standard deviation from three independent experiments. Different lower-case letters indicate that means are statistically different ($P < 0.01$)

The 5-min time point is not quantitatively different from the longest light induction of 120 min (Fig. 2). However, observing the plates revealed that *M. acridum* benefited from longer induction times in terms of growth delay (Fig. 3).

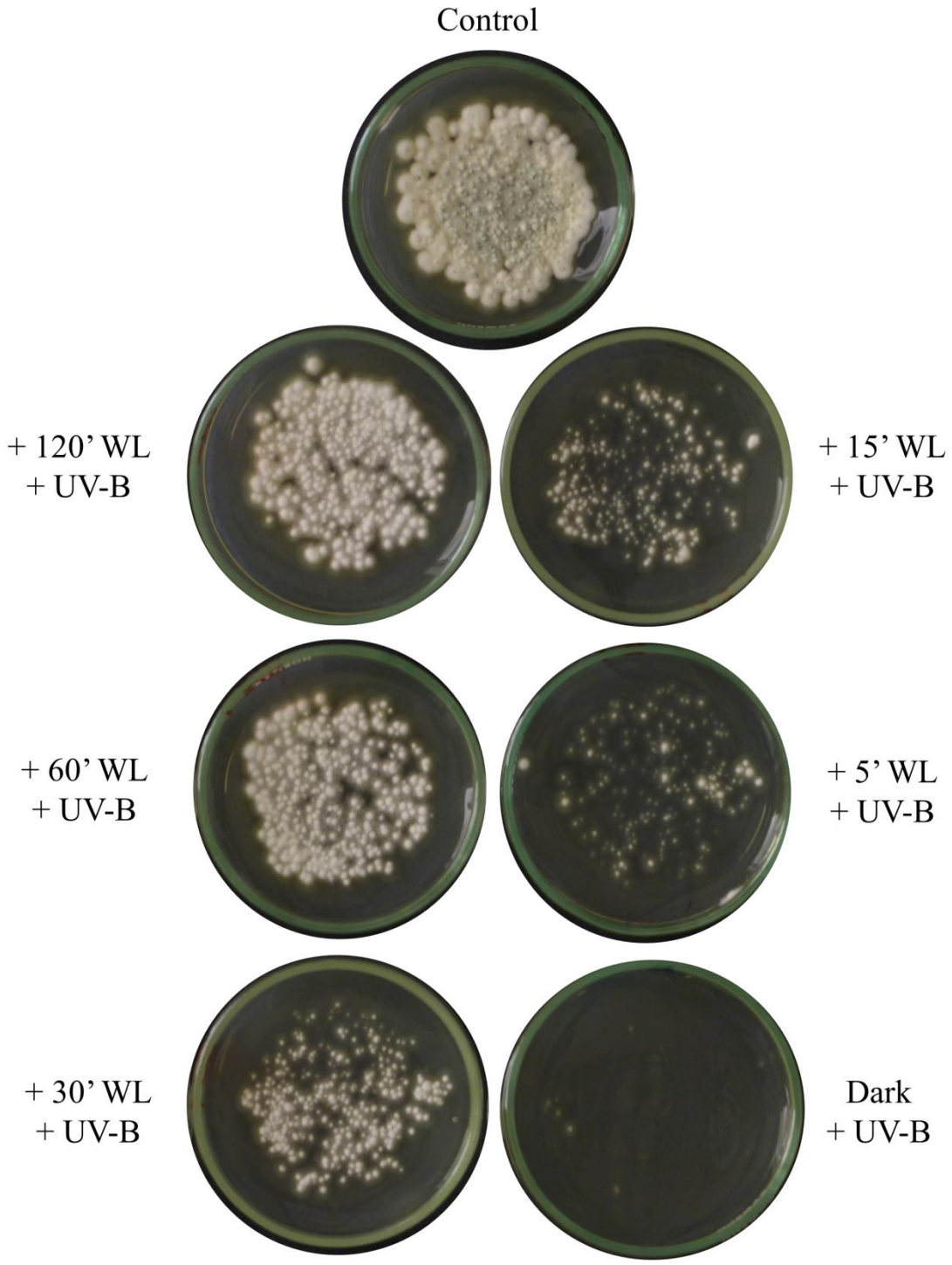

Fig. 3 – Effect of white-light (WL) induction for different illumination periods on *M. acridum* development after UV-B exposure. Twenty-six-hour old colonies were induced under white light for 0 (dark), 5, 15, 30, 60 or 120 minutes. After light induction and before UV-B exposure, colonies were incubated in the dark for the remaining time to complete 2 h (see Materials and Methods). Control group was light-induced for 120 minutes but was not exposed to UV-B. Images were obtained 72 h after UV-B exposure

Since a 5-minute exposure to white light was enough to induce UV-B tolerance, we performed a separate experiment in which we evaluated whether a 1-minute exposure could produce the same effect. Indeed, exposing colonies to white light for 1 minute followed by incubation in the dark for 119 minutes resulted in an increased tolerance to UV-B radiation and in a reduced growth delay after UV-B treatment (Supplementary Figure S3). This result is consistent with the fast light induction of a wide range of biological processes in *N. crassa* and *A. nidulans* (Crosthwaite; Loros; Dunlap, 1995; Chen et al., 2009; Ruger-Herreros et al., 2011).

Given that only few minutes of light exposure were enough to elicit UV tolerance we designed an experiment to evaluate the minimum time of dark incubation following light induction necessary to achieve the increased tolerance to UV-B. Colonies were light-induced for 5 minutes and were then incubated in the dark for 0, 10, 25, 55 or 115 minutes before UV-B exposure. Results are shown in Fig. 4. We observed that the colonies that received only 5 minutes of illumination and no incubation in the dark were already more tolerant to UV-B radiation when compared to colonies that were not induced by light (dark). However, these same colonies displayed reduced tolerance when compared to colonies that received at least some time of dark incubation before UV-B exposure (Fig. 4).

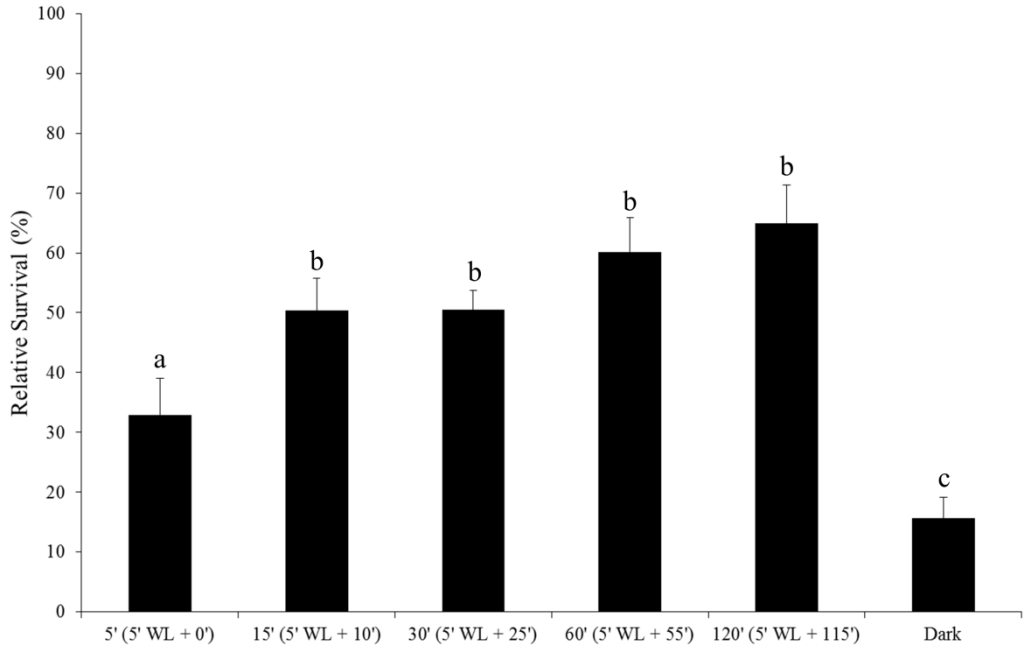

Fig. 4 – Effect of different dark-incubation periods after 5 min white light (WL) on the survival of *M. acridum* to UV-B exposure. Twenty-six-hour old colonies were light-induced for 5 minutes and then were dark-incubated for 0, 10, 25, 55 or 115 minutes before UV-B exposure. Dark treatment received no light induction before UV-B stress. Colonies were counted 48 and 72 h after UV-B treatment. Error bars represent standard deviation from three independent experiments. Different lower-case letters indicate that means are statistically different ($P < 0.05$)

Also, increasing the dark incubation time reduced the growth delay resulting from UV-B exposure (Fig. 5). These results are consistent with a model in which light regulates gene expression to protect *M. acridum* from UV-B damage. It is plausible that increasing the dark incubation time allows for better and more complete transcription and translation of light-regulated genes, thus rendering the fungus more tolerant to UV-B radiation.

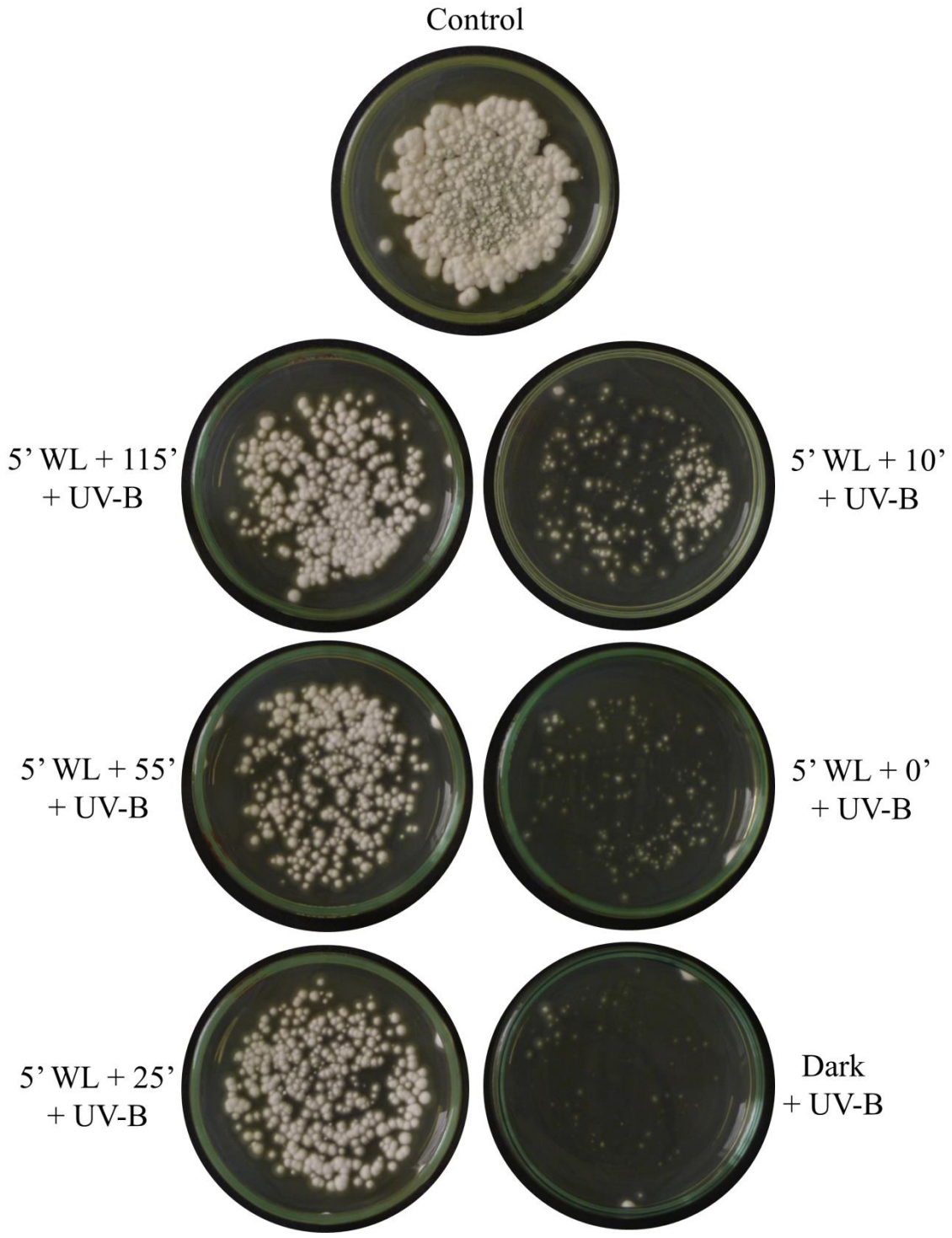

Fig. 5 – Effect of different dark-incubation periods after 5 min white light (WL) on *M. acridum* colony development after UV-B exposure. Twenty-six-hour old colonies were light-induced for 5 minutes and then were dark-incubated for 0, 10, 25, 55 or 115 minutes before UV-B exposure. Dark treatment received no light induction before UV-B radiation and control plates were light-induced for 5 minutes but were not exposed to UV-B. Images were obtained 72 h after UV-B exposure

We also investigated whether it was possible to induce tolerance to UV-B radiation in younger colonies and germlings. For this experiment, colonies were allowed to grow for 12, 18 and 26 h (control) before being induced for 2 h under white light. Colonies were then exposed to UV-B. The results showed that younger 12 and 18-h-old colonies responded to light and increased their tolerance to UV-B to the same extent as the 26-h-old colonies (Fig. 6). Surprisingly, colony age was unrelated to UV-B tolerance. It would be expected that older colonies, for having a larger number of nuclei, should be more resistant to UV-B inactivation. However, 12-, 18- and 26-h-old colonies all had the same survival under UV-B radiation (Fig. 6).

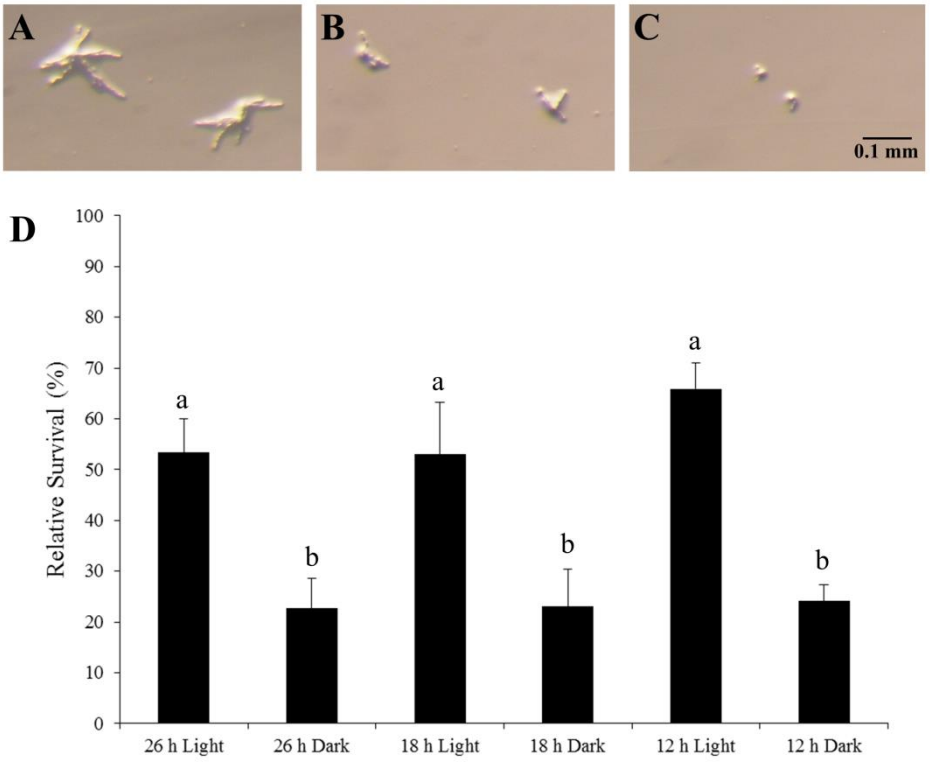

Fig. 6 – Effect of *M. acridum* colony age on light induction of UV-B tolerance. (A) Colonies were allowed to develop for 12, 18 or 26 h before being light-induced for 2 h and exposed to UV-B radiation. (B) Relative survival after UV-B exposure for colonies aged 12, 18 or 26 h. Dark treatment received no light induction before UV-B stress. Error bars represent standard deviation from three independent experiments. Different lower-case letters indicate that means are statistically different ($P < 0.01$)

Although we cannot explain this result at the present moment, we gathered some information as to how nuclei number can affect UV inactivation. In *N. crassa*, Norman (1951) has shown that uninucleated microconidia and multinucleated macroconidia averaging 2.3 nuclei per conidium diverge in their susceptibility to UV with microconidia being less tolerant to radiation (Norman, 1951). The author has also shown that the inactivation kinetics per nucleus is different when the nuclei of micro and macroconidia are compared. Overall, the sole nucleus in microconidia is twice as much susceptible to UV radiation as any nuclei in macroconidia. The author attributes this effect to nuclei interaction: an organism containing a single nucleus is more likely to be UV-inactivated because mutations leading to recessive lethals are necessarily expressed. Conversely, the presence of more than one nucleus compensates this effect because a mutation otherwise leading to a recessive lethal is complemented by another nucleus or other nuclei. Later, Norman (1954) compared the UV inactivation kinetics for uninucleated microconidia and for macroconidia averaging 2.3, 4.2 and 5.9 nuclei per conidium (Norman, 1954). The author concluded that nuclei interaction is already present when the number of nuclei is two. Increasing the nuclei number beyond this point does not improve the interaction of the nuclei. Nonetheless, increasing the average number of nuclei from 2.3 to 4.2 and from that to 5.9 did result in higher survival under UV radiation. However, the inactivation difference between 4.2 and 5.9 nuclei per conidium is smaller than the difference between 2.3 and 4.2. In our experiments, even the youngest colony (12-h old) has a number of nuclei that far exceeds that of the macroconidia contemplated in the works of Norman. Also, the traffic of nuclei and other organelles throughout hyphae must be considered. Therefore, despite the correlation between UV survival and number of nuclei, we are unsure as to whether increasing an already great nuclei number in colonies could produce an appreciable

difference in UV tolerance and also whether this difference could be detected by our experiment in which colonies were exposed to a single UV-B dose. A possibility to investigate this phenomenon is a high UV dose reaching close to 100% mortality of the youngest colonies. In this regimen, the survival difference between younger and older colonies, if existing, would have a better chance of being observed.

Taken together, our results indicate that light could regulate the expression of repair genes in order to protect the fungus from DNA damage caused by UV-B radiation. A rapid blue light induction of DNA repair enzymes has previously been shown in *Trichoderma harzianum* and *Aspergillus fumigatus* (Berrocal-Tito et al., 1999; Berrocal-Tito et al., 2000; Fuller et al., 2013). In *T. harzianum*, blue light induces the accumulation of photolyase mRNA in conidiophores, yielding conidia with increased photoreactivation capacity (Berrocal-Tito et al., 1999; Berrocal-Tito et al., 2000). However, the authors observed that, in the absence of photoreactivating light (as is the case with our experiments) there was no increase in intrinsic survival to UV exposure (Berrocal-Tito et al., 2000). Even if these results seem obvious, one should remember that photolyases were previously implicated in "dark repair" (Yamamoto; Fujiwara; Shinagawa, 1983; Yamamoto et al., 1983; Sancar and Smith, 1989) and this effect was recently reported in *Trichoderma reesei* (Guzmán-Moreno et al., 2014). Verma and Idnurm (2013) observed that light is responsible for inducing the transcription of the endonuclease *uve1* gene in *Cryptococcus neoformans* and that this induction protected the fungus from UV damage (Verma and Idnurm, 2013). The authors further observed that homologous genes in *N. crassa* and *Phycomyces blakesleeanus* are also light-regulated, this regulation being dependent on the White Collar Complex. In *A. fumigatus*, the effects of blue and red light in inducing UV tolerance are very similar to those observed in our experiments (Fuller et al., 2013). The authors also reported that

light induces the expression of genes for a photolyase and a UV endonuclease. Deleting the gene for the blue photoreceptor resulted in loss of photolyase induction by light. However, even a double mutant for the blue and red photoreceptors still displayed light induction of UV tolerance (Fuller et al., 2013). The authors state that other photoreceptors, possibly cryptochromes, could be involved in the tolerance induction process. The Cryptochrome Database (http://www.dbcryptochrome.org) indicates that the *M. acridum* genome bears – aside from the classical blue- and red-light photoreceptors – two genes in the photolyase/cryptochrome family: a Class I photolyase and a Cry-DASH, both of which could play major roles in light signalling and UV tolerance (Veluchamy and Rollins, 2008; Guzmán-Moreno et al., 2014). Recently it was shown that the *P. blakesleeanus* cry-DASH is capable of repairing UV-B-induced cyclobutane pyrimidine dimers lesions in double-strand DNA (Tagua et al., 2015). However, it should be borne in mind that *P. blakesleeanus* is a photolyase-lacking fungus while the *M. acridum* genome bears a photolyase gene.

Here we have shown that a rapid light exposure induces UV-B tolerance in *M. acridum* and that blue/UV-A light alone is capable of producing the same effect. Further experiments should elucidate whether this response is dependent on the classical blue photoreceptor, the cyptochromes, or a combination of both. Furthermore, the results open the prospect of using short light exposures to obtain a more UV-B resistant mycelium in mycelium-based formulations (Pereira and Roberts, 1990; Booth; Tanigoshi; Dewes, 2000). This could be a valid alternative to the continuous or photoperiod-like illumination commonly used to produce conidia with increased stress tolerance.

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Chapter 2

Exposing *Metarhizium acridum* mycelium to visible light up-regulates a photolyase gene and increases photoreactivating ability

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ABSTRACT

Metarhizium acridum is an entomopathogen currently used against acridids. We have previously reported that exposing mycelium to visible light increases *M. acridum* tolerance to ultraviolet-B (UV-B) radiation. Here we evaluated if light could also increase tolerance to ultraviolet-C (UV-C) radiation. We observed that, as opposed to UV-B radiation, light did not increase tolerance to UV-C radiation under dark repair conditions. However, light did increase tolerance to UV-C radiation if photoreactivating light was present after UV-C exposure. Quantitative PCR experiments revealed that light up-regulates a photolyase gene. This is the first report showing that light regulates photoreactivating ability in *M. acridum*.

Keywords: photoreactivation; ultraviolet; *Metarhizium*; light; tolerance; entomopathogen

Short Communication

Metarhizium acridum is an important entomopathogenic fungus currently used for the biological control of acridids (Lacey et al., 2015). Success of this alternative approach is a direct consequence of fungal survival under harsh field conditions. Among these, we can cite exposure to solar ultraviolet radiation as one determining factor (Braga et al., 2001; Braga et al., 2015). Therefore, methods increasing *M. acridum* tolerance to ultraviolet radiation are highly sought-after (Rangel et al., 2015).

Repair of UV-damaged DNA in fungi is mostly carried out by three different pathways: 1) nucleotide excision repair (Goldman; McGuire; Harris, 2002), 2) UV-endonuclease repair (Yajima et al., 1995), and 3) photoreactivation (Sancar, 1990; Essen, 2006). The first two are processes that can occur in the absence of light and are

sometimes collectively referred to as “dark repair” processes. Photoreactivation, however, is only functional in the presence of photoreactivating light (320-500 nm) (Sancar, 1990). Photoreactivation is carried out by enzymes called photolyases, which use light energy to lyse the UV-induced bond between adjacent pyrimidine bases in DNA (Sancar, 1990; Essen, 2006).

Many biological processes in fungi are regulated by light (Fischer et al., 2016). In *Metarhizium robertsii*, visible light during growth has been shown to induce the production of conidia with increased tolerance to ultraviolet-B (UV-B, 280-315 nm) radiation (Rangel et al., 2011). Also, we have previously reported that pre-illuminating 24-h-old mycelium of *M. acridum* to visible light induces its tolerance to UV-B radiation (Brancini; Rangel; Braga, 2016). However, UV-B lamps commonly used in such studies emit both UV-B and residual ultraviolet-A (UV-A, 315-400 nm) radiation (De Menezes et al., 2015). While both UV-A and UV-B have been shown to produce DNA damage, UV-A also has a role in photoreactivation (Sancar, 1990). One way to partly overcome this photoreactivation issue during exposure to UV-B lamps is to use ultraviolet-C (UV-C, 100-280 nm) radiation instead of UV-B. UV-C lamps emit at 254 nm and this radiation is strongly absorbed by DNA, thus making exposure times very short and reducing the possible interference of photoreactivating wavelengths.

Here we evaluated if pre-illumination of 24-h-old mycelium with visible light can induce its tolerance to UV-C radiation in the same way observed for UV-B radiation. By allowing mycelium to repair UV-C damage both in the dark and under photoreactivating light, we also investigated whether pre-illumination regulates photoreactivating ability in *M. acridum*.

Conidia of *M. acridum* ARSEF 324 (USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA) were obtained by growing the

fungus on Potato Dextrose Agar (Difco) supplemented with 0.5% yeast extract (Difco) (PDAY) in the dark at 28 °C for 12 days. Conidia were scrapped from plates and used to prepare suspensions at 10^6, 10^5, and 10^4 conidia ml^{-1} in 0.01% Tween 80® (Sigma). Five microliters of these suspensions were used to inoculate PDAY plates. Then, these plates were incubated in the dark at 28 °C for 24 h. The light induction experiment plates were then placed under white light from fluorescent lamps (Osram, Germany) (5.3 W m^{-2}) for 1 h. Dark control plates were kept in the dark. Finally, both light and dark plates were exposed to UV-C radiation from a laminar flow cabinet lamp (irradiance at 254 nm = 22.5 mW m^{-2} nm^{-1}) for 2 min. After exposure, plates were incubated in the dark and photographed after 72 h to evaluate growth.

To evaluate the effects of pre-illumination on photoreactivating capacity, we prepared both light and dark treatments exactly as described above and exposed them to UV-C radiation for 2 min. However, instead of incubating the plates in the dark afterwards, both light and dark treatments were placed under white light (5.3 W m^{-2}) for 2 h. After this time, plates were moved to the dark and observed at 48 and 72 h. In a different experiment, photoreactivation under white light was replaced by photoreactivation under radiation from UV-B lamps filtered by a Mylar film (JSC Industries). This film is capable of blocking UV-B radiation from the lamps, allowing only residual UV-A radiation to reach the plates (Rangel; Anderson; Roberts, 2008). Three independent experiments were performed.

We also evaluated if visible light could regulate the expression of a photolyase gene in *M. acridum*. To do this, conidia obtained from PDAY plates were used to prepare a suspension at 2.5 × 10^7 conidia ml^{-1} in 0.01% (v/v) Tween® 80. Four milliliters of this suspension were used to inoculate 100 ml Sabouraud Dextrose Broth (Difco) in Erlenmeyer flasks. The culture was incubated in a shaker at 125 rpm, 28 °C

under complete darkness for 24 h. Then, light-treatment flasks were placed under white fluorescent lamps at an irradiance of 5.3 W m^{-2} for 60 or 120 min. Mycelia from the cultures were filtered using filter paper, washed with distilled water and immediately frozen in liquid nitrogen. A parallel dark control was also prepared. Frozen mycelia, stored at –70 °C, were disrupted by grinding in liquid nitrogen until a fine powder was obtained. Fifty milligrams of grounded powder, still frozen, were transferred to 450 µl RLT buffer from the RNeasy Plant Mini Kit (Qiagen) and were homogenized immediately by vortexing. RNA purification was performed according to manufacturer's instructions. Quantification was performed with NanoVue (GE Healthcare). Ten micrograms total RNA was used for DNase treatment with RQ1 RNase-free DNase (Promega) following manufacturer's instructions in a reaction volume of 10 µl. After treatment, samples were purified with phenol:chloroform and ressuspended in 10.5 µl nuclease-free water. These samples were then used for cDNA synthesis by ImProm™ Reverse Transcriptase (Promega) according to manufacturer's instructions. After cDNA synthesis, the final reaction volume (25 µl) was diluted by adding 21 µl nuclease-free water.

Quantitative RT-PCR (qRT-PCR) was used to assess the expression of a photolyase gene (*phr*, MAC_05491) after visible light exposure. Gene expression was quantified through a TaqMan® Universal PCR Master Mix Kit assay using the following probes: [*phr*, F: 5'-GGCGAGGAAGCAGTTTAAAGAC-3', R: 5'-CTTTGTAAGCTGCTTGTTTGATGGT-3', Reporter: 5'-AACAGCGCATTCAAAC-3'], [*gapdh*, F: 5'-GCTTCCTGCACCACCAACT-3', R: 5'-AGACCCTCAACAATGGTGAACTTG-3', Reporter: 5'-CTCGCCAAGGTCATCC-3']. Reaction mix consisted of 1 µl cDNA, 5 µl Master Mix, 0.5 µl TaqMan® probe, and 3.5 µl nuclease-free water for a total of 10 µl. Reactions were carried out on an Applied

Biosystems 7500 Fast Real-Time PCR Systems (Applied Biosystems). Thermal cycling conditions were as follows: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The number of copies for each gene was calculated by a standard curve constructed with genomic DNA (i. e. C_T values versus $\log_{10}$ gene copy number). The measured quantity of *phr* mRNA was normalized using the values obtained for the glyceraldehyde 3-phosphate dehydrogenase gene (*gapdh*), an adequate reference gene in *Metarhizium* (Fang and Bidochka, 2006), run in the same plate. Four independent experiments were performed. Statistical analyses were performed with GraphPad Prism 5.01 software (GraphPad Software).

Unlike previously observed for UV-B radiation (Brancini; Rangel; Braga, 2016), pre-illumination with visible light did not increase *M. acridum* tolerance to UV-C radiation under dark repair conditions (Fig. 1) even if light did up-regulate a UV-endonuclease gene (Fig. S1). The explanation for this could be the use of rich media in our experiments. A previous work has shown that entomopathogenic fungi need a period of liquid holding in poor medium in order to efficiently repair UV-damaged DNA (Chelico; Haughian; Khachatourians, 2006). Since pre-illumination increases photoreactivating ability in *Trichoderma* (Berrocal-Tito et al., 2000; Berrocal-Tito et al., 2007), we analyzed if this was also the case for *M. acridum*. Indeed, a one-hour pre-illumination with visible light did increase survival if photoreactivating light was provided after UV-C exposure (Fig. 1). This result led us to suppose that residual UV-A radiation emitted by UV-B lamps could also be used for photoreactivation. To test this, we exposed 24-h-old mycelium to UV-C radiation and then provided it with Mylar-filtered radiation from UV-B lamps as photoreactivating light. Under these conditions, residual UV-A light from UV-B lamps could be used by the fungus to repair UV-damaged DNA (Fig. 1).

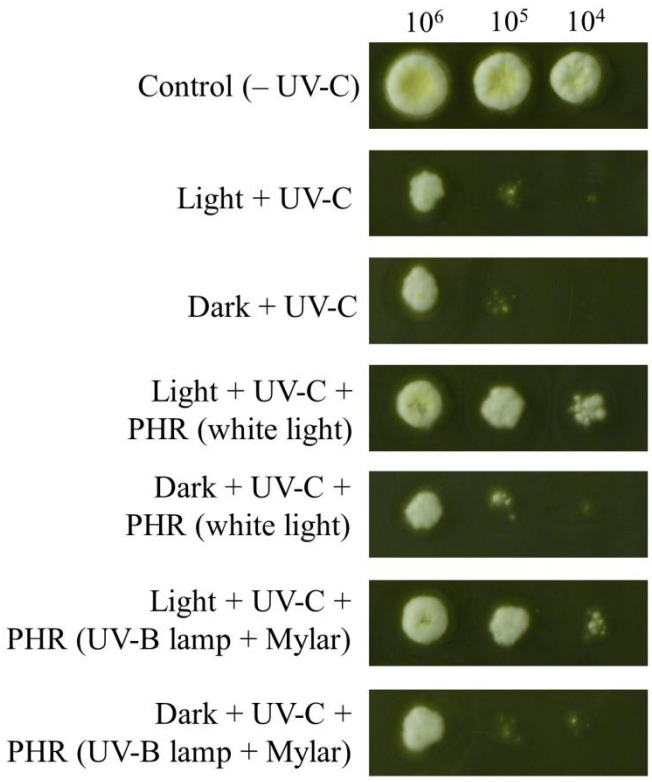

Fig. 1 – Effects of pre-illumination on UV-C tolerance and photoreactivating ability in *M. acridum*. Twenty-four-hour-old colonies were exposed to light for 60 min (Light) or kept in the dark (Dark) before being exposed to UV-C radiation for 2 min. Then, both Light and Dark treatments were exposed to either white light [PHR (white light)] or to Mylar-filtered UV-B lamps [PHR (UV-B lamp + Mylar)] for 2 h. Note that Light treatment uses either white light or residual UV-A radiation from the UV-B lamps to photoreactivate DNA lesions, leading to an increase in survival. Images were obtained 72 h after UV-C exposure and are representative of three independent experiments

Since the increase in photoreactivating ability was caused by pre-illumination with visible light, we investigated if light could regulate a photolyase gene in *M. acridum*. After 60 and 120 min of visible light exposure, *phr* was about 10-fold up-regulated relative to dark. Light regulation of a photolyase gene has been previously shown in *Trichoderma* (Berrocal-Tito et al., 2000; Berrocal-Tito et al., 2007), *Aspergillus fumigatus* (Fuller et al., 2013), *Aspergillus nidulans* (Ruger-Herreros et al., 2011), *Neurospora crassa* (Chen et al., 2009), and *Ustilago maydis* (Brych et al., 2016). In many of these organisms, light directly drives photolyase gene expression via activation of blue light photoreceptors, which are homologue to the White Collar-1

protein first described in *N. crassa* (Ballario et al., 1996). We speculate that the same mechanism is responsible for controlling photolyase gene expression in *M. acridum*.

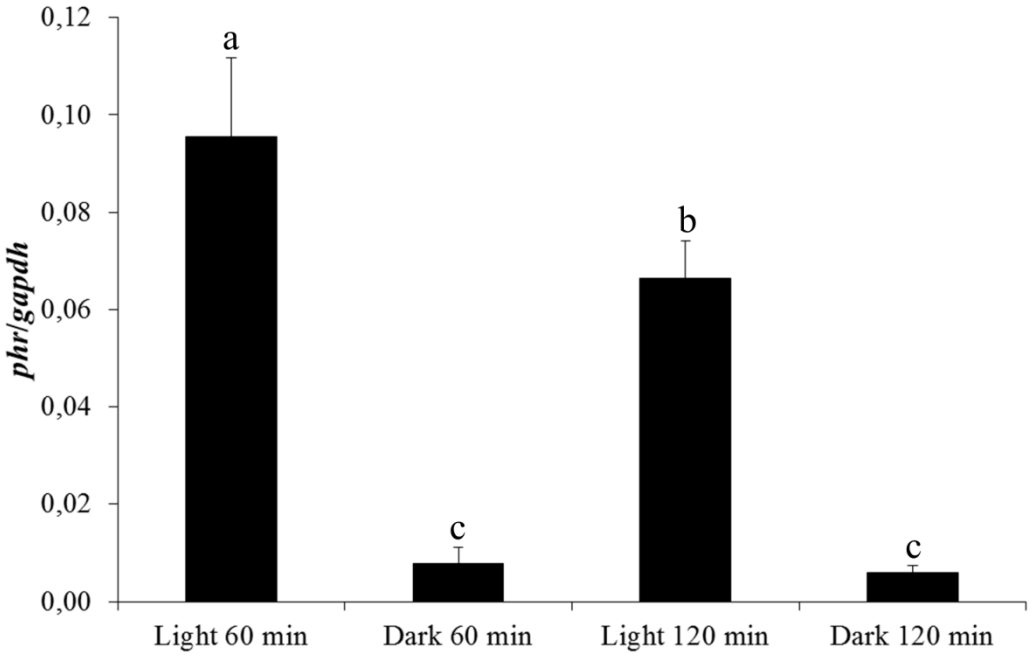

Fig. 2 – Light regulation of a photolyase gene (*phr*) in *M. acridum*. Quantitative PCR experiments were carried out after white light induction for 60 or 120 min. Dark controls for each time point were also analysed. Light strongly up-regulates *phr*. Four independent experiments were performed. Different lowercase letters indicate that means are statistically different (One-way ANOVA, Tukey's post-test, $P < 0.01$)

It was previously shown that overexpressing a photolyase gene in *M. robertsii* leads to increased tolerance to UV-B and solar radiation and that these modified strains could be more suitable for biological control (Fang and St Leger, 2012). The authors also showed that photolyase null-mutants displayed reduced UV tolerance and could thus be useful for biocontrol in areas where direct sunlight exposure is not an issue (Fang and St Leger, 2012). However, the authors did not evaluate the effects of pre-illumination on UV tolerance or photolyase gene expression in wild type strains.

Here we show that light up-regulates the expression of a photolyase gene and increases *M. acridum* ability to photoreactivate. This increased photoreactivating ability allows the fungus to repair UV-damaged DNA using either visible light or residual UV-

A radiation emitted by UV-B lamps. Since this residual UV-A radiation was found to be enough for photoreactivation, researchers employing UV-B lamps to assess tolerance should be aware that photoreactivation during the experiment is a possibility and should be taken into account. In this regard, the light-induced tolerance to UV-B radiation observed in our previous work (Brancini; Rangel; Braga, 2016) could be a consequence of increased photoreactivating ability that then allowed the residual UV-A radiation emitted by the UV-B lamps to serve as photoreactivating light. To our knowledge, this is the first work showing that visible light both up-regulates a photolyase gene in *M. acridum* and increases photoreactivating ability, which are crucial phenomena for survival given the abundance of photoreactivating light in nature.

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Chapter 3

Combining transcriptomics and proteomics reveals potential post-transcriptional control of gene expression after light exposure in *Metarhizium acridum*

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Running title: Light-regulated gene expression in *Metarhizium*

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ABSTRACT

Light is an important stimulus for fungi as it regulates many diverse and important biological processes. *Metarhizium acridum* is an entomopathogenic fungus currently used for the biological control of insect pests. The success of this approach is heavily dependent on tolerance to environmental stresses. It was previously reported that light exposure increases tolerance to ultraviolet radiation in *M. acridum*. There is no information in the literature about how light globally influences gene expression in this fungus. We employed a combination of mRNA-Sequencing and high-throughput proteomics to study how light regulates gene expression both transcriptionally and post-transcriptionally. Mycelium was exposed to light for 5 min and changes at the mRNA and protein levels were followed in time-course experiments for two and four hours, respectively. After light exposure, changes in mRNA abundance were observed for as much as 1128 genes or 11.3% of the genome. However, only 57 proteins changed in abundance and at least 347 significant changes at the mRNA level were not translated to the protein level. We observed that light downregulated subunits of the eukaryotic translation initiation factor 3, the eIF5A-activating enzyme deoxyhypusine hydroxylase, and ribosomal proteins. We hypothesize that light is perceived as a stress by the cell that responds to it by reducing translational activity. Overall, our results indicate that light acts both as a signal and a stressor to *M. acridum* and highlight the importance of measuring protein levels in order to fully understand light responses in fungi.

Introduction

Metarhizium acridum (Ascomycota: Sordariomycetes) is a soil-inhabiting entomopathogenic fungus currently used for the biological control of Orthoptera insects, mostly locusts and grasshoppers (Lacey et al., 2015). The success of biological control

is heavily dependent on survival under harsh environmental conditions. Among these, heat and ultraviolet-B radiation (UV-B, 280-315 nm) are among the most stressful. The effects of UV-B radiation range from delayed conidia germination to complete inactivation (Braga et al., 2001; Braga et al., 2015). In this scenario, methods increasing *M. acridum* tolerance to UV-B radiation are highly sought after. Previous studies have shown that many physical and chemical factors can modulate stress tolerance in *Metarhizium* and other fungi (Rangel et al., 2011; Rangel et al., 2015; Dias et al., 2019). One of such factors is exposure to visible light.

Light is an important stimulus that regulates many biological processes in fungi. Depending upon the organism, light can regulate processes as diverse as development, secondary metabolite production, entrainment to circadian oscillators, and phototropism (Yu and Fischer, 2019). Importantly, light responses are normally fast and transient with hierarchical signaling (Chen et al., 2009). Fungi respond to light by using photoreceptors capable of sensing mostly blue (phototropins), green (opsins), and red (phytochromes) light, although distinct fungi will differ in their ability to sense each of these wavelengths (Yu and Fischer, 2019). In *Metarhizium robertsii*, growth under white light results in the production of conidia that germinate faster and are more virulent when compared to conidia produced in the dark (Oliveira; Braga; Rangel, 2018). Also, using blue light during growth resulted in increased conidia yield (Oliveira; Braga; Rangel, 2018). Regarding stress tolerance, we have previously reported that exposing *M. acridum* mycelium to white or blue light leads to increased tolerance to UV-B radiation (Brancini; Rangel; Braga, 2016). We have also shown that light induces the expression of a photolyase gene and we and others have reported that photoreactivation is probably involved in UV-B radiation tolerance (Fang and St Leger,

2012; Brancini et al., 2018). Nevertheless, we have no information about how light regulates gene expression genome-wide.

Genome-wide regulation after light exposure was evaluated in the ascomycete model *Neurospora crassa* and light was found to modulate the expression of as much as 24% of all predicted genes (Wu et al., 2014). However, the authors did not measure protein levels and therefore the number of changes at the mRNA level that are effectively translated to the protein level is still unknown. In this regard, a recent study focused on combining mRNA-Seq and high-throughput proteomics to study clock-controlled genes in *N. crassa* (Hurley et al., 2018). The authors observed that circadian output is highly influenced by post-transcriptional regulation, especially translational control, thus emphasizing the need to measure protein levels. Here we combined mRNA-Seq and Tandem Mass Tag (TMT)-based high-throughput proteomics to study how light regulates gene expression both transcriptionally and post-transcriptionally in *M. acridum*.

Materials and Methods

Strains and growth conditions

Metarhizium acridum ARSEF 324 was obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA). The culture was maintained in Potato Dextrose Agar (Difco) supplemented with 0.5% yeast extract (Difco). Conidia were obtained by growing at 28 °C in complete darkness for 12 days.

Light exposure

Conidia were scraped from plates and used to prepare a suspension at 2.5×10^7 cells ml^{-1} in Tween 80® 0.05% (Sigma). Four milliliters of this suspension were used to

inoculate 100 ml of Potato Dextrose Broth (Difco) in 250 ml Erlenmeyer flasks. For each experiment, a total of six cultures were prepared. These cultures were grown in complete darkness at 28 °C under agitation (125 rpm) for 24 h to produce mycelium. Then, five of the six culture flasks were exposed to white light from fluorescent lamps (irradiance = 5.3 W m^{-2}; photon flux = 24.7 µmol m^{-2} s) for 5 min. Flasks were moved back to dark for different lengths of time depending on experiment type. For transcriptomics, dark incubations after light exposure were for 0 (5L 0D), 10 (5L 10D), 25 (5L 25D), 55 (5L 55D), and 115 (5L 115D) min. For proteomics, these incubations were for 10 (5L 10D), 25 (5L 25D), 55 (5L 55D), 115 (5L 115D), and 235 (5L 235D) min. In both cases a control was always kept in the dark (DD). After the incubation was over, mycelium was vacuum filtered, washed with distilled water, and immediately frozen in liquid nitrogen. Frozen mycelia were stored at −70 °C until RNA or protein extraction. Three independent experiments were performed for mRNA-Seq and three independents experiments for high-throughput proteomics.

Effects of light on the transcriptome

Frozen mycelia were ground with mortar and pestle under liquid nitrogen to obtain a fine powder. Approximately 50 mg of frozen powder were added to 450 µl RLT buffer from the RNeasy Plant Mini Kit (Qiagen). Purification was performed following manufacturer's instructions and total RNA was eluted with nuclease-free water. Quality assessment was performed on an Agilent Bioanalyzer 2100 and all samples presented with RNA Integrity Number ≥ 7. Libraries were constructed with the TruSeq Stranded mRNA v4 (Illumina) following manufacturer's instructions. Library quantification was performed via quantitative PCR and sequencing was run on HiSeq 2500 equipment. Three independent experiments were performed separately and

sequenced together in the same lane. Because each experiment consisted of six samples, a total of 18 samples were sequenced yielding approximately 20 million reads per sample.

Sequencing data were aligned to *M. acridum* genome (Gao et al., 2011) with Hisat2 (Kim; Langmead; Salzberg, 2015). The alignments were then analyzed with Cufflinks (Trapnell et al., 2010) using the -G option (no Reference Annotation Based Transcript assembly). Differential expression and statistical testing were performed with Cuffdiff 2 (Trapnell et al., 2013). Finally, Cuffdiff output was analyzed with cummeRbund (Trapnell et al., 2012). Differences between light treatments and DD were considered significant if they could satisfy $P < 0.01$ and a 2-fold cutoff. Gene clustering by expression pattern was performed with *clust* (Abu-Jamous and Kelly, 2018), heat maps were built with TM4 MeV (Saeed et al., 2003), and principal component analysis was achieved with ClustVis (Metsalu and Vilo, 2015). Gene ontology analyses were performed on the Blast2GO suite (Gotz et al., 2008).

Validation of mRNA-Seq data was performed for photolyase (MAC_05491) and UV-endonuclease (MAC_07337) coding genes with quantitative reverse transcription PCR (qRT-PCR). Total RNA extraction was performed exactly as described for mRNA-Seq and the downstream protocol for cDNA synthesis and gene quantification was as previously described (Brancini et al., 2018).

Effects of light on the proteome

Frozen mycelia were ground with mortar and pestle under liquid nitrogen to obtain a fine powder. Approximately 50 mg of frozen powder were added to 500 µl of extraction buffer [7M urea, 2M thiourea, 4% CHAPS (Sigma)] and the mixture was vortexed for 2 min. Samples were then centrifuged at 10,000 × *g* and 4 °C for 5 min.

The supernatant was collected and total protein was quantified with the 2-D Quant Kit (GE Healthcare). Protein purification was performed with a methanol/chloroform protocol as previously described (Wessel and Flugge, 1984).

Proteins were reduced with dithiothreitol, alkylated with iodoacetamide, and finally digested with trypsin. Resulting peptides were labelled with TMT 10-plex (Thermo Scientific) with one tag for each condition according to manufacturer's instructions. After isobaric tagging, the six conditions in each experiment were pooled and fractionated by reverse phase chromatography (C_{18}, 1 × 100 mm, 3.5 µm, 130 Å, Waters). Elution was performed at 0.1 ml/min using a gradient of A (20 mM pH 10 ammonium formate) and B (acetonitrile) from 1 to 37.5% over 61 min. A total of 12 fractions were collected. These were dried in a vacuum centrifuge and solubilized in 0.1% formic acid.

Tandem mass spectrometry (MS/MS) analyses were performed as previously described (Becher et al., 2018). Briefly, peptides from each of the 12 fractions were analyzed on a nanoLC (UltiMate 300 RSLC, Thermo) equipped with a C_{18} pre-column (Precolumn C_{18} PepMap 100, 300 µm × 5 mm, 5 µm , 100 Å) and an analytical column (Acclaim C_{18} PepMap 100, 75 mm × 50 cm, 3 mm, 100 Å). The nanoLC equipment was coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo). Elution was always performed with solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). Peptides were loaded into the column at 30 µl/min solvent A for 3 min. Peptides were eluted from the column with an elution gradient adjusted to 0.3 ml/min over 120 min. The concentration of B in the gradient was ramped to 4% over 4 min, to 8% in 2 min, to 26% over 96 min, and to 40% over 10 min. Eluted peptides were analyzed in positive mode and data-dependent method. Full scan spectra

were obtained in the 375-12,000 m/z range. The top ten precursors in MS were selected for MS/MS.

Raw spectra were processed with IsobarQuant (Franken et al., 2015) and protein identification was performed with MASCOT (Matrix Science). Identification was based on the *M. acridum* genome (Gao et al., 2011). MASCOT search parameters were as follows: enzyme trypsin; up to three missed cleavages; peptide tolerance 10 ppm; MS/MS tolerance 0.02 Da; carbamidomethyl (Cys) and TMT10plex (Lys) as fixed modifications; TMT10plex on N-terminus, oxidation (Met), and N-acetylation as variable modifications. Batch effects were removed using limma (Ritchie et al., 2015) and results were normalized via the vsn strategy of variance normalization (Huber et al., 2002). Quantitative information was only analyzed when a given protein was found in two or three experiments. If the protein was identified in two experiments, missing data for the third experiment were imputed with the k-nearest neighbor algorithm. Changes at the protein level were considered significant if they could satisfy a 2-fold cutoff relative to DD at False Discovery Rate < 0.05. Combined mRNA/protein graphs were plotted with Origin 8.0 software (OriginLab Corporation).

Data availability statement

All supplementary material files have been uploaded to fig**share**. https://doi.org/10.25387/g3.8115998.

Results

Effects of light on the transcriptome

To evaluate light-regulated gene expression, we performed mRNA-Seq of RNA extracted from mycelia exposed to light for 5 min followed by incubation in the dark for

different lengths of time (0, 10, 25, 55, and 115 min). A control was kept in complete darkness (DD). Our analysis encompassed 9514 genes corresponding to 95.4% of the genome (Table S1). A gene was considered light-regulated if significant mRNA change was observed in at least one time point relative to DD. Light regulated the expression of 4819 genes at $P < 0.01$. Because many genes were only weakly regulated, we applied a 2-fold cutoff and observed that 1128 transcripts changed in abundance under these criteria (Table S2). Of these, 719 (64%) were upregulated and 409 (36%) were downregulated. Principal component analysis revealed that the majority of changes occurred at the initial time points (especially 5L 0D, 5L 10D, and 5L 25D) and not at later time points (Fig. 1).

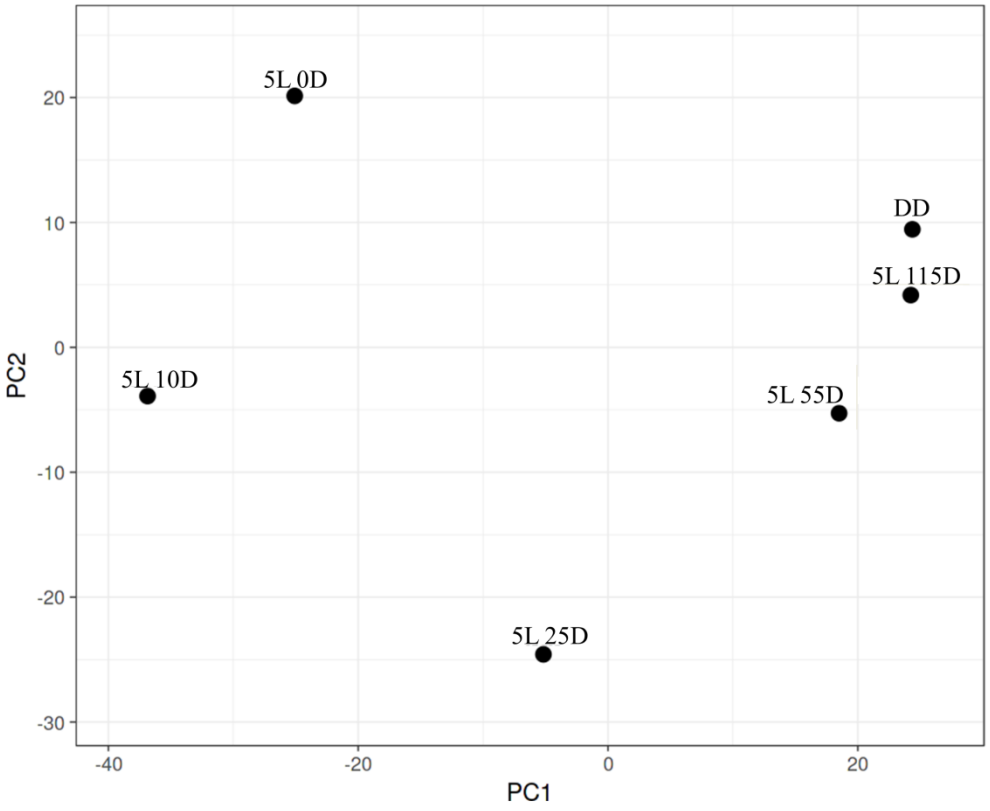

Fig. 1 – Principal component analysis for the 1128 light-regulated genes. Changes at mRNA level occurred mostly in the first 30 min after light exposure and not at later time points

To understand the kinetics of gene regulation after light exposure, we clustered the 1128 light-regulated genes according to their expression profile by using *clust* (Abu-Jamous and Kelly, 2018). *clust* deals with the clustering problem with a data extraction approach instead of the more traditional data partitioning. On the one hand, this generates tight clusters with little to no ambiguity in gene assignment. On the other hand, only about 50% of all genes are clustered (Abu-Jamous and Kelly, 2018). For our data set, *clust* generated 13 clusters comprising 619 genes (54.9%) with an average cluster size of 47.6 genes (Fig. 2 and Table S3).

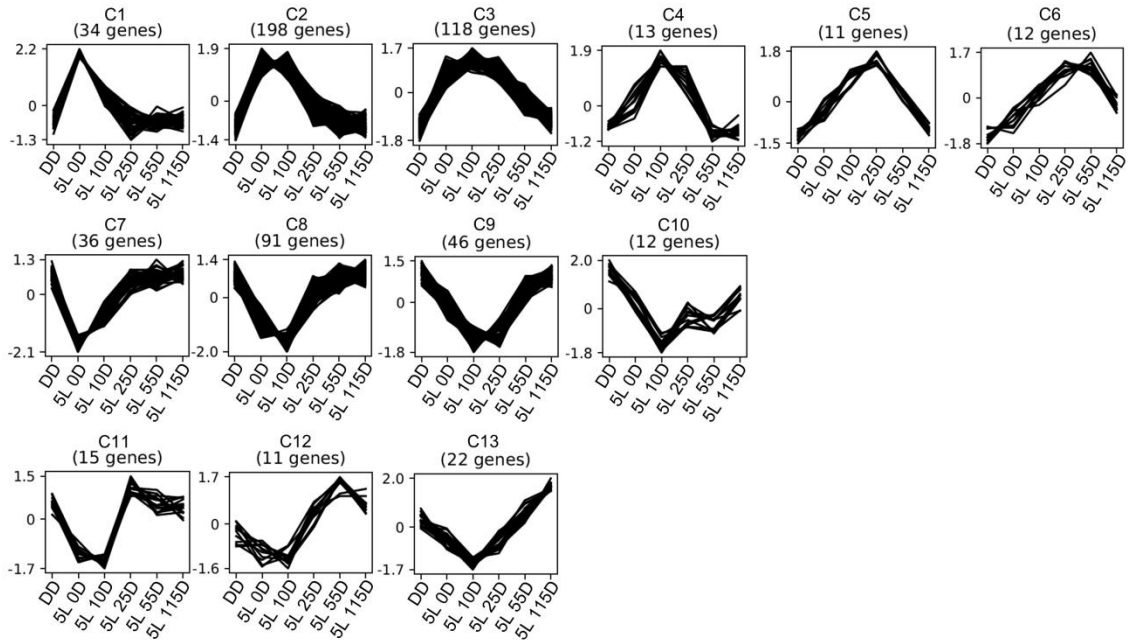

Fig. 2 – Clustering analysis for light-regulated genes resulted in 13 clusters depicting upregulated (C1 through C6), downregulated (C7 through C10), and oscillatory (C11 through C13) genes. Values in y-axis are Z-scores

The upregulated clusters (C1 through C6) showed that light can regulate gene expression at multiple time points, thus allowing us to classify genes as early- (5L 0D, 5L 10D, and 5L 25D; clusters C1 through C5) and late- (5L 55D and 5L 115D; cluster C6) regulated according to their peak expression (Fig. 2). Also, this revealed a potential hierarchical model in which light initially drives the expression of genes coding for

transcription factors that will then act on downstream genes. Approximately the same phenomenon was observed for downregulated gene clusters (C7 through C10), although late downregulated genes were not observed (Fig. 2). Finally, some gene clusters presented an oscillatory pattern characterized by initial downregulation followed by late upregulation (C11 through C13) (Fig. 2).

To gain better insight into which biological processes were regulated by light, we performed Gene Ontology analyses on clusters C2 and C8 which are the largest up and downregulated gene clusters, respectively. Overall, light upregulated genes involved in cellular response to stress and cellular protein localization (Fig. 3A) and downregulated genes involved in transmembrane transport (Fig. 3B). Some biological processes, such as 'oxidation-reduction process' and 'regulation of transcription from RNA polymerase II promoter', were shared by both clusters. Because response to stress and transcriptional regulation were enriched in cluster C2, we looked for genes belonging to known oxidative stress response pathways. We observed that a stress-activated MAPK gene (MAC_08084) homologue to *N. crassa os-2* and *Aspergillus nidulans hogA* was upregulated together with the bZip transcription factor *asl-1* homologue (MAC_03844).

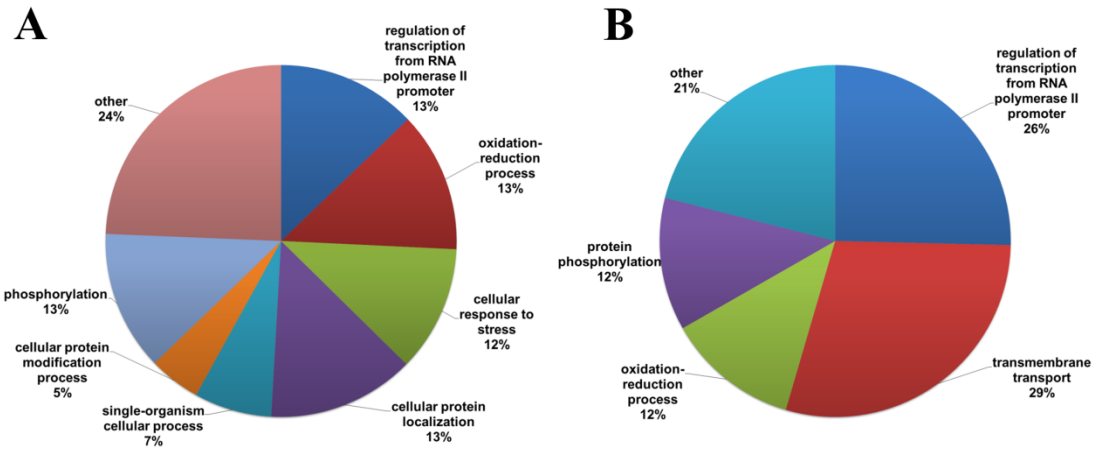

Fig. 3 – Gene Ontology analysis for genes on clusters (A) C2 and (B) C8 which are the largest up and downregulated gene clusters, respectively

Because transcriptional regulators were abundant in clusters C2 and C8, we performed a separate analysis for such light-regulated genes (Fig. 4). Among these, we found homologue genes for the core circadian oscillator *frq* (MAC_01916) and the circadian transcriptional repressor *csp-1* (MAC_07134) both of which are also regulated by light in *N. crassa* (Froehlich et al., 2002; Sancar et al., 2011). Future experiments should elucidate whether *M. acridum* possesses a circadian clock.

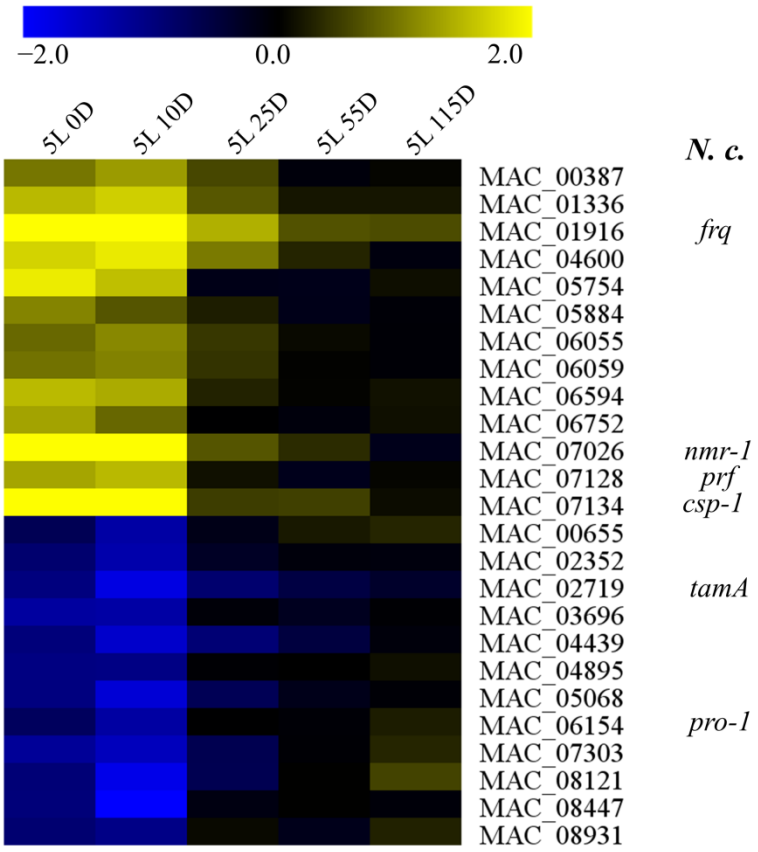

Fig. 4 – Heat map depicting the early regulation of genes coding for transcriptional regulators on clusters C2 and C8. Transcriptional regulator activity was according to Gene Ontology. Values in scale bar are $\log_2$ fold-change relative to DD. *N . c.* = *Neurospora crassa* known homologue genes

For validation purposes, we have evaluated the expression of genes encoding for a photolyase (MAC_05491) and a UV-endonuclease (MAC_07337) by qRT-PCR.

Similar patterns of light regulation in mRNA-Seq and qRT-PCR experiments were observed for both genes (Fig. 5).

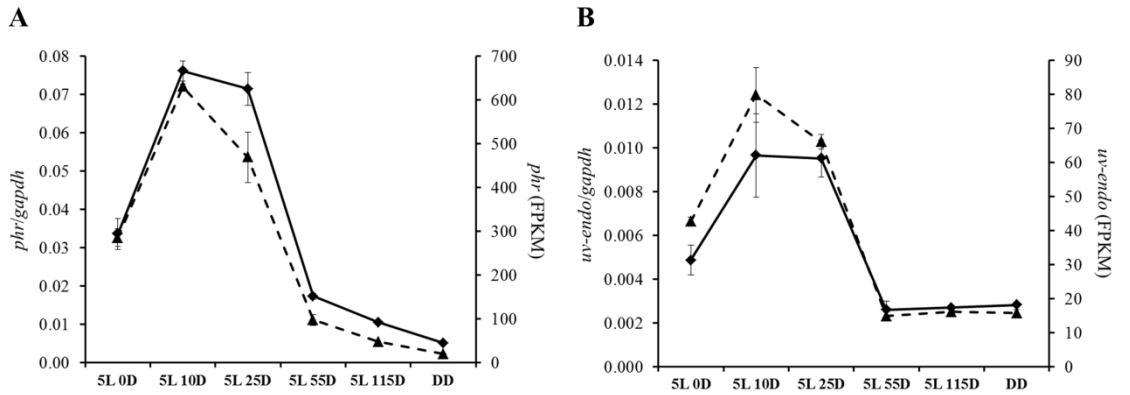

Fig. 5 – Validation of mRNA-Seq data for the (**A**) *phr* (MAC_05491) and (**B**) *uv-endo* (MAC_07337) genes with quantitative reverse transcription PCR (qRT-PCR). Solid lines refer to qRT-PCR (primary *y*-axis) and dashed lines refer to mRNA-Seq data (secondary *y*-axis). Error bars are standard deviation from three independent experiments

Effects of light on the proteome

For high-throughput proteomics experiments, we analyzed a longer time point (5L 235D) in order to better account for the expected delay between mRNA and protein. We also removed the very short 5L 0D time point from proteomics analyses. Our proteomics data showed good agreement between the three experiments and quantitative information was used only if a protein was present in at least two experiments (Fig. 6).

Our analysis encompassed 3852 proteins representing 38.6% of all predicted gene products. Of these, only 57 were regulated by light at least 2-fold, with 41 upregulated and 16 downregulated proteins. Changes in abundance at the protein level peaked at 5L 235D for 89.5% of regulated proteins, with only six proteins changing at earlier time points (Fig. 7).

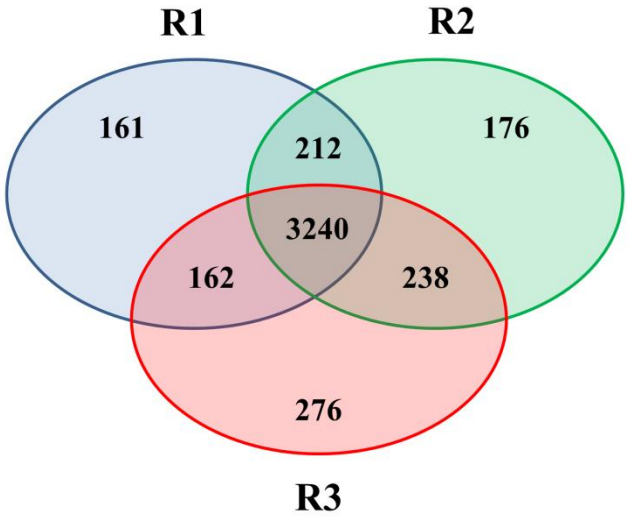

Fig. 6 – Venn diagram showing the number of identified proteins in experiments R1, R2, and R3. Quantitative data was only used if a given protein was identified in at least two experiments, resulting in quantitative information for 3852 proteins

We then analyzed the top 10 highest up and downregulated proteins after light exposure (Table 1 and Table 2). The strongest upregulated protein, acid sphingomyelinase (MAC_02084), is involved in sphingolipid metabolism. SignalP-5.0 (Almagro Armenteros et al., 2019) sequence analysis revealed the presence of an N-terminal secretory signal peptide that could indicate the protein has a role in insect pathogenicity. Five out of the ten most upregulated proteins are currently uncharacterized and two of these (MAC_09637, MAC_02991) have no homologues in *N. crassa*. One of the uncharacterized proteins (MAC_09800) is annotated as a flavin-binding monooxygenase in *M. guizhouense*, *M. brunneum*, and *M. majus*. We also observed the accumulation of two other flavin-binding monooxygenases (MAC_09799, MAC_09164) after light exposure (Table S4). Furthermore, MAC_09799 and MAC_09800 are neighboring genes and presented the same protein accumulation profile (Table S4). We also observed the upregulation of heat shock protein 30 (MAC_07554) and photolyase (MAC_05491), both probably involved in light-induced stress tolerance.

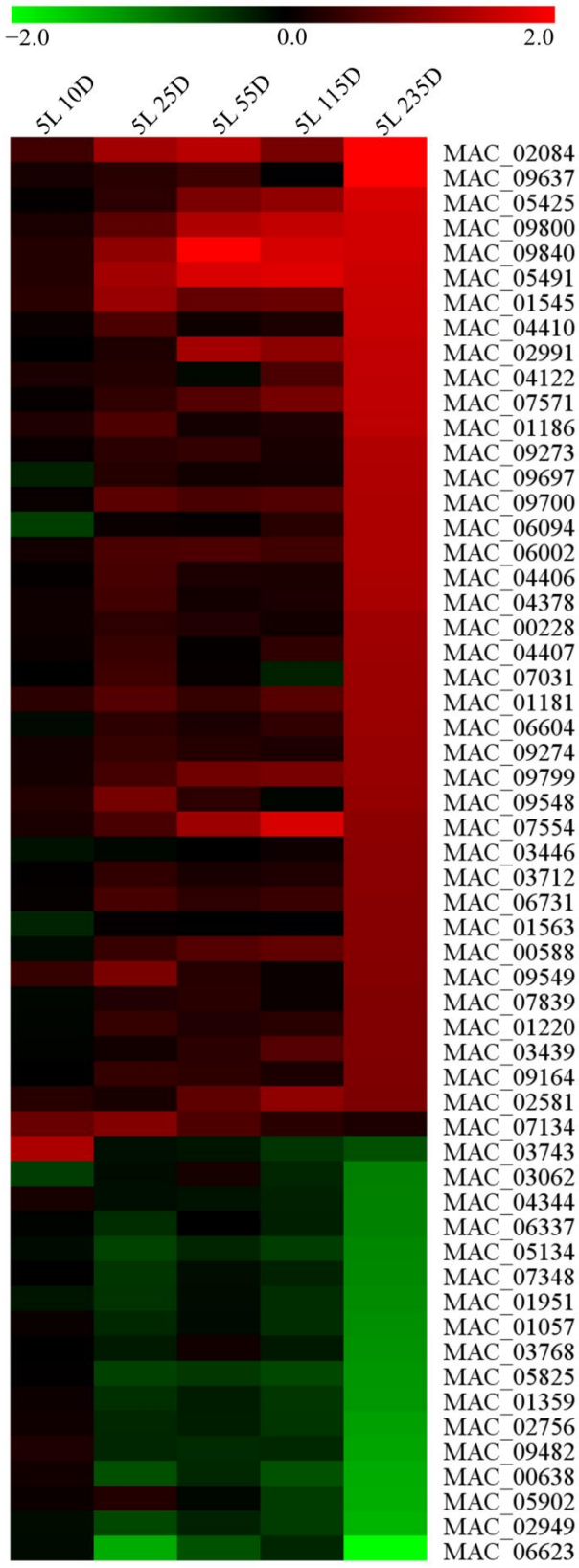

Fig. 7 – Heat map depicting changes at the protein level after light exposure. Almost 90% of all proteins were regulated at 5L 235D. Values in scale bar are $\log_2$ fold-change relative to DD

Table 1 – Ten most upregulated proteins after light exposure
[a]peak log_2 fold-change relative to DD

	Name	Gene ID	Protein Entry	log_2 fold-change[a] (time point)
1	Acid sphingomyelinase, putative	MAC_02084	E9DWT6	2.40 (5L 235D)
2	Uncharacterized protein	MAC_09637	E9EID9	2.09 (5L 235D)
3	Membrane protein, putative	MAC_09840	E9EIZ2	2.00 (5L 55D)
4	Photolyase	MAC_05491	E9E6J3	1.76 (5L 115D)
5	Heat shock protein 30	MAC_07554	E9ECF6	1.70 (5L 115D)
6	Uncharacterized protein	MAC_05425	E9E6C7	1.69 (5L 235D)
7	Uncharacterized protein	MAC_09800	E9EIV2	1.64 (5L 235D)
8	Uncharacterized protein	MAC_01545	E9DV97	1.57 (5L 235D)
9	Lysine amidinotransferase	MAC_04410	E9E3G2	1.56 (5L 235D)
10	Uncharacterized protein	MAC_02991	E9DZE3	1.51 (5L 235D)

Table 2 – Ten most downregulated proteins after light exposure
[a]peak log_2 fold-change relative to DD

	Name	Gene ID	Protein Entry	log_2 fold-change[a] (time point)
1	Cytochrome P450 phenylacetate 2-hydroxylase, putative	MAC_06623	E9E9S5	−2.17 (5L 235D)
2	Amino acid transporter, putative	MAC_02949	E9DZA1	−1.42 (5L 235D)
3	Carboxyphosphonoenolpyruvate phosphonomutase, putative	MAC_05902	E9E7Q4	−1.37 (5L 235D)
4	54S ribosomal protein L12	MAC_00638	E9DSP0	−1.35 (5L 235D)
5	Eukaryotic translation initiation factor 3 subunit E	MAC_09482	E9EHY4	−1.30 (5L 235D)
6	GNAT family N-acetyltransferase, putative	MAC_02756	E9DYQ9	−1.26 (5L 235D)
7	Deoxyhypusine hydroxylase	MAC_01359	E9DUR1	−1.18 (5L 235D)
8	Vitamin B6 transporter, putative	MAC_05825	E9E7H7	−1.18 (5L 235D)
9	Rhomboid family protein	MAC_03768	E9E1M0	−1.15 (5L 235D)
10	Eukaryotic translation initiation factor 3 subunit M	MAC_01057	E9DTV9	−1.12 (5L 235D)

Among downregulated proteins, subunits E and M of eukaryotic translation initiation factor 3 (eIF3) were at least 2-fold regulated after light exposure (Table 2). The downregulation of two eIF3 subunits prompted us to lower the 2-fold cutoff in the search for other regulated eIF3 subunits. We found eIF3 subunit K to be 1.8-fold and eIF3 subunit F to be 1.4-fold downregulated (Fig. 8A). This observation was specific to eIF3 as subunits for other translation initiation factors were unchanged (Table S5). However, the enzyme deoxyhypusine hydroxylase (MAC_01359) was downregulated at the protein level (Table 2 and Fig. 8B). This protein is one of two enzymes required for the post-translational modification that activates eukaryotic initiation factor 5A (eIF5A) which has a role in translation elongation (Saini et al., 2009).

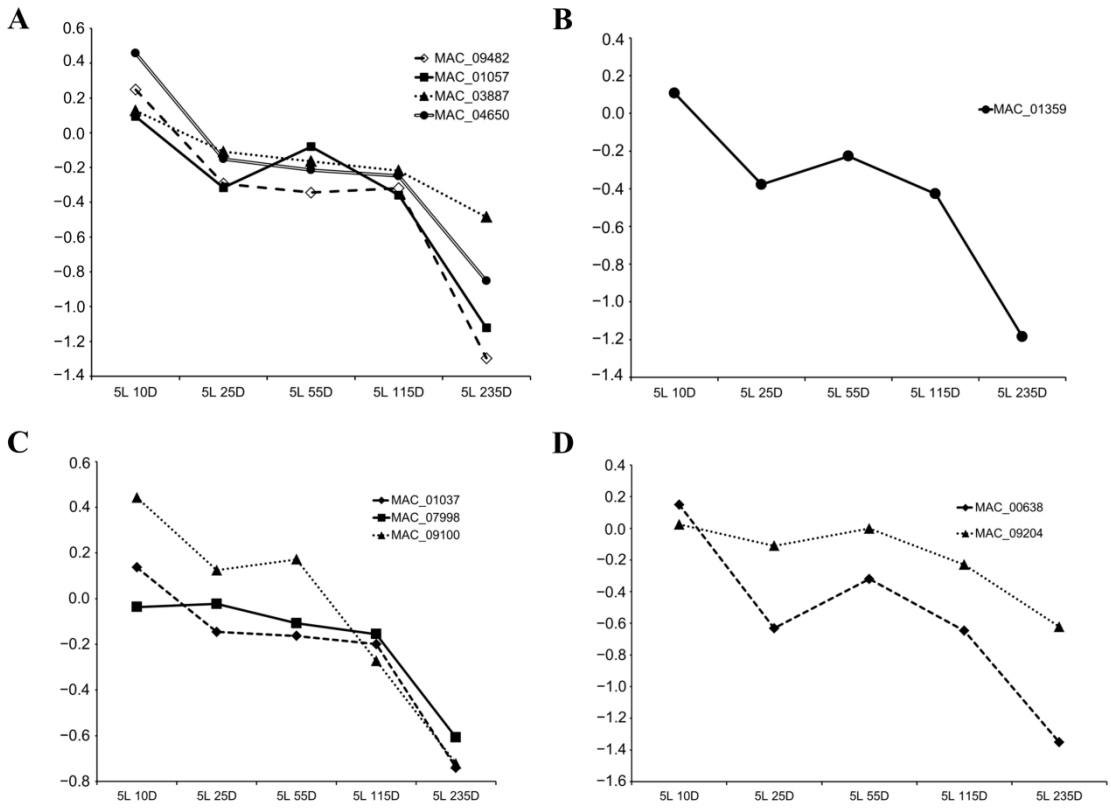

Fig. 8 – Light downregulated proteins involved in translation, including (**A**) eIF3 subunits M (MAC_01057), E (MAC_09482), K (MAC_04650), and F (MAC_03887); (**B**) the eIF5A-activating enzyme deoxyhypusine hydroxylase (MAC_01359); (**C**) cytosolic ribosomal proteins P0 (MAC_01037), S14 (MAC_07998), and S29 (MAC_09100); and (**D**) mitochondrial ribosomal proteins L12 (MAC_00638) and MRP2 (MAC_09204). Values in y-axis are $\log_2$ fold-change relative to DD

The decreased translation initiation/elongation caused by light exposure prompted us to look for regulated ribosomal proteins. We observed downregulation of 40S ribosomal proteins S14 and S29 (MAC_07998, MAC_09100) and 60S ribosomal protein P0 (MAC_01037), although these only satisfied a 1.5-fold cutoff (Fig. 8C). Furthermore, mitochondrial ribosomal proteins 54S L12 (MAC_00638) and 40S MRP2 (MAC_09204) were 2.5- and 1.5-fold downregulated, respectively (Table 2 and Fig. 8D).

Combining proteomics and mRNA-Seq data to find post-transcriptional regulatory mechanisms

After light exposure, 1128 mRNAs (out of 9514 evaluated) changed in abundance while only 57 proteins (out of 3852 evaluated) did so. Combining both data sets resulted in 34 light-regulated mRNA/protein pairs. We used these pairs to elucidate the average time required to go from peak mRNA to peak protein change. This was done by calculating R^2 for $\log_2$-$\log_2$ correlation plots. Overall, mRNA change at any time point best correlated with protein change 1-2 h later (Table 3 and Table S6).

Table 3 – Person correlation coefficient for changes at the mRNA and protein levels. Correlation was calculated based on the 34 light-regulated mRNA/protein pairs. Values above a 0.60 cutoff are highlighted in red

		Proteomics				
		5L 10D	5L 25D	5L 55D	5L 115D	5L 235D
mRNA-Seq	5L 0D	0.29	0.26	0.74	0.73	0.10
	5L 10D	0.15	0.11	0.71	0.70	0.02
	5L 25D	0.11	0.19	0.79	0.76	0.16
	5L 55D	0.18	0.21	0.78	0.81	0.36
	5L 115D	0.04	0.28	0.55	0.59	0.65

The majority of pairs followed this 1-2 h delay as observed for the photolyase (Fig. 9A). A very early regulated gene coding for a C2H2 transcription factor (= *N.*

crassa CSP-1) presented an accompanying early protein accumulation and was one of the fastest regulated protein in the data set, perhaps a requirement to fulfill its biological role (Fig. 9B). In at least two instances there was protein accumulation after gene downregulation, such as observed for a polyketide synthase (Fig. 9C).

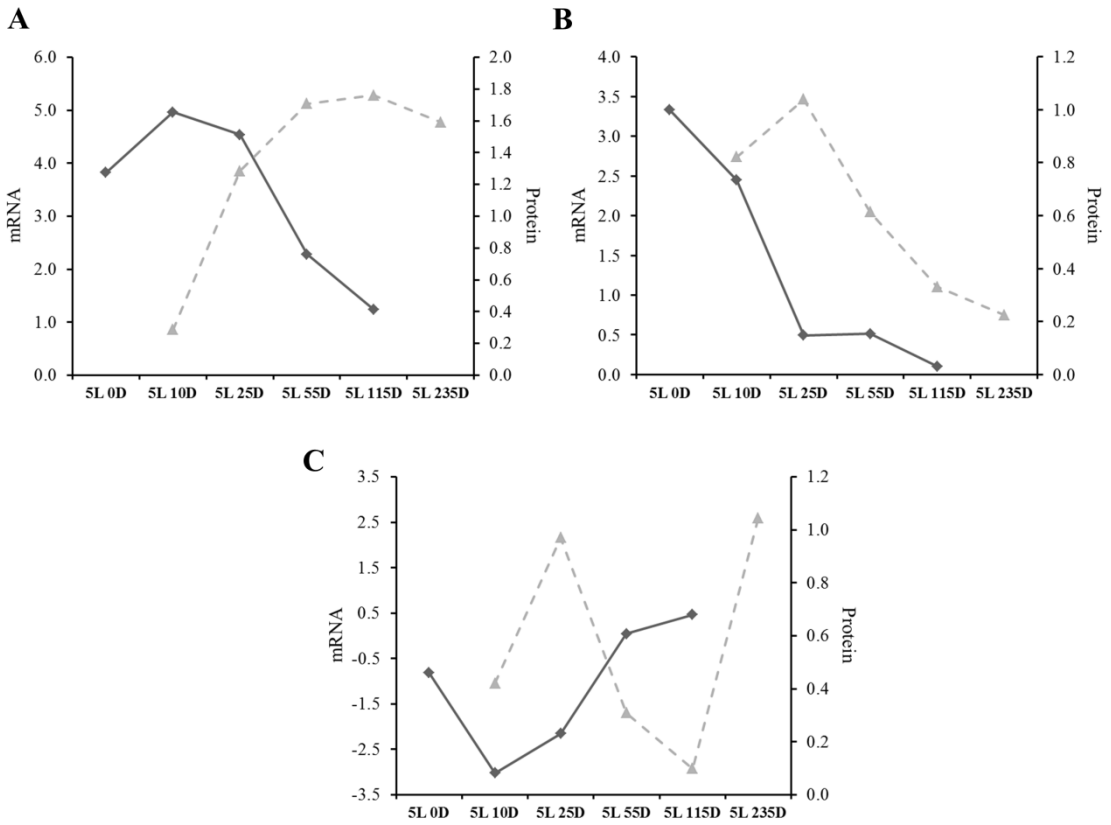

Fig. 9 – Combined mRNA/protein plots showing good correlation for (**A**) photolyase (MAC_05491) and (**B**) C2H2 transcription factor (= *N. crassa* CSP-1, MAC_07134) with an inverse and poor correlation for (**C**) polyketide synthase (MAC_09549). Solid lines refer to mRNA and dashed lines refer to protein. Values in *y*-axes are $\log_2$ fold-change relative to DD

A consequence of having only 34 mRNA/protein pairs is that 23 proteins changed abundance in the absence of mRNA regulation (Table 4). In principle, this would leave us with 1094 mRNAs for which there was no protein change. However, we need to take into account that mRNA-Seq and proteomic data sets are different sizes (9514 vs 3852, Table 4). Therefore, the number of mRNAs changing after light exposure without an accompanying protein change is actually 347, while the remaining

747 present no protein quantitative data (Table 4). This means that a large number of changes at the mRNA level are not translated into changes at the protein level. More importantly, it was not possible to predict, based on mRNA fold change or expression pattern, whether regulation at the transcript level would lead to changes at the protein level. Some mRNAs were upregulated by as much as 18-fold and downregulated by as much as 5.7-fold without any accompanying changes in protein expression levels (Table S6).

Table 4 – Combination of mRNA-Seq and proteomics data sets based on the number of regulated mRNAs/proteins
[a]$P < 0.01$ and at least 2-fold regulation
[b]False Discovery Rate < 0.05 and at least 2-fold regulation

	mRNA-Seq		Proteomics
Genome		9974	
Evaluated mRNAs/proteins	9514		3852
Light-regulated	1128[a]		57[b]
Upregulated	719 (64%)		41 (72%)
Downregulated	409 (36%)		16 (28%)
mRNA/protein pairs		34	
protein change without mRNA change		23	
mRNA change without protein change		347	

Discussion

The success of biological control with *M. acridum* depends on the fungus surviving the stresses imposed by the environment. Among these, heat and UV-B radiation can be cited as the most relevant. We previously observed that a 5-min exposure to light will increase tolerance to UV-B radiation in a time-dependent manner (Brancini; Rangel; Braga, 2016). Therefore, we combined transcriptomics (mRNA-Seq) and high-throughput proteomics to understand how light regulates gene expression both transcriptionally and post-transcriptionally. Our experiments were performed by exposing mycelium to a 5-min pulse of light and then incubating it in the dark for

different lengths of time. Conversely, most studies evaluating light responses in fungi expose mycelium to light for different lengths of time with no incubation in the dark afterwards (Chen et al., 2009; Ruger-Herreros et al., 2011; Fuller et al., 2013; Schumacher et al., 2014; Wu et al., 2014). Because light is also regarded as a stress to fungi, different exposures to light would inevitably lead to varying amounts of stress based on the length of light exposure. We have therefore tried to mitigate this effect by using the same exposure for all time points in our analysis. Furthermore, the 5-min exposure was chosen based on prior work (Brancini et al. 2016) in which we observed that this exposure was sufficient to result in increased tolerance to UV-B radiation.

Light transcriptionally regulated 1128 genes or 11.3% of the genome after a 5-min exposure. Most genes were regulated in the first 30 min after light exposure with only few genes being late regulated (Fig. 1 and 2). According to the hierarchical model of gene regulation by light uncovered in *N. crassa* (Chen et al., 2009), the White Collar Complex (WCC) initially drives the expression of its target genes and some of these are transcription factors that will then act downstream of the WCC to regulate other genes. Accordingly, we observed early regulation of many genes coding for transcriptional regulators (Fig. 4). We hypothesize that the reduced number of late regulated genes is a consequence of the short initial exposure to light. Longer exposures would keep gene expression levels higher instead of creating a quick rise and fall in mRNA abundance as observed in our data (Fig. 2) and this could be essential for the induction of late regulated genes. A previous work employed mRNA-Seq to study light regulation in *N. crassa* by exposing mycelium to light for 0, 15, 60, 120, and 240 min (Wu et al., 2014). Light was kept on throughout the experiment (no dark incubation afterwards) and the authors observed consistent gene regulation at all time points. This supports the

hypothesis that late gene regulation could also be dependent on longer exposures to light in *M. acridum*.

The strongest upregulated protein in our data set was an acid sphingomyelinase (Table 1), responsible for the breakdown of sphingomyelin to ceramide and phosphorylcholine. A gene coding for a sphingomyelinase was upregulated in *Ophiocordyceps unilateralis s.l.* during ant infection (De Bekker et al., 2015). The authors speculate that a secreted sphingomyelinase could be involved in insect behavior manipulation by regulating sphingolipid metabolism. Insect behavior control by *M. acridum* has never been observed, but the strong accumulation of a sphingomyelinase containing an export signal peptide could indicate it has a role in insect killing as observed for *Bacillus cereus* (Doll; Ehling-Schulz; Vogelmann, 2013). The accumulation of the enzyme in response to light is in accordance with the host lifestyle, as locusts are known to engage in behavioral fever by basking in the sun, a phenomenon beginning on day 1 post-infection (Clancy et al., 2018).

Proteins also upregulated after light exposure were a photolyase (Table 1) and a Cry-DASH (Table S4). We have previously reported that light increased photoreactivating ability in *M. acridum* and hypothesized that photoreactivation mediated UV-B radiation tolerance (Brancini et al., 2018). While both proteins were upregulated, the photolyase accumulated much faster than the cryptochrome. The former surpassed the 2-fold cutoff at 5L 25D whereas the latter only did so at 5L 235D. Because tolerance to UV-B radiation increases quickly after light exposure, the photolyase is possibly the better candidate enzyme for mediating photoreactivation and UV-B tolerance with Cry-DASH fulfilling other regulatory roles as observed in *A. nidulans* and *Fusarium fujikuroi* (Bayram et al., 2008; Castrillo; García-Martinez; Avalos, 2013). It is important to note that the photolyase accumulating after light

exposure is the same for which we observed photoinduction at the mRNA level in our previous publication (Brancini et al., 2018).

Interestingly, many proteins negatively regulated by light were found to be involved in translation (Table 2 and Table S4). Downregulation of eIF3 subunits (Fig. 8A) and of the eIF5A-activating enzyme deoxyhypusine hydroxylase (Fig. 8B) suggests that light exposure reduces translational activity by acting on both translation initiation and elongation. Furthermore, some cytosolic and mitochondrial ribosomal proteins were also downregulated (Fig. 8C and 8D). Surprisingly, this potential reduction in translational activity peaked at 5L 235D, when almost all changes in protein abundance were observed (Fig. 7). Translation reduction and reprogramming are known cellular responses to stress (Yamasaki and Anderson, 2008; Spriggs; Bushell; Willis, 2010; Crawford and Pavitt, 2019). Also, decreased expression for genes coding for ribosomal proteins was observed after *N. crassa* mycelium was exposed to light (Wu et al., 2014). Here we show that this phenomenon is also observable at the protein level which is in agreement with the idea that light serves as both a signal and a stress to the cell (Wu et al., 2014). Recently, Hurley and coworkers have shown that translation in *N. crassa* is under the influence of the circadian clock and occurs preferentially after dusk and not during the day (Hurley et al., 2018), which is line with prior work reporting that translational activity is decreased at late subjective morning (Caster et al., 2016). It seems reasonable to say that light reduces translational activity in *N. crassa* by resetting the clock to subjective morning. It should be noted that the aforementioned downregulated proteins did not present downregulation of their corresponding mRNAs in our data set. This could be due to these proteins being post-transcriptionally regulated or it could be a consequence of late gene regulation that is beyond our last time point (5L 115D).

Combining our transcriptomic and proteomic data sets revealed an interesting phenomenon: while 1128 mRNAs changed in abundance in response to light, only 57 proteins did so (Table 4). These values correspond to 11.8% of all 9514 evaluated transcripts and 1.48% of all 3852 evaluated proteins. No more than 34 mRNA-protein pairs could be formed that were regulated in both data sets (Table S6). These pairs were used to calculate the 1-2 h delay required to go from mRNA peak regulation to protein peak regulation (Fig. 9 and Table 3).

As mentioned above, we observed that the majority of changes at the mRNA level were not translated to changes at the protein level. Lack of protein change in the event of gene downregulation could be explained by protein stability: stable proteins will last many hours inside the cell and our experiment only encompassed the first four hours following light exposure. However, if proteins are stable, then accumulation would be expected from gene upregulation. The negative effect light apparently had on translation initiation and elongation could perhaps help explain such a phenomenon.

Under conditions of decreased translational activity, there should be a mechanism allowing specific mRNAs to bypass this overall decrease. Light downregulated eIF3 subunits E (eIF3e), M (eIF3m), K (eIF3k), and F (eIF3f) but it did not regulate any other subunit. eIF3 is thought to mediate 43S pre-initiation complex assembly and attachment to mRNA, scanning, and start codon selection (Hinnebusch, 2017). In *Schizosaccharomyces pombe*, there are two distinct eIF3 complexes formed with either eIF3m or eIF3e (Zhou et al., 2005). On the one hand, the complex formed with eIF3m binds to the bulk of cellular mRNA and is responsible for overall translation. This makes *eIF3m* an essential gene. On the other hand, the complex formed with eIF3e is more restricted and regulates the translation of specific mRNAs (Zhou et al., 2005). In *N. crassa*, mutants for all the known eIF3 subunits were

analyzed. In accordance with *S. pombe*, *eIF3m* was found to be an essential gene whereas *eIF3e* and *eIF3k* mutants were viable (Smith et al., 2013). Therefore, different eIF3 subunits are probably involved in the translation of distinct mRNA molecules and their regulation constitutes an additional layer of post-transcriptional control (Genuth and Barna, 2018). We speculate that light can affect the translation of specific mRNAs by regulating eIF3 subunits and therefore translation initiation.

In line with this hypothesis, light also downregulated some ribosomal proteins (Fig. 8C and 8D) while the majority remained unchanged (Table S5). It was previously shown in mouse embryonic stem cells that active ribosomes are heterogeneous with respect to ribosomal proteins (Shi et al., 2017). These heterogeneous ribosomes translate different pools of mRNAs involved in different biological processes such as metabolism, proliferation, and cell survival. For instance, RPL10A was found to be required for the translation of specific mRNAs. This regulation was mediated, at least in part, by 5' UTR internal ribosome entry site (IRES) elements (Shi et al., 2017). It seems a natural consequence that regulating the abundance of ribosomal proteins could lead to differences in mRNA translation for specific genes sets. This "ribosome code" has been speculated and discussed for the past 60 years, but it is only recently gaining more attention (Emmott; Jovanovic; Slavov, 2019).

Taken together, our results indicate that light acts as both a signal and a stress in *M. acridum*. When acting as a signal, light regulates the expression of as much as 11.3% of the genome. Because it is also perceived as a stress, light ultimately causes a decrease in translational activity by downregulating some eIF3 subunits, the eIF5A-activating enzyme deoxyhypusine hydroxylase, and ribosomal proteins. We hypothesize the downregulation of these proteins buffers the changes at the mRNA level and ultimately results in the small number of regulated proteins observed. Therefore, our results show

that changes at the mRNA level are not necessarily translated to changes at the protein level and highlight the importance of analyzing the proteome in order to fully understand light responses in fungi.

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3 – DISCUSSION

Understanding how light regulates biological processes in general, and stress tolerance in particular, is essential if we are to use this stimulus to increase the efficiency of biological control employing *M. acridum*. The fungus genome bears genes coding for many of the classical photoreceptors (Table 1). Therefore an investigation on the effects of different light qualities was warranted and it was observed that white and blue light, and not red light, increase tolerance to UV-B radiation. Recently, it was shown that growing *M. robertsii* under white or blue light induces the fungus to produce conidia with increased tolerance to UV-B radiation when compared to conidia obtained from dark grown cultures (Dias et al., 2019). Also, growing under red light decreased conidia tolerance when compared to dark cultures (Dias et al., 2019). A tendency toward reduced tolerance was also observed when *M. acridum* mycelium was exposed to red light (Chapter 1), but the effect was not statistically significant. Taken together, these results indicate that the same molecular mechanism could be responsible for the acquisition of UV-B tolerance on both conidia and mycelia.

In this regard, it was observed that light exposure induces the expression of a photolyase-coding gene (Chapter 2) and the enzyme itself rapidly accumulates in mycelium (Chapter 3). The roles of photolyases on UV-B radiation tolerance have been reported here and elsewhere (Fang and St Leger, 2012). It is tempting to suggest that growing the fungus under light ultimately leads to photolyase accumulation in conidia, which would be good evidence that the acquisition of UV-B tolerance in conidia and mycelium has the same underlying mechanism. Experiments aiming at quantifying photolyase accumulation in conidia could also unveil at which point during development the enzyme reaches maximum concentration in conidia. At this point, it is possible that light exposure could be interrupted given that proteins in dormant conidia

are likely stable. This would represent a reduction in both cost and complexity for industrial production of *Metarhizium* conidia.

As previously mentioned, the increase in UV-B tolerance was brought about by blue light and not by red light. However, the exact photoreceptor responsible for this induction remains unknown. It would be tempting to speculate that *M. acridum*, as a Sordariomycete like *N. crassa*, has all its blue-light responses dependent on the WCC (WC-1/WC-2). However, knowledge about photoreception in *Trichoderma atroviride*, a hypocrealean fungus closer than *Neurospora* to *Metarhizium*, prevents such speculation. In *T. atroviride*, genes coding for a photolyase (*phr1*) and a cryptochrome (*cry1*) are upregulated after blue light exposure and this regulation is dependent on the WC-1 homologue Blr1. Photoreactivation itself is also increased after exposure to blue light and is drastically reduced in a Δ*blr1* background (Garcia-Esquivel et al., 2016). Surprisingly however, the fungus is still capable of responding to blue light even in the absence of Blr1. It was then unveiled that Cry1 and Phr1 also display photoreceptor activities and are capable of regulating the expression of some blue light responsive genes (Garcia-Esquivel et al., 2016). Furthermore, the photolyase Phr1 was previously shown to regulate its own photoinduction (Berrocal-Tito et al., 2007). In *M. acridum*, light does not regulate the expression of either WC-1 or WC-2, but it does upregulate the transcription of genes coding for a photolyase and a putative cryptochrome DASH, resulting in protein accumulation (Chapter 3). Because WC-1, photolyase, and cryptochrome are all potential blue light photoreceptors, it is not possible to determine which of these is involved in blue light sensing in *M. acridum*.

Light exposure of *M. acridum* mycelium also induced the expression of a homologue to the core circadian oscillator gene *frequency* (*frq*) (Chapter 3). *frq* is a direct target of the WCC and its photoinduction is essential to proper clock resetting in

N. crassa (Crosthwaite; Loros; Dunlap, 1995; Hurley; Loros; Dunlap, 2016). It is currently not known if *M. acridum* (or any *Metarhizium* species) has a circadian clock. It was recently reported that a circadian oscillator in the phytopathogenic fungus *Botrytis cinerea* regulates virulence when infecting plants (Hevia et al., 2015). Conidia of the fungus obtained at dawn or dusk were used to infect *Arabidopsis thaliana* leaves and lesion size was larger when infection was performed at dusk (Hevia et al., 2015). Given that *Metarhizium* conidia are used in the field for biological control, it would be advantageous to unveil if the fungus has a circadian clock and whether it affects virulence.

The most striking result observed when combining transcriptomics and proteomics was the reduced number of light-regulated proteins (less than 60) when as much as 11% of the genome was light-regulated at the transcript level. In Chapter 3 we discussed how light is interpreted as a stress by the cell and translational activity decreases as a consequence. We have not, however, discussed the implications of very fast and short-lived changes at the mRNA level on translation. As shown, light causes a quick rise in mRNA levels of light-upregulated genes. This increase, however, lasts very shortly (typically 30 min) and then mRNA levels drop to control (DD) levels. This creates a time constraint: in order for protein levels to change after such fast and short-lived regulation, translation needs to be equally fast or the information is lost. To date, there is no information as to how fast translation in *Metarhizium* can be and therefore this "translational limitation" needs to be taken into account when gene transcription presents the fast and short-lived regulatory pattern. Dealing with this issue could be achieved via ribosome profiling or Ribo-Seq, a technique that focusses on actively-translated mRNA. This type of experiment could unveil whether and how light exposure changes the dynamics of translation. As an example, Ribo-Seq could elucidate if peaks

at the mRNA level (observed in mRNA-Seq experiments) actually result in peaks at the ribosome-associated mRNA levels. Furthermore, changes at the ribosome-associated mRNA levels would have a better chance of correlating with changes at the protein level and therefore would serve as a better predictor of gene regulation when compared to standard mRNA-Seq.

4 – CONCLUSIONS

Light induces tolerance to UV-B radiation in the mycelium of *M. acridum*. This induction in dependent on light quality and only blue light achieves the increase. Light exposure also results in increased photoreactivation ability which is crucial to UV-B radiation tolerance. The molecular mechanism behind this phenomenon is possibly the photoinduction of a photolyase-coding gene with subsequent protein accumulation. Finally, light also affects the transcription of as much as 11% of the genome. However, these changes at the mRNA level are frequently not translated to the protein level. This is potentially due to light also acting as a stress and ultimately causing a reduction in translational activity.

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