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RESEARCH ARTICLE

Light exposure during growth increases riboflavin production, reactive oxygen species accumulation and DNA damage in *Ashbya gossypii* riboflavin-overproducing strains

Rui Silva[1], Tatiana Q. Aguiar[1,*,†], Rui Oliveira[1,2] and Lucília Domingues[1]

[1]CEB—Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal and [2]CITAB—Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Department of Biology, University of Minho, 4710-057 Braga, Portugal

[*]**Corresponding author:** CEB—Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal. Tel: +351 253 601 968; Fax: +351 253 604429; E-mail: tatiana.aguiar@deb.uminho.pt

One sentence summary: Light exposure during growth affects riboflavin production in *Ashbya gossypii* overproducing strains by increasing the cellular oxidation state, which in turn is accompanied by DNA damage.

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[†]Tatiana Q. Aguiar, http://orcid.org/0000-0003-4368-7597

ABSTRACT

The overproduction of riboflavin (vitamin B_2) by *Ashbya gossypii*, one of the most distinctive traits of this filamentous hemiascomycete, has been proposed to act as an ecological defense mechanism, since it is triggered by environmental stress. The interaction of endogenous riboflavin with light generates reactive oxygen species (ROS) and induces oxidative DNA damage in mammalian cells, but exogenous riboflavin was shown to protect *A. gossypii* spores against ultraviolet light. Envisioning a better understanding of this biotechnologically relevant trait, here we investigated the putative genotoxic effects associated with the overproduction of riboflavin by *A. gossypii*. For assessing that we developed the *Ashbya* Comet Assay, which was able to reproducibly measure oxidative (H_2O_2/menadione-mediated) and non-oxidative (camptothecin-mediated) DNA damage in *A. gossypii*. Using this protocol, we determined that exposure to sunlight-mimicking light during growth significantly increased the DNA damage accumulation in riboflavin-overproducing cells, but not in non-overproducing ones. The exposure of overproducing cells to light induced the intracellular accumulation of ROS and increased the production of riboflavin 1.5-fold. These results show that riboflavin-overproducing strains are highly susceptible to photo-induced oxidative DNA damage and draw attention for the importance of controlling the exposure to light of biotechnological riboflavin production processes with *A. gossypii*.

Keywords: *Ashbya gossypii*; riboflavin; comet assay; photosensitizer; light-induced DNA damage; ROS

INTRODUCTION

Ashbya gossypii occurrence in nature is as a plant wound pathogen in the warmer parts of the planet (e.g. cotton plants in the tropics), usually in close relationship with certain plant-feeding insects (Aguiar, Silva and Domingues 2015). The overproduction of riboflavin (vitamin B_2) by *A. gossypii*, which is one of the most distinctive traits of this filamentous hemiascomycete,

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has been connected with sporulation and stress, and is thought to represent an ecological advantage for both the fungus and its insect vectors, by conferring them protection against plant defenses. Both nutritional (Schlösser *et al.* 2007) and oxidative stress (Walther and Wendland 2012; Kavitha and Chandra 2014) can trigger riboflavin production in *A. gossypii*. Oxidative stress-inducing agents, such as H_2O_2 and menadione, have been shown to concomitantly increase riboflavin production and standard antioxidant defense mechanisms (Kavitha and Chandra 2014), which are driven by an *Yap1*-dependent response (Walther and Wendland 2012). Moreover, riboflavin was reported to have a protective effect for the spores of *A. gossypii* against ultraviolet (UV) light (Stahmann *et al.* 2001), as its addition to spore suspensions was shown to improve their UV resistance. However, riboflavin is a strong photosensitizer that upon interaction with UV light generates reactive oxygen species (ROS), which can cause oxidative damage to both DNA and proteins (Alam, Iqbal and Naseem 2015). Therefore, endogenous riboflavin in excess can become harmful to the cells, an effect that was already reported in several organisms, such as bacteria and mammalians (Lloyd *et al.* 1990; Besaratinia *et al.* 2007; Alam, Iqbal and Naseem 2015). Nevertheless, despite this well-recognized photo-induced cytotoxicity of riboflavin, until the date there are no studies investigating the potential genotoxic effects of riboflavin in *A. gossypii* overproducing strains.

The Comet Assay (Single Cell Gel Electrophoresis) is a simple, sensitive and versatile technique for measuring DNA damage (Collins 2004). It has been applied to a wide variety of organisms (such as human cells, insects, plants and fungi) as a standard procedure for a wide range of applications, such as compounds' genotoxicity testing, ecological monitoring (environmental risk assessment), human studies (biomonitoring and nutritional studies), DNA repair studies, among others (Collins 2004; Azevedo *et al.* 2011; Langie, Azqueta and Collins 2015). The first report of this methodology is dated of 1984, when Ostling and Johanson used the Comet Assay to investigate the effect of radiation on the DNA damage of individual mammalian cells (Ostling and Johanson 1984). The theoretical scientific basis of this technique consists of the relaxation of the negative supercoiling of genomic DNA when lesions (such as single strand breaks) are present, which increases its mobility when an electric field is applied. Thus, it is possible to establish a direct correlation between the electrophoretic mobility of the DNA and the level of damage present (Collins 2004; Oliveira and Johansson 2012).

Given the photosensitizer role of riboflavin and the connection of its overproduction by *A. gossypii* with environmental stress, it was our objective to understand whether riboflavin itself may act as a stressor for this filamentous fungus and under what conditions. Therefore, envisioning a better understanding of this relevant trait, here we investigated the putative genotoxic effects associated with the interaction between riboflavin and light in overproducing and non-overproducing *A. gossypii* strains. In this context, a Comet Assay protocol was developed for assessing DNA damage in *A. gossypii*.

MATERIAL AND METHODS

Strains and media

The *A. gossypii* strains ATCC10895, kindly provided by Prof. Peter Philippsen (University of Basel), and A8 (Ledesma-Amaro, Buey and Revuelta 2015), kindly provided by Prof. Jose Luis Revuelta (University of Salamanca), were used throughout this study. Stock cultures of these strains were kept as spores suspended in spore buffer containing 200 g L^{-1} glycerol, 8 g L^{-1} NaCl and 0.25% (v v^{-1}) Tween 20 and stored at −80°C. Spores were prepared as previously described (Magalhães *et al.* 2014) with slight modifications. The mycelium was digested with 4 mg mL^{-1} of lysing enzymes from *Trichoderma harzanium* (Sigma-Aldrich Quimica, Sintra, Portugal) for 2–3 h at 37°C. Agar-solidified Ashbya Full Medium (AFM; 10 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 20 g L^{-1} glucose, 1 g L^{-1} myo-inositol) was used for the cultivation of *A. gossypii* strains.

Radial growth conditions and cell treatments

Agar-solidified AFM plates (90 mm diameter) with or without supplementation of stress agents were inoculated with 10 μL of a spore suspension of *A. gossypii* (10^7 spores mL^{-1}) and incubated at 30°C for 3 days. When indicated, the following stress agents were added to AFM: hydrogen peroxide (H_2O_2; 5, 10 or 25 mM), menadione (1, 5 or 10 μM) or camptothecin (CPT; Abcam, Cambridge, UK; 10, 20 or 40 μM). All agents were of analytical grade and diluted or dissolved in ultra-pure water for use at specified concentrations. When indicated, growth under light exposure was done using a fluorescent lamp (LUMILUX T5 HE, OSRAM, Berlim, Germany) with an irradiance level of 16 μmol m^{-2} s^{-1}, measured by a LI-250 Light Meter with a LI-190 quantum sensor (LI-COR Biosciences, Lincoln, USA). Final colony radial growth (Brancato and Golding 1953) was determined by measuring the diameter of colonies in two perpendicular directions, through two guide lines previously drawn on the lower outer face of the plates. Images of the colony morphology were recorded at the end of the growth. Total riboflavin quantification was done as previously described (Silva, Aguiar and Domingues 2015).

The *Ashbya gossypii* comet assay

The *Ashbya* Comet Assay was developed using as basis the protocol of the Yeast Comet Assay (Azevedo *et al.* 2011; Oliveira and Johansson 2012), which was adapted and optimized for the filamentous fungus *A. gossypii*. The resulting protocol comprised the three following steps: (1) protoplast generation, (2) buffer incubations and electrophoresis, and (3) comets' analysis.

For protoplast generation, the protocol described by Nieland and Stahmann (2013) was used with slight modifications. Mycelium from *A. gossypii* strains was harvested with a sterile loop from the areas near the edges of the colonies growing on agar-solidified AFM under different conditions and suspended in solution A (1.2 M $MgSO_4$, 10 mM Na-phosphate buffer, pH 5.8) with lysing enzymes (5 mg mL^{-1}). This mixture was incubated at 30°C for 1 h in order to release the protoplasts. Total liberation of protoplasts was verified by microscopic analysis. Afterwards, the mixture was centrifuged at 3000 rpm for 10 min (4°C), washed with solution B (1 M sorbitol, 10 mM Tris-HCl, pH 7.5), and the protoplasts resuspended in the same solution. To avoid the contamination of the protoplast suspension with high amounts of spores, 1 mL of paraffin oil light (Applicchem, Darmstadt, Germany) was added, mixed by vortexing and left resting until two phases were formed. Finally, the lower phase, containing the protoplasts, was collected and diluted (1:4) in solution B.

For the buffer incubations and electrophoresis, the protoplast suspension was harvested by centrifugation at maximum speed for 5 min at 4°C and resuspended in 1.5% (w v^{-1}) low melting agarose (LMA; GRS Agarose LMT, grisp, Porto, Portugal) at 35°C. Immediately after, the suspensions were spread onto microscopic glass slides pre-coated with 0.5% (w v^{-1}) melted multipurpose agarose. Next, the suspensions were covered with

coverslips and the microscopic slides were kept on ice for 5 min in order to solidify the LMA, thus creating a microgel. The coverslips were removed and the slides were then submerged in lysing buffer (30 mM NaOH, 1 M NaCl, 50 mM EDTA, 10 mM Tris-HCl, 0.05% w v^{-1} lauroylsarcosine, pH 10) for 2 h at 4°C. Then, the slides were transferred into pre-chilled electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris-HCl, pH 10) and incubated for 20 min. Subsequently, in the same buffer, an electric field of 0.7 V cm^{-1} was applied for 10 min to the slides. Finally, the samples were neutralized (10 mM Tris-HCl, pH 7.4) for 10 min, fixated in ethanol 96% (v v^{-1}) for another 10 min and left to air-dry overnight at room temperature. All the buffers were prepared just before use.

For the analysis of the comets, the microgels were stained with 10 μL of 3300-fold diluted GelRed (Biotium, Fremont, USA) and several representative images from the total area of the microgel were acquired by fluorescence microscopy (Leica Microsystems DM fluorescence microscope), using a magnification of 200× or 400×. The images were analyzed with the CometScore software and the tail length of the comets (μm) was chosen as the parameter to measure DNA damage. Alternatively, the same software was used to determine the percentage of DNA in the comet tail, another parameter used to determine DNA damage.

Analysis of intracellular ROS accumulation

Cells from the areas near the edges of *A. gossypii* ATCC 10895 and A8 colonies grown in the dark or exposed to light for 3 days on agar-solidified AFM at 30°C were harvested and suspended in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) at a concentration of 0.04 $g_{fresh\ biomass}$ mL^{-1}. The mixtures were then vortexed. Subsequently, the cell suspensions were loaded with a solution of 5 mM 2',7'-dichlorofluorescein diacetate (H_2DCFDA) to obtain a final probe concentration of 50 μM. These cell suspensions were incubated at 30°C and 200 rpm during 1 h in the dark. In co-incubation experiments, a control was used for each condition, which consisted of the cell suspensions without the addition of H_2DCFDA. Finally, the cell suspensions were diluted five times and transferred to 96-well black microplates in order to measure the fluorescence intensity of the samples using a Synergy Mx/Cytation 3 microplate reader (BioTek Instruments, Winooski, USA) with the filters set to an excitation/emission of 494/522 nm. The controls (cell suspensions without H_2DCFDA) of each sample were used as blanks, and their fluorescence intensities were subtracted from those of the samples (cell suspensions with H_2DCFDA) in order to remove any response background caused by cells or reagents. Simultaneously, aliquots of the same cell suspensions were also placed on microscopic slides and analyzed independently by fluorescence microscopy (Leica Microsystems DM fluorescence microscope).

Statistical analysis

For the comet assay experiments, two independent assays were performed for each condition. The cells for the assays were collected from three independent cell cultures that were resuspended together in solution A. The mean of the tail length was obtained from at least 40 comets (minimum of 20 comets analyzed from each duplicate, as recommended by the protocol of Oliveira and Johansson 2012). GraphPad Prism for IOS version 6.0 was used to carry out the statistical analysis. Differences were tested by one-way ANOVA followed by post-hoc Dunnett's and Tukey's multiple comparison tests (comet tail length, colony radial growth and fluorescence intensity) and by *t*-test (specific riboflavin production). Statistical significance was established at $P < 0.05$ for the comparisons.

RESULTS AND DISCUSSION

The *Ashbya* Comet Assay is able to measure oxidative and non-oxidative DNA damage

The Comet Assay (Single Cell Gel Electrophoresis) is a technique that is based on the increased electrophoretic mobility of damaged DNA, allowing the establishment of a direct correlation between this parameter and the level of damage present in the sample (Collins 2004; Oliveira and Johansson 2012). Contrasting with its extensive use in higher eukaryotes for genotoxicity studies (Langie, Azqueta and Collins 2015), it has been poorly explored and applied to microorganisms, notwithstanding the valuable outcomes obtained in the few studies published (Carmello *et al.* 2015; Cruz *et al.* 2016). In this work, a Comet Assay based on the protocol developed for *S. cerevisiae* (Azevedo *et al.* 2011; Oliveira and Johansson 2012) was adapted and optimized for use in *A. gossypii*. In order to validate this protocol, standard stress agents routinely used in this technique were used to induce oxidative (H_2O_2 and menadione) and non-oxidative (CPT) DNA damage in *A. gossypii*. Since *A. gossypii* grows exclusively in filamentous form, the two main alterations made to the protocol were related to life cycle differences between the two microorganisms. Therefore, for the successful application of this assay in *A. gossypii* some of the technical adaptations performed proved to be of major importance.

(i) Growth of *A. gossypii* cultures on solid media with the stress agent present from the inoculation start, contrasting with stress induction in *S. cerevisiae* that is carried out after an initial growth period in liquid medium and for a shorter period of time. This adaptation facilitated the monitoring of morphological and physiological alterations caused by the stress agents in the filamentous fungus.

(ii) During growth, spore formation starts to occur in older parts of the *A. gossypii* mycelia, which hampers the obtaining of a pure protoplasts suspension (required for the comet assay). Therefore, after protoplast formation, paraffin was added to the suspension in order to create two phases, the upper one with the hydrophobic spores and the lower with the protoplasts (Nieland and Stahmann 2013). The omission of this step from the protocol completely compromised the assay, as the presence of spores in the sample obstructed the visualization of the comets (data not shown).

(iii) The variability observed in the control samples (e.g. existence of comet tails) still captures much attention and is a central topic of discussion among the users of the comet assay (Møller *et al.* 2014). This variability is often associated with the presence of replication forks in the sample or with older cells, as the culture advances towards the stationary phase (Azevedo *et al.* 2011). In the specific case of *A. gossypii*, not much can be done regarding the former, since this fungus owns multinucleated mycelia that divide asynchronously (Anderson *et al.* 2013). However, regarding the latter, collection of the older zones of the colonies (the central area corresponding to the inoculum halo) was avoided.

With the newly developed *Ashbya* Comet Assay, we were able to measure the DNA damage in cells of the *A. gossypii* strain ATCC10895 growing on media containing different concentrations of H_2O_2 (Fig. 1). Cells exposed to this stress agent

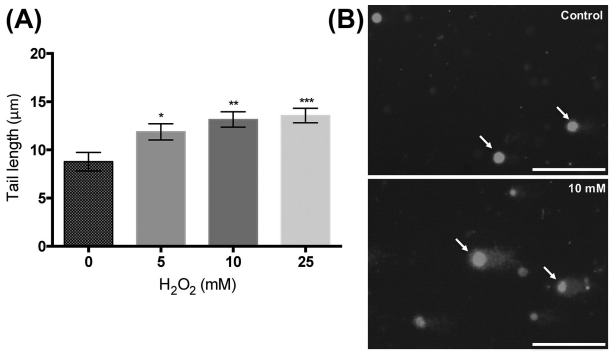

Figure 1. Measurement of H_2O_2-mediated oxidative DNA damage in *A. gossypii* ATCC 10895 with the *Ashbya* Comet Assay. **(A)** Data are presented as mean ± standard error of the mean of the tail length of at least 40 comets obtained from two independent experiments for each concentration (as described in Materials and Methods). Before harvesting, cells were grown for 3 days at 30°C on agar-solidified AFM containing different concentrations of H_2O_2 (0, 5, 10 or 25 mM). Asterisks represent significant differences compared to the control (0 mM H_2O_2). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. **(B)** Representative images of the comets obtained with the Comet Assay protocol adapted in this work for control cells (0 mm H_2O_2) and cells exposed to 10 mM H_2O_2. DNA was stained with GelRed. White bar = 50 μm.

Table 1. Colony radial growth of *A. gossypii* ATCC 10895 after 3 days of growth at 30°C on agar-solidified AFM containing different concentrations of H_2O_2. Data are presented as mean ± standard deviation of three biological replicates. Superscript letters represent significant differences compared to the control (0 mM H_2O_2).

Condition (mM H_2O_2)	Radial growth (mm)
0	31 ± 1[a]
5	28 ± 0[b]
10	24 ± 0[b]
25	16 ± 1[c]

[a,b]$P < 0.001$; [a,c]$P < 0.0001$.

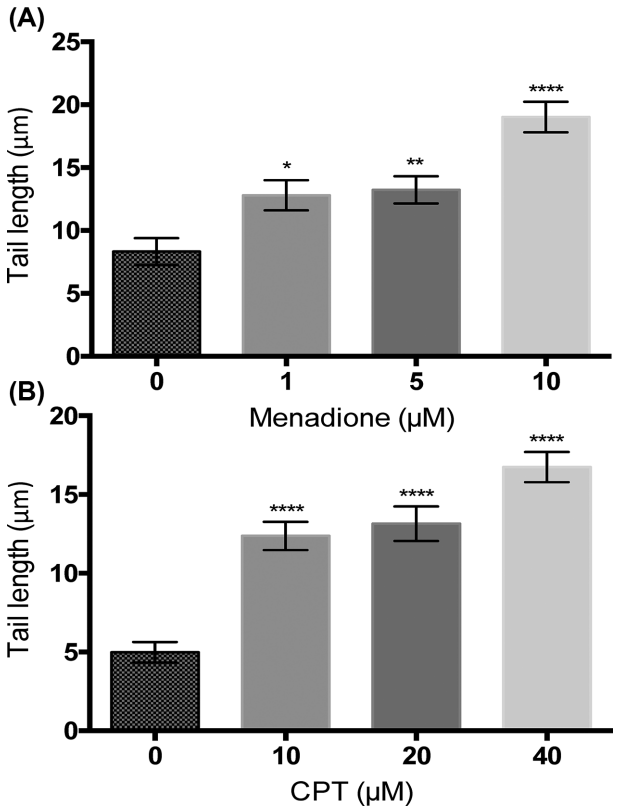

Figure 2. Exposure to **(A)** menadione (oxidative agent) or **(B)** camptothecin (CPT; non-oxidative agent) induces DNA damage in *A. gossypii* ATCC 10895. Data are presented as mean ± standard error of the mean of the tail length of at least 40 comets obtained from two independent experiments for each concentration (as described in Materials and Methods). Before harvesting, cells were grown for 3 days at 30°C on agar-solidified AFM containing different concentrations of menadione (1, 5 or 10 μM) and CPT (10, 20 or 40 μM). Asterisks represent significant differences compared to the control (0 μM menadione/CPT). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$.

presented significantly longer comet tail lengths than the control (cells growing in the absence of H_2O_2), indicating increased DNA damage. Besides the comet tail length, the percentage of DNA in the comet tail (another independent DNA damage parameter) was measured as well, indicating also increased DNA damage in cells growing on media containing H_2O_2 (data not shown). Exposure of *A. gossypii* to increasing concentrations of H_2O_2 proportionally affected its growth, as the radial growth in the presence of this compound was 92% (5 mM), 79% (10 mM) and 51% (25 mM) of that of the control condition (Table 1).

DNA damage caused by two additional stress agents, menadione (oxidative) and CPT (non-oxidative), could also be measured with the *Ashbya* Comet Assay. Similar to H_2O_2, menadione causes DNA damage through a ROS mechanism, whereas CPT causes strand breaks through the inhibition of DNA topoisomerase I (Liu *et al.* 2000). Longer comet tails were detected in cells exposed to these two types of stress agents, with all the concentrations tested presenting significant differences compared to the control (Fig. 2). Once again, the percentage of DNA in the comet tail corroborated the results obtained with the comet tail length, with the single exception for the concentration of 1 μM of menadione (data not shown). In this condition, although a higher percentage of DNA in the comet tail (21.0 ± 11.6%) was observed, the difference to the control (14.1 ± 10.6%) was not statistically significant. Contrary to H_2O_2, these stress agents did not affect the radial growth of *A. gossypii* at the concentrations tested (data not shown). Altogether, these results support the reproducibility of this new protocol, validating it for measuring oxidative and non-oxidative DNA damage.

The Comet Assay protocol optimized here is the first developed for use in *A. gossypii*, offering a reliable and flexible way to study chemicals' genotoxicity. To the extent of our knowledge, only two other protocols were developed for filamentous fungi over the last 15 years, *Neurospora crassa* (Bhanoori and Venkateswerlu 1998) and *Sordaria macrospora* (Hahn and Hock 1999). Since *A. gossypii* is one of the most sensitive fungal species to oxidative stress (Nikolaou *et al.* 2009), a trait that is closely related with riboflavin overproduction (Walther and Wendland 2012; Kavitha and Chandra 2009), this protocol becomes a very useful tool to study the unexplored link between genome integrity and riboflavin overproduction in this filamentous fungus.

Light exposure during growth affects riboflavin production and increases DNA damage and intracellular oxidation in *A. gossypii*

Aiming its biotechnological exploitation, the key reactions and pathways for riboflavin overproduction in *A. gossypii* have been studied at extremely comprehensive levels (Aguiar, Silva and Domingues 2015). On the other hand, the regulatory and

Table 2. Colony radial growth and specific riboflavin production for the *A. gossypii* strains ATCC 10895 and A8, measured after 3 days of growth on agar-solidified AFM at 30°C, in the dark or exposed to light. Data are presented as mean ± standard deviation of at least five biological replicates. Superscript letters represent significant differences between specific riboflavin productions by the same strain under different conditions.

Strain (condition)	Radial growth (mm)	Riboflavin (mg $g_{dry\ weight}^{-1}$)
ATCC 10895	28 ± 1	2 ± 1
ATCC 10895 (light)	26 ± 2	2 ± 1
A8	16 ± 2	15 ± 3[a]
A8 (light)	17 ± 1	22 ± 3[b]

[a,b]$P < 0.01$.

ecological mechanisms behind this trait remain largely unknown. The overproduction of riboflavin by *A. gossypii* has been assumed as a stress response of the fungus due to its connection with oxidative stress (Walther and Wendland 2012; Kavitha and Chandra 2014) and has also been suggested to benefit the fungus by protecting its spores against UV irradiation (Stahmann *et al.* 2001). However, the interaction of endogenous riboflavin with UV light is known to cause cellular damages, particularly at the DNA level (Lloyd *et al.* 1990; Besaratinia *et al.* 2007; Alam, Iqbal and Naseem 2015). Thus, to assess the effects of riboflavin overproduction on the *A. gossypii* genome integrity, after validation of the *Ashbya* Comet Assay, it was used to measure the DNA damage in two different strains: the sequenced strain ATCC 10895 and the riboflavin-overproducing strain A8, which is able to produce up to 10-fold more riboflavin than strain ATCC 10895 (Table 2). To investigate the influence of light on DNA damage, these strains were incubated in the dark or under exposure to a fluorescent lamp emitting light with a spectral wavelength distribution close to that of the sunlight (Tarrant 1968). While no differences were detected between the comet tail length of the two strains when they were grown in the dark (Fig. 3A), significantly longer comet tails were observed in strain A8 when exposed to light in comparison with strain ATCC 10895 under the same condition (Fig. 3A).

The filamentous fungus *A. gossypii* occurs in nature mainly in the warmer parts of the planet and therefore its exposure to light (sunlight) should be high (Stahmann *et al.* 2001; Dietrich *et al.* 2013). The results obtained here with light, at intensity near to that used to maintain microalgae cultures (Fernandes *et al.* 2013), strongly indicate that the overproduction of riboflavin increases the accumulation of DNA damage. The role of riboflavin as a photosensitizer with effects at the physiological and molecular levels was already reported in microorganisms (Lloyd *et al.* 1990) and in higher eukaryotes (Besaratinia *et al.* 2007; Alam, Iqbal and Naseem 2015). In their work with *Escherichia coli*, Lloyd and colleagues (Lloyd *et al.* 1990) demonstrated that a riboflavin auxotrophic strain exposed to a broad spectrum near-UV light (320–400 nm) presented decreased survival rates when subjected to non-limiting riboflavin supplementation, as compared to limiting riboflavin supplementation. In turn, the survival rates of the parental strain (prototrophic for riboflavin) were practically unaffected by this type of light exposure when subjected to the same supplementation conditions. Therefore, the susceptibility of this auxotrophic strain to the photosensitizer effects of riboflavin should derive from its necessity to uptake riboflavin from the medium (Lloyd *et al.* 1990). When exposed to a similar light spectrum, embryonic fibroblasts of mice treated with riboflavin displayed increased DNA damage compared to non-treated ones (Besaratinia *et al.* 2007). Moreover, diabetic mice treated with riboflavin and exposed to a fluorescent lamp also accumulated more DNA damage than those without treatment (Alam, Iqbal and Naseem 2015). Thus, one can assume that the accumulation of riboflavin by vegetative cells of *A. gossypii* may act as a stressor when light exposure is not avoided.

Following the reported indications that riboflavin acts as a photosensitizer through the generation of ROS from the excitation of its isoalloxazine ring (Alam, Iqbal and Naseem 2015), evidence of its photo-induced oxidative effects in *A. gossypii* was found using the fluorescent redox-sensitive probe H_2DCFDA. This lipophilic probe has the ability to permeate the cells. Therein, it is deacetylated to H_2DCF by esterases, becoming hydrophilic and consequently held inside the cells. When oxidants are present it oxidizes to DCF, whose fluorescence can be detected at an excitation/emission range of 492–495/517–527 nm (Cruz *et al.* 2016). As shown in Fig. 4A, exposure to light induced a significant increase in the fluorescence intensity of riboflavin-overproducing cells (from strain A8) loaded with H_2DCFDA, revealing an increased intracellular oxidation (higher ROS accumulation) under this condition. Conversely, the non-overproducing strain ATCC 10895 presented residual

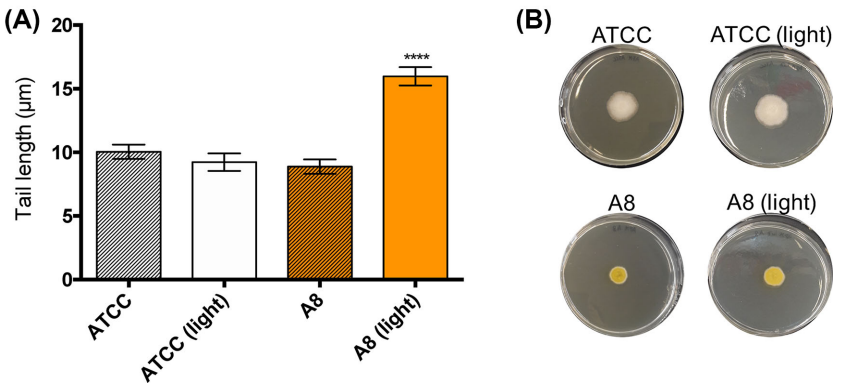

Figure 3. Exposure to light during growth induces DNA damage in the *A. gossypii* riboflavin-overproducing strain A8, but not in strain ATCC 10895. **(A)** Data are presented as mean ± standard error of the mean of the tail length of at least 40 comets obtained from two independent experiments for each condition (as described in Materials and Methods). Before harvesting, cells were grown for 3 days on agar-solidified AFM at 30°C, in the dark or exposed to light. Asterisks represent significant differences between strains and conditions. ****$P < 0.0001$. **(B)** Representative images of the colonies from where cells were harvested for DNA damage measurement with the *Ashbya* Comet Assay.

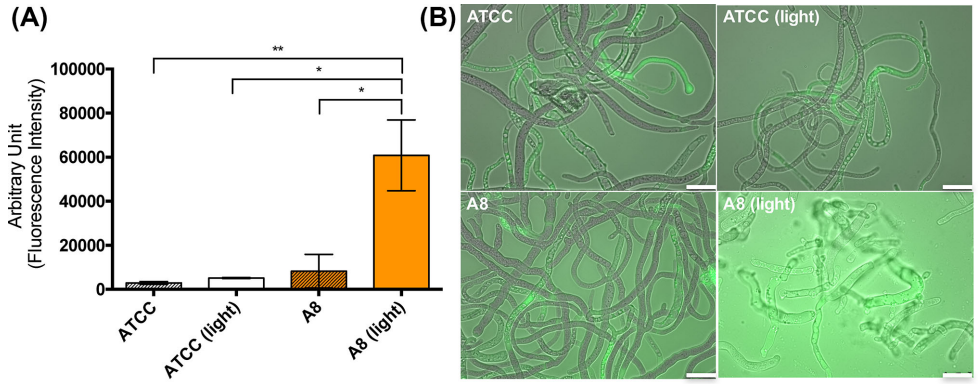

Figure 4. Exposure to light during growth induces intracellular ROS accumulation in the *A. gossypii* riboflavin-overproducing strain A8, but not in strain ATCC 10895. **(A)** Fluorescence intensity of cell suspensions loaded with H_2DCFDA determined by fluorimetry (as described in Material and Methods) presented as mean ± standard deviation of two biological replicates. Asterisks represent significant differences between strains and conditions. $^*P < 0.05$ and $^{**}P < 0.01$. **(B)** Representative overlaid images (obtained from bright-field and fluorescence microscopy) of *A. gossypii* cell suspensions loaded with H_2DCFDA. White bar = 25 μm. Before harvesting, cells were grown for 3 days on agar-solidified AFM at 30°C, in the dark or exposed to light.

intracellular oxidation in both conditions. Additionally, the representative images obtained by microscopy clearly depicted the high oxidation level of the riboflavin-overproducing strain A8 when exposed to light (Fig. 4B). A strain-related bias of these results can be excluded, since these effects were exclusively found with light exposure, having the riboflavin-overproducing strain presented statistically similar values to the non-overproducing strain when it was cultivated in the dark. Taking this into account, these results indicate that riboflavin, accumulated intracellularly by *A. gossypii*, acts as a photosensitizer agent that causes DNA damage through a mechanism triggered by oxidative stress.

Over the years, riboflavin overproduction has been interconnected with sporulation and environmental stress. The link of this trait with sporulation has been established either through the addition of external second messengers (cAMP) to the medium (Stahmann *et al.* 2001) or through the deletion of positive regulators of sporulation, such as the gene *AgSOK2* (Wasserstrom *et al.* 2017). In both cases, a concomitant negative effect towards sporulation and riboflavin production was observed. In turn, environmental conditions, in the form of nutritional (Schlösser *et al.* 2007) or oxidative (Walther and Wendland 2012; Kavitha and Chandra 2014) stresses have also been connected with riboflavin overproduction. In the latter case, addition to the medium of exogenous oxidative agents (H_2O_2 and menadione) increased riboflavin production through the oxidative stress-responsive transcription factor AgYap1 (Walther and Wendland 2012). In light of these observations, the overproduction of riboflavin by *A. gossypii* was proposed to serve as an ecological defense mechanism for this insect-dependent plant pathogen against the harsh environmental conditions (rich in ROS and alkaloids) it may face in its natural habitats (Walther and Wendland 2012; Dietrich *et al.* 2013). Our results show that riboflavin-overproducing strains are highly susceptible to photo-induced oxidative DNA damage (Figs 3 and 4), an event accompanied by increased riboflavin production (Table 2). The fact that this increase in riboflavin production was only observed in cells presenting high intracellular ROS accumulation (Fig. 4) reinforces the link between oxidative stress and riboflavin overproduction by *A. gossypii*. Since the production of ROS, via consumption of oxygen in a so-called oxidative burst, is one of the earliest plant's defense strategies against pathogenic infections, the overproduction of riboflavin may act as a scavenging mechanism that protects both *A. gossypii* and its insect vectors against this plant's attack (Walther and Wendland 2012; Dietrich *et al.* 2013). While oxidative stress has been shown to trigger riboflavin production (Walther and Wendland 2012; Kavitha and Chandra 2014), our results suggest that the interaction of riboflavin with light leads to ROS-mediated oxidative stress and DNA damage in overproducing cells. Previously, riboflavin overproduction was suggested to play a photoprotectant role for the spores of *A. gossypii*, as the addition of external riboflavin to spore suspensions produced a photo-protective effect against UV light (Stahmann *et al.* 2001). However, here we show that riboflavin acts as a photosensitizer in *A. gossypii* cells. While the ability of *A. gossypii* spores to uptake riboflavin is unknown, their hydrophobic nature may prevent such mechanism (Stahmann *et al.* 2001). Therefore, one can assume that riboflavin exerts negative photosensitizing effects mainly when it is accumulated intracellularly. Supporting evidence for this can also be found in *E. coli* (Lloyd *et al.* 1990). Since riboflavin produced by *A. gossypii* is predominantly stored in the vacuole or excreted to the extracellular medium (Förster *et al.* 1999), one may presume that *A. gossypii* evolved this adaptation to prevent the photosensitizing effects of riboflavin here described, as already hypothesized by Stahmann *et al.* (2001).

Finally, it is worth to note that exposure to light led to a 1.5-fold increase in riboflavin production by the overproducing strain A8 in comparison with the same strain cultivated in the dark (Table 2). Exploration of the oxidative trigger to design strategies to improve the biotechnological production of riboflavin by *A. gossypii* was already reported (Walther and Wendland 2012; Kavitha and Chandra 2009). These strategies focused in promoting oxidative stress with external agents (H_2O_2 and menadione) at doses that are not limiting for growth but sufficient to induce a response in riboflavin production. Here, using a similar rationale, we show that light can also be used to positively impact riboflavin production in riboflavin-overproducing *A. gossypii* strains, with the advantage that no extra chemical compound needs to be added to the medium. Altogether, the results reported here draw attention for the importance of controlling the exposure to light of biotechnological riboflavin production processes with *A. gossypii*. From now on, one must bear in mind that testing riboflavin-overproducing strains in dark steel fermenters or in glass bioreactors under random-light conditions may retrieve quite different results.

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REFERENCES

Aguiar TQ, Silva R, Domingues L. *Ashbya gossypii* beyond industrial riboflavin production: a historical perspective and emerging biotechnological applications. *Biotechnol Adv* 2015;**33**:1774–86.

Alam MM, Iqbal S, Naseem I. Ameliorative effect of riboflavin on hyperglycemia, oxidative stress and DNA damage in type-2 diabetic mice: Mechanistic and therapeutic strategies. *Arch Biochem Biophys* 2015;**584**:10–9.

Anderson CA, Umut K, Korndorf T *et al.*. Nuclear repulsion enables division autonomy in a single cytoplasm. *Curr Biol* 2013;**23**:1999–2010.

Azevedo F, Marques F, Fokt H *et al.* Measuring oxidative DNA damage and DNA repair using the yeast comet assay. *Yeast* 2011;**28**:55–61.

Besaratinia A, Kim S, Bates SE *et al.* Riboflavin activated by ultraviolet A1 irradiation induces oxidative DNA damage-mediated mutations inhibited by vitamin C. *Proc Natl Acad Sci* 2007;**104**:5953–8.

Bhanoori M, Venkateswerlu G. The alkaline single cell gel electrophoresis: a new test for assessing DNA single strand breaks in *Neurospora crassa*. *Mutat Res* 1998;**405**:29–34.

Brancato FP, Golding NS. The diameter of the mold colony as a reliable measure of growth. *Mycologia* 1953;**45**: 848–64.

Carmello JC, Pavarina AC, Oliveira R *et al.* Genotoxic effect of photodynamic therapy mediated by curcumin on *Candida albicans*. *FEMS Yeast Res* 2015;**15**:fov018.

Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol* 2004;**26**:249–61.

Cruz M, Antunes P, Paulo L *et al.* Antioxidant and dual dose-dependent antigenotoxic and genotoxic properties of an ethanol extract of propolis. *RSC Adv* 2016;**6**:49806–16.

Dietrich FS, Voegelli S, Kuo S *et al.* Genomes of *Ashbya* fungi isolated from insects reveal four mating-type loci, numerous translocations, lack of transposons, and distinct gene duplications. *G3 (Bethesda)* 2013;**3**:1225–39.

Fernandes B, Teixeira J, Dragone G *et al.* Relationship between starch and lipid accumulation induced by nutrient depletion and replenishment in the microalga *Parachlorella kessleri*. *Bioresour Technol* 2013;**144**:268–74.

Förster C, Santos MA, Ruffert S *et al.* Physiological consequence of disruption of the *VMA1* gene in the riboflavin overproducer *Ashbya gossypii*. *J Biol Chem* 1999;**274**: 9442–8.

Hahn A, Hock B. Assessment of DNA damage in filamentous fungi by single cell gel electrophoresis, comet assay. *Environ Toxicol Chem* 1999;**18**:1421–4.

Kavitha S, Chandra TS. Effect of vitamin E and menadione supplementation on riboflavin production and stress parameters in *Ashbya gossypii*. *Process Biochem* 2009;**44**:934–8.

Kavitha S, Chandra TS. Oxidative stress protection and glutathione metabolism in response to hydrogen peroxide and menadione in riboflavinogenic fungus *Ashbya gossypii*. *Appl Biochem Biotechnol* 2014;**174**:2307–25.

Langie S, Azqueta A, Collins AR. The comet assay: past, present, and future. *Front Genet* 2015;**6**:266.

Ledesma-Amaro R, Buey RM, Revuelta JL. Increased production of inosine and guanosine by means of metabolic engineering of the purine pathway in *Ashbya gossypii*. *Microb Cell Fact* 2015;**14**:58.

Liu LF, Desai SD, Li TK *et al.* Mechanism of action of camptothecin. *Ann N Y Acad Sci* 2000;**922**:1–10.

Lloyd RE, Rinkenberger JL, Hug BA *et al.* Growing *Escherichia coli* mutants deficient in riboflavin biosynthesis with non-limiting riboflavin results in sensitization to inactivation by broad-spectrum near-ultraviolet light (320-400 nm). *Photochem Photobiol* 1990;**52**:897–901.

Magalhães F, Aguiar TQ, Oliveira C *et al.* High-level expression of *Aspergillus niger* β-galactosidase in *Ashbya gossypii*. *Biotechnol Progr* 2014;**30**:261–8.

Møller P, Loft S, Ersson C *et al.* On the search for an intelligible comet assay descriptor. *Front Genet* 2014;**5**:217.

Nieland S, Stahmann KP. A developmental stage of hyphal cells shows riboflavin overproduction instead of sporulation in *Ashbya gossypii*. *Appl Microbiol Biotechnol* 2013;**97**:10143–53.

Nikolaou E, Agrafioti I, Stumpf M *et al.* Phylogenetic diversity of stress signaling pathways in fungi. *BMC Evol Biol* 2009;**9**:44.

Oliveira R, Johansson B. Quantitative DNA damage and repair measurement with the yeast comet assay. In: L. Bjergbæk (ed). *DNA Repair Protocols, Methods in Molecular Biology*. 2012. New York: Springer Science + Business Media, 101–9.

Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun* 1984;**123**:291–8.

Schlösser T, Wiesenburg A, Gätgens C *et al.* Growth stress triggers riboflavin overproduction in *Ashbya gossypii*. *Appl Microbiol Biotechnol* 2007;**76**:569–78.

Silva R, Aguiar TQ, Domingues L. Blockage of the pyrimidine biosynthetic pathway affects riboflavin production in *Ashbya gossypii*. *J Biotechnol* 2015;**193**:37–40.

Stahmann KP, Arst HN, Jr, Althöfer H *et al.* Riboflavin, overproduced during sporulation of *Ashbya gossypii*, protects its hyaline spores against ultraviolet light. *Environ Microbiol* 2001;**3**:545–50.

Tarrant AWS. The spectral power distribution of daylight. *Lighting Res Technol* 1968;**33**:75–82.

Walther A, Wendland J. *Yap1*-dependent oxidative stress response provides a link to riboflavin production in *Ashbya gossypii*. *Fungal Genet Biol* 2012;**49**:697–707.

Wasserstrom L, Dünkler A, Walther A *et al.* The APSES protein Sok2 is a positive regulator of sporulation in *Ashbya gossypii*. *Mol Microbiol* 2017;**106**:949–60.