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Rhamnolipids inhibit aflatoxins production in *Aspergillus flavus* by causing structural damages in the fungal hyphae and down-regulating the expression of their biosynthetic genes

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ABSTRACT

Aflatoxins are hepatotoxic and carcinogenic fungal secondary metabolites that usually contaminate crops and represent a serious health hazard for humans and animals worldwide. In this work, the effect of rhamnolipids (RLs) produced by *Pseudomonas aeruginosa* #112 on the growth and aflatoxins production by *Aspergillus flavus* MUM 17.14 was studied *in vitro*. At concentrations between 45 and 1500 mg/L, RLs reduced the mycelial growth of *A. flavus* by 23–40% and the production of aflatoxins by 93.9–99.5%. Purified mono-RLs and di-RLs exhibited a similar inhibitory activity on fungal growth. However, the RL mixture had a stronger inhibitory effect on aflatoxins production at concentrations up to 190 mg/L, probably due to a synergistic effect resulting from the combination of both congeners. Using transmission electron microscopy, it was demonstrated that RLs damaged the cell wall and the cytoplasmic membrane of the fungus, leading to the loss of intracellular content. This disruptive phenomenon explains the growth inhibition observed. Furthermore, RLs down-regulated the expression of genes *aflC*, *aflE*, *aflP* and *aflQ* involved in the aflatoxins biosynthetic pathway (6.4, 44.3, 38.1 and 2.0-fold, respectively), which is in agreement with the almost complete inhibition of aflatoxins production. Overall, the results herein gathered demonstrate for the first time that RLs could be used against aflatoxigenic fungi to attenuate the production of aflatoxins, and unraveled some of their mechanisms of action.

1. Introduction

The contamination of foodstuffs and agricultural commodities by mycotoxin-producing fungi is a severe problem that causes substantial economic and health impacts due to losses in crop yield and quality, and their toxicity to humans and animals (Ren et al., 2020). Mycotoxins are a heterogeneous group of extremely toxic secondary metabolites synthesized by certain filamentous fungi that exhibit carcinogenic, mutagenic, immunosuppressive and teratogenic activities. Species belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* are among the most frequently reported agents of food and feed contamination, and also the primary source of mycotoxins in a wide variety of agricultural products (Lee and Ryu, 2017). The primary cause of human exposure to mycotoxins is the consumption of contaminated crops or foods prepared from them. For instance, it is estimated that approximately 25% of the cereals produced worldwide are contaminated with mycotoxins (Liew and Mohd-Redzwan, 2018). Consequently, maximum acceptable concentrations for specific mycotoxins in food and feed are usually established, although the permissible threshold levels vary widely for different crops in different countries (Lee and Ryu, 2017).

Aflatoxins are one of the most relevant groups of mycotoxins, being *Aspergillus flavus* and *Aspergillus parasiticus* the main producers (Zhao et al., 2020). Among other relevant crops, rice and corn, which represent a significant fraction of the staple food diet in several countries, are usually contaminated with aflatoxins. In developing countries, outbreaks of acute intoxications (which can lead to death), due to a short exposure to a high concentration of aflatoxins have been reported (Wild and Gong, 2010). In developed countries, the most common is chronic toxicity, due to the continuous consumption of food and feed containing low concentrations of aflatoxins, which results in immunosuppression and the development of different types of cancer (Marchese et al., 2018). Aflatoxin B_1 (AFB_1) is considered the most toxic aflatoxin and the most

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potent carcinogen found in nature, being classified as a Group 1 carcinogen by the International Agency for Research on Cancer. Although the liver is the main target of AFB_1, it has also been associated to the development of lung, colon, breast, esophageal and gastric cancer (Marchese et al., 2018; Ren et al., 2020; Wu et al., 2017).

The levels of aflatoxins in food and feed commodities should be reduced to the lowest technologically possible. Accordingly, several strategies (including chemical, physical and biological methods) have been investigated to reduce fungal and aflatoxins contamination in crops. However, such methods are usually limited by their partial efficiency, impairment of some food quality parameters or for leaving toxic residues in the products (Hernández-Falcón et al., 2018; Zhao et al., 2020). Furthermore, due to the difficulty of removing aflatoxins from contaminated products, the prevention of their biosynthesis remains the best solution to avoid food contamination. The use of synthetic fungicides may be a useful strategy to control fungal contamination at pre- and post-harvest stages in crops. However, these compounds can be detrimental to human and environmental health, and their indiscriminate use can promote the development of resistant fungal strains (Moon et al., 2018; Ren et al., 2020; Sha et al., 2012; Tleuova et al., 2020; Zhao et al., 2020). In that sense, biological control appears to be the most promising approach to manage aflatoxins contamination in crops. The applicability of antagonistic microbial strains for this purpose has been widely demonstrated in the last years, and it is based in four main activities: exclusion of fungal pathogens due to competition for nutrients and space; production of metabolites that inhibit the growth of pathogens; production of metabolites that inhibit the production of aflatoxins; and degradation of produced aflatoxins (Veras et al., 2016). As recently reviewed by Ren et al. (2020), around 50 different microbial species (including bacteria, yeasts and filamentous fungi) have been reported as biocontrol agents against aflatoxigenic fungi. Among the most studied are *Bacillus* and *Lactobacillus* spp., which produce a wide variety of inhibitory compounds (*e.g.* antibiotics, biosurfactants, bacteriocins, organic acids and enzymes) and are usually recognized as safe microorganisms. Due to their ability to reduce the fungal growth and the production of aflatoxins, lipopeptide biosurfactants produced by *Bacillus* spp. have been widely studied for application as biopesticides for plant and post-harvest protection. These compounds are easily biodegradable in soils, being a healthier and environmentally-friendly alternative to synthetic fungicides, as they display a good balance between efficacy and preservation of the environment (Crouzet et al., 2021; González Pereyra et al., 2018; Veras et al., 2016). Furthermore, lipopeptide biosurfactants can also stimulate the plant immune system, improving plant protection against fungal pathogens (Crouzet et al., 2021), and they are already included in the formulation of fungicides (*e.g.* Serenade ASO, commercialized by Bayer Crop Science).

Rhamnolipids (RLs) are a class of glycolipid biosurfactants produced mainly by strains of *Pseudomonas aeruginosa*. RLs comprise a hydrophilic moiety, consisting of one (mono-RLs) or two (di-RLs) rhamnose molecules, linked to a hydrophobic chain, consisting of one or two β-hydroxy fatty acids. Different RL congeners can be found, depending on the length of the fatty acid units (8–16 carbon atoms) and their degree of saturation (Fig. 1). RLs are usually produced as mixtures of different congeners, and to date more than 60 different RLs have been identified. Besides their excellent surface-active properties, RLs have been reported to exhibit interesting biological activities, including antifungal activity (Borah et al., 2015; Robineau et al., 2020; Rodrigues et al., 2017). However, their effect on the production of aflatoxins has not been reported yet.

The aim of this work was to study the effect of RLs on the growth and aflatoxins production in *A. flavus* MUM 17.14. For that purpose, the RL mixture produced by *P. aeruginosa* #112, as well as the purified mono-RL and di-RL congeners were evaluated. In order to study the possible mechanisms involved in growth and aflatoxins production inhibition, the ultrastructural damages caused by RLs on *A. flavus* MUM 17.14 were studied through transmission electron microscopy (TEM). Finally, the effect of RLs in the expression of some genes involved in fungal sporulation and aflatoxins production was also assessed.

2. Material and methods

2.1. Fungal strain

A. flavus MUM 17.14, which produced AFB_1, was obtained from the culture collection of Micoteca da Universidade do Minho (MUM), Portugal. It was maintained at −80 °C in sterile demineralized water supplemented with glycerol (20%, *v*/v). Whenever required, frozen stocks were streaked on Malt Extract Agar (MEA, Blakeslee's formula) plates and incubated at 25 °C for 7 days. The agar plates were stored at 4 °C no longer than 3 weeks. The composition of MEA medium was (g/L): malt extract, 20; glucose, 20; peptone, 1; agar, 20; pH 5.5.

2.2. Rhamnolipids

The RLs used in this study were purified and characterized in our previous works (Gudiña et al., 2016; Rodrigues et al., 2017). Briefly, the RL mixture produced by *P. aeruginosa* #112 was purified through liquid column chromatography using the polystyrene resin Amberlite XAD-2 (Sigma-Aldrich Co., USA). The purity of the RL mixture, estimated through HPLC (Hruzová et al., 2020) and using 90% pure RLs (R90,

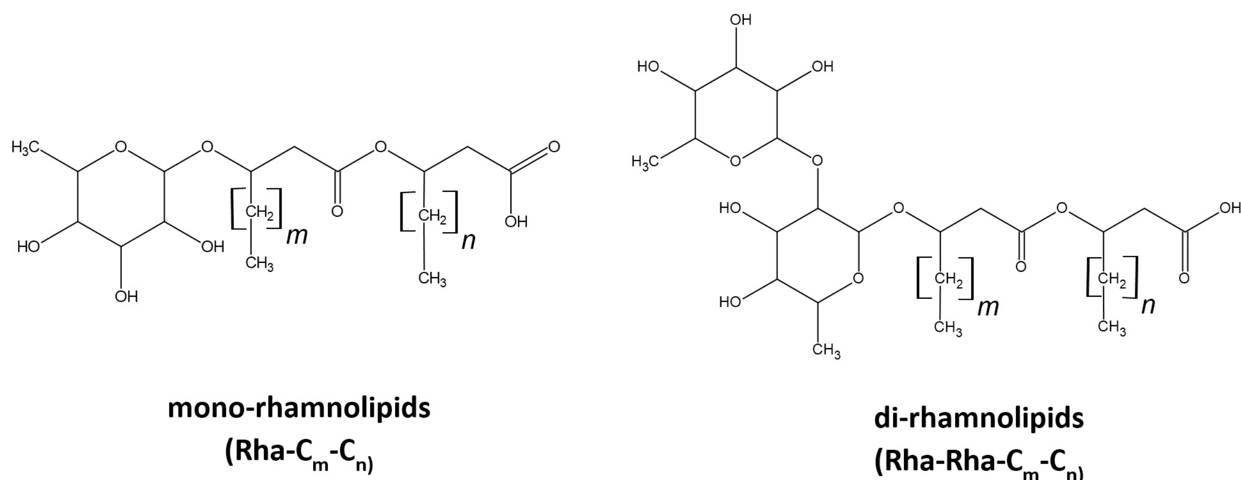

Fig. 1. Structure of mono- and di-rhamnolipids. n, m = 4, 6, 8, 10 or 12.

Sigma-Aldrich Co., USA) as reference was 92%. The purified RL mixture contained mono-RLs (Rha-C_{10}-C_{10} 41%; Rha-C_{10} 16%; Rha-C_{10}-C_{12} 5%) and di-RLs (Rha-Rha-C_{10}-C_{10} 25%; Rha-Rha-C_{10}-C_{12} 6%; Rha-Rha-C_{10} 5%; Rha-Rha-C_{10}-C_8 2%) (Gudiña et al., 2016). The mono- and di-RL congeners present in the RL mixture were subsequently separated through silica gel column chromatography (Gudiña et al., 2016; Rodrigues et al., 2017).

2.3. Effect of RLs on fungal growth and aflatoxins production

The antifungal activity of the RLs produced by *P. aeruginosa* #112 against *A. flavus* MUM 17.14 was evaluated in Petri dishes (55 mm diameter) containing MEA medium supplemented with the freeze-dried RLs (RL mixture and purified mono- and di-RL congeners) at different concentrations (45–1500 mg/L). The agar plates were inoculated with 10 μL of a spore suspension of *A. flavus* MUM 17.14 (prepared in sterile demineralized water with a concentration of 1×10^5 spores/mL, according to Rodrigues et al. (2017)) in the center of the plate. Subsequently, the plates were incubated at 25 °C for 5 days. Control assays were performed using MEA medium without RLs. The fungal radial growth was determined by measuring the diameter of the growth zone. The percentage of radial growth inhibition relative to the control was calculated as follows:

$$Growth\ inhibition\ x\ (\%) = \left(1 - \frac{diameter\ x}{diameter\ c}\right) \times 100 \quad (1)$$

where *diameter x* (cm) represents the diameter of the mycelial growth in the medium with the treatment *x*, and *diameter c* represents the diameter of the fungal growth in the control. All the experiments were performed in triplicate.

The effect of RLs on the production of aflatoxins by *A. flavus* MUM 17.14 was studied using the same plates at the end of the incubation period (5 days). The entire content of each plate was cut into pieces, transferred to a 50 mL tube and extracted with 20 mL of a mixture containing acetonitrile:methanol:acetic acid (78:20:2, v/v/v) by agitation with vortex at high speed. The extracts were left overnight at room temperature in the dark. Subsequently, 2 mL of each extraction were filtered through a 0.2 μm syringe filter and analyzed by HPLC with fluorescence detection according to the methodology described by Guimarães et al. (2018). The detection (S/N of 2:1) and quantification limit (S/N of 10:1) of the method were 3.3 and 16.5 ng/mL, respectively. Repeatability was evaluated by calculating a pooled RSD using 24 samples performed in triplicate ($RSD_{pooled} = 19\%$). Mean recoveries (98 ± 9%) were evaluated by extracting plates containing MEA medium supplemented with 3 μg of AFB_1 in triplicate. The HPLC system used was equipped with a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a photochemical post-column derivatization reactor (PHRED unit, Aura Industries, USA) and a Jasco FP-920 fluorescence detector. The instrument and the chromatographic data were managed by a Varian 850-MIB data system interface and a Galaxie chromatography data system, respectively. A C_{18} reversed-phase YMC-Pack ODS-A analytical column (250 × 4.6 mm i.d., 5 μm particle size) connected to a guard column with the same stationary phase was used. The mobile phase used was a mixture of water:acetonitrile:methanol (3:1:1, v/v/v), and the column oven temperature was set to 30 °C. The injection volume was 30 μL, and the compounds were eluted at a flow rate of 1.0 mL/min for a 20 min isocratic run. The fluorescence of aflatoxins was recorded at excitation and emission wavelengths of 365 and 435 nm, respectively. Calibration curves of aflatoxins were prepared in mobile phase using a certified aflatoxins standard solution mixture (46304-U, Sigma-Aldrich, USA) at a concentration range of 50–1000 ng/mL. Aflatoxins were quantified by comparing the peak areas in the samples with those of the calibration curves. All the analyses were performed in triplicate.

2.4. Effect of RLs on sporulation

To analyze the effect of RLs in the production of spores in *A. flavus* MUM 17.14, an aliquot containing 1×10^5 spores was spread onto 5 mL of MEA solid medium (containing 1500 mg RL mixture/L) in 15 mL tubes. The tubes were incubated in the dark at 25 °C for 5 days. At the end of the growth period, the spores were harvested by washing them out from the agar surface through the successive addition of 1 mL of a Tween 20 solution (0.01%, *v*/v) until a final volume of 10 mL. The number of spores in the suspensions was counted using a Neubauer improved cell counter (Marienfeld GmbH, Germany). MEA without RLs was used as control. The assays were performed in triplicate.

2.5. Isolation of total RNA and gene expression assays

100 mL flasks containing 50 mL of MEA liquid medium supplemented with 1500 mg RL mixture/L were inoculated with 1×10^6 spores of *A. flavus* MUM 17.14. MEA medium without RLs was used as control. The cultures were incubated at 28 °C and 150 rpm for 3 days. Subsequently, the cultures were centrifuged (9000 ×*g*, 20 min), the supernatants were removed and the mycelia were collected and immediately frozen at −80 °C for total RNA extraction. The supernatants were stored at −20 °C and used to evaluate the production of aflatoxins through HPLC, as described above. Four replicates of each condition were performed.

The mycelia recovered from the different assays were ground to a fine powder using a mortar and a pestle under liquid nitrogen. Subsequently, total RNA was extracted using the Plant/Fungi Total RNA Purification Kit (NORGEN Biotek Corporation, Canada), followed by treatment with RNase-free DNase I (Thermo Fisher Scientific Inc., USA) to degrade contaminating genomic DNA, according to the manufacturer's instructions. The concentration and purity of the extracted RNA samples was assessed by measuring the absorbance at 260/280 nm using a NanoDrop™ One/One^C Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific Inc., USA). The integrity of the RNA was evaluated through agarose gel (0.8%, *w/v*) electrophoresis. The purified RNA was stored at −80 °C until use. The complementary DNA (cDNA) was synthesized using the Xpert cDNA Synthesis Mastermix (Grisp Lda., Portugal), following the manufacturer's instructions. Real-Time PCR (qPCR) assays were performed in a CFX96™ thermal cycler (Bio-Rad, USA). Gene-specific primers synthesized by Metabion International AG (Germany) and Eurofins Genomics (Germany) were used to analyze the expression of some of the genes involved in aflatoxins biosynthesis (*aflC*, *aflE*, *aflP*, *aflQ*, *aflR* and *aflS*) and sporulation (*brlA*, *abaA* and *wetA*) (Table 1). Each reaction contained the optimized concentrations of cDNA and primers, and 5 μL of Xpert Fast SYBR (Grisp Lda., Portugal), in a final volume of 10 μL. Subsequently, they were incubated at 95 °C for 2 min, followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. A melting curve (65 to 95 °C, 1 °C/min) was generated at the end of the reaction to assess the specificity of the amplification and discard the presence of primer dimers. The β-*tubulin* gene was used to normalize the expression of the target genes. Appropriate controls (no template control, no reverse transcriptase control and negative control) were performed. The efficiency (*E*) of each set of primers was calculated by the Bio-Rad CFX96 Manager Software™ using a calibration curve obtained using 10-fold serial dilutions of cDNA from the control assays as a template, and the thermal cycling conditions described above. Relative expression levels and fold differences were calculated with the mathematical models proposed by Pfaffl (2001) using the threshold cycle (Ct) values obtained and the corresponding primer efficiencies. Four independent replicates of each condition were analyzed in triplicate.

2.6. Transmission electron microscopy (TEM) analysis

Samples of *A. flavus* MUM 17.14 grown in agar plates (MEA medium and MEA medium containing 1500 mg RL mixture/L) for 5 days were

Table 1
Primers used to study the expression of genes related to aflatoxins biosynthesis and sporulation through qPCR. *E* (%): primers efficiency. Tm (°C): primer melting temperature.

Target gene	Nucleotide sequence (5′ - 3′)	*E* (%)	Tm (°C)	Reference
aflC	Fw: TGCATGGCGATGTGGTAGTT Rv: GTAAGGCCGCGAGAGAAAG	103	60.04 58.33	Moon et al. (2018)
aflE	Fw: TCTAGCGCCGGTGTTCGT Rv: TTACCCCTTTCCAGCCATTG	101	61.37 57.77	Moon et al. (2018)
aflP	Fw: CGATCTTTTGGCCCAGGAT Rv: CAACCGTCATGTCGATCTGATT	98	63.00 63.60	Chang et al. (2012a)
aflQ	Fw: GTCGCATATGCCCCGGTCGG Rv: GGCAACCAGTCGGGTTCCGG	101	65.72 65.79	Wang et al. (2017)
aflR	Fw: GGGAACAAGAGGGCTACCGA Rv: TGCCAGCACCTTGAGAACG	103	61.26 60.60	Kong et al. (2010)
aflS	Fw: GGTCGTGCATGTGCGAATC Rv: GAGGGCAACAACCAGTGAGG	100	59.94 60.89	Kong et al. (2010)
abaA	Fw: TCTTCGGTTGATGGATGATTTC Rv: CCGTTGGGAGGCTGGGT	101	56.50 61.36	Han et al. (2016)
brlA	Fw: TATCCAGACATTCAAGACGCACAG Rv: GATAATAGAGGGCAAGTTCTCCAAAG	97	60.92 59.52	Chang et al. (2012a)
wetA	Fw: CCACAGCAGCCGATCCA Rv: CCCCTTGCAGGATGTCATG	102	59.35 58.20	Chang et al. (2012b)
β-*tubulin*	Fw: TTGAGCCCTACAACGCCACT Rv: TGGTTCAGGTCACCGTAAGAGG	101	61.76 61.67	Wang et al. (2017)

fixed by immersion in 2.5% glutaraldehyde and 2% paraformaldehyde (prepared in 0.1 M sodium cacodylate buffer (pH 7.4)) solution for 5 days. After washing and 2 h in post-fixating 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) solution, the samples were washed in buffer, incubated with 1% uranyl acetate overnight, washed in buffer, dehydrated through graded series of ethanol, and finally embedded in Epon (EMS). Ultrathin sections were cut at 50 nm on an RMC Ultramicrotome (PowerTome, USA) using a diamond knife and recovered to 200 mesh Formvar Ni-grids, followed by post staining using 2% uranyl acetate and saturated lead citrate solution. Visualization was performed at 80 kV in a JEM-1400 microscope (JEOL, Japan) and digital images were acquired using a CCD digital camera Orious 1100 W (Japan).

2.7. Statistical analysis

All data were expressed as the means ± standard deviation of at least three independent replicates. Results from aflatoxins production and fungal growth were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's HSD multiple comparisons test. Results from qPCR and sporulation assays were analyzed using the Student's *t*-test. All statistical analyses were performed at a significance level of 0.05 using the software GraphPad Prism 7 for Windows (GraphPad Software, La Jolla California, USA).

3. Results and discussion

3.1. Antifungal activity of RLs against Aspergillus flavus MUM 17.14

The development of new environmentally-friendly treatments to reduce or eliminate mycotoxins in food is of utmost importance; hence

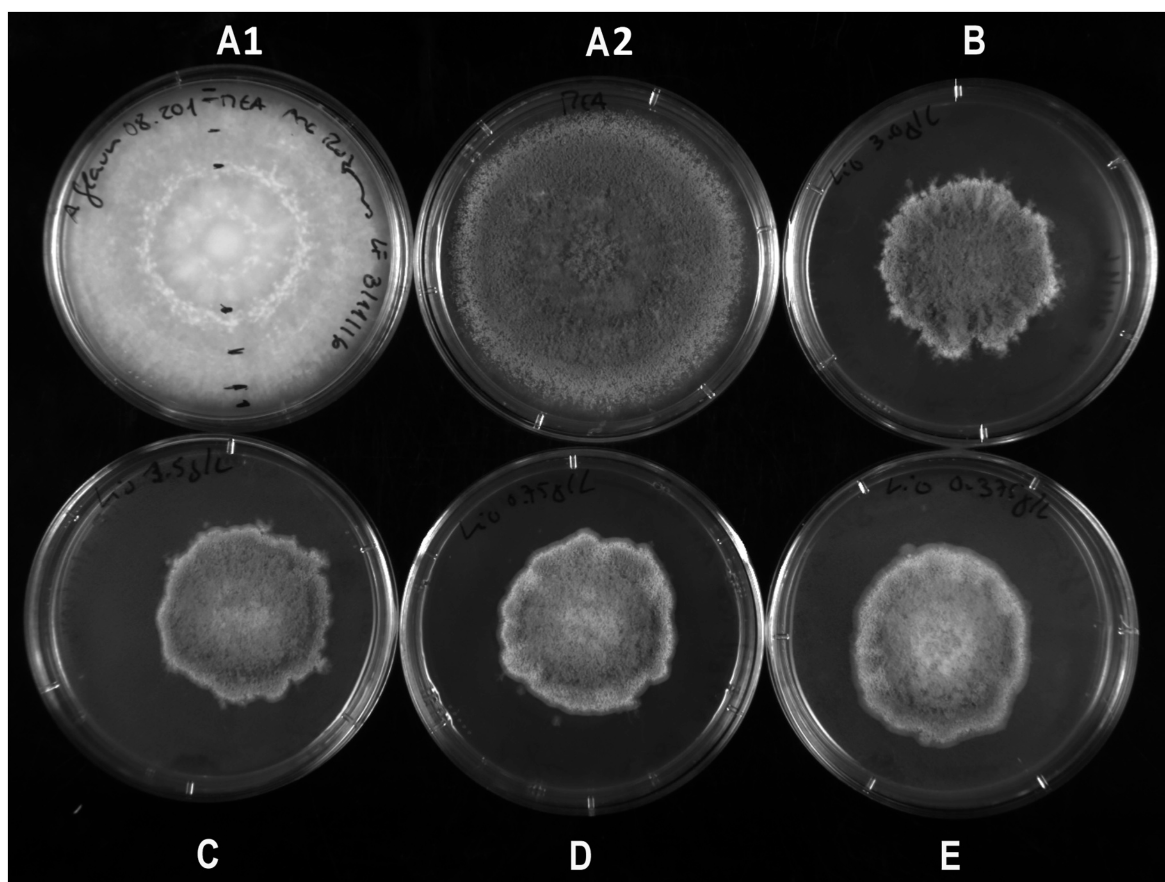

Fig. 2. Antifungal activity of the rhamnolipid (RL) mixture produced by *Pseudomonas aeruginosa* #112 at different concentrations against *Aspergillus flavus* MUM 17.14. A1: 0 mg_{RL}/L (reverse view); A2: 0 mg_{RL}/L (top view); B: 3000 mg_{RL}/L; C: 1500 mg_{RL}/L; D: 750 mg_{RL}/L; E: 375 mg_{RL}/L.

in this work, the effect of RLs on the growth of *A. flavus* MUM 17.14, as well as on its aflatoxins production, was studied. The antagonistic activity of RLs against *A. flavus* MUM 17.14 was first evaluated using the RL mixture at different concentrations (Fig. 2). As it can be seen in Table 2, similar growth inhibition percentages (34–40%) were obtained with the RL mixture at concentrations between 90 and 1500 mg/L. These results agree with previous studies that reported antifungal activity of RLs produced by *P. aeruginosa* and synthetic RL-derivatives against other genera of fungi, including *Botrytis*, *Colletotrichum*, *Fusarium* and *Phytophthora*, at concentrations between 25 and 450 mg/L (Borah et al., 2015; Kim et al., 2000; Reddy et al., 2016; Robineau et al., 2020; Sha et al., 2012). Regarding the purified mono- and di-RL congeners, they exhibited a similar inhibitory activity against *A. flavus* MUM 17.14, although other authors reported different activities for different RL congeners (Borah et al., 2015; Robineau et al., 2020). Only at the lowest and the highest concentrations tested, di-RLs exhibited a statistically significant ($p < 0.05$) higher antagonistic activity comparing to mono-RLs (Table 2).

As it can be seen from Table 2, RLs only partially inhibited the growth of *A. flavus* MUM 17.14, and a relationship between RLs concentration and growth inhibition was not observed. Similar results were obtained by Rodrigues et al. (2017) regarding the antifungal activity of RLs against *Aspergillus niger* and *Aspergillus carbonarius*. In that case, it was demonstrated that the antifungal activity of RLs against those fungi was related to their aggregation behavior, which can be modified by changing the pH or the ionic strength of the culture medium (Rodrigues et al., 2017).

Contrary to the mycelial growth, the production of spores was significantly increased ($p < 0.05$) by the RL mixture at a concentration of 1500 mg/L: from $6.57 \times 10^6 \pm 0.67 \times 10^6$ spores/mL to $1.82 \times 10^7 \pm 0.18 \times 10^7$ spores/mL. This can be explained as a stress response of *A. flavus* to the presence of RLs.

The internal structures of hyphae and spores of *A. flavus* MUM 17.14 grown in the presence of RLs (1500 mg RL mixture/L) were analyzed by TEM. Regarding the spores, TEM images from cultures grown in the presence of RLs (Fig. 3C) showed that they remained intact, electron-opaque, and their cell components were well arranged, as for the spores obtained from cultures grown without RLs (Fig. 3A). However, it could be observed a slight deformation on the spore structure compared to the round shape observed in control. In the non-treated hyphae (Fig. 3B), it was possible to observe a uniform shape, as well as an intact cell wall and membrane, a good internal organization and the presence of organelles, including Golgi complex, mitochondria and vacuoles with normal appearance and regular distribution. Contrarily, RLs seriously damaged the hyphal cell structure. In hyphae grown in the presence of RLs, it was possible to observe considerable damages in the cell wall, lysis and disappearance of the cell membrane, loss of mitochondria and other organelles, increased lipid content and lack of intracellular content (Fig. 3D).

Table 2
Growth inhibition percentages obtained for *Aspergillus flavus* MUM 17.14 with the rhamnolipid mixture and with the purified mono- and di-rhamnolipid congeners at different concentrations. The assays were performed at 25 °C for 5 days. The results represent the average of three independent experiments ± standard deviation.

	Growth Inhibition (%)		
[RL] mg/L	RL mixture	Mono-RL	Di-RL
0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
45	23.5 ± 2.8^{bB}	27.5 ± 1.5^{bB}	37.8 ± 0.0^{bA}
90	38.9 ± 1.8^{cA}	36.8 ± 3.1^{cA}	40.9 ± 2.3^{bA}
190	40.9 ± 1.8^{cB}	43.5 ± 2.3^{dAB}	47.2 ± 1.0^{cA}
375	34.7 ± 2.0^{cB}	47.2 ± 1.0^{dA}	46.1 ± 2.5^{cA}
750	39.5 ± 6.2^{cA}	46.6 ± 0.9^{dA}	46.1 ± 2.5^{cA}
1500	40.1 ± 2.4^{cB}	36.1 ± 4.5^{cB}	49.5 ± 2.7^{cA}

Different lower case letters within the same column and different capital letters within the same row indicate statistically significant differences ($p < 0.05$).

The mechanisms involved in the antifungal activity of RLs are not well established, and it is usually assumed that due to their detergent-like effect they interact with the biological membranes, disturbing their integrity and permeability by inducing the formation of pores and ion channels, which ultimately results in cell lysis (Sánchez et al., 2007). This is in accordance with the damages caused by RLs in the *A. flavus* hyphae herein observed (Fig. 3B and D), which supports that mechanism of action. The fact that RLs displayed more damages in the hyphae than in the spores can be due to the differences in the thickness of their cell walls.

Lipopeptide biosurfactants produced by *Bacillus* species have been reported to exhibit strong antifungal activity against aflatoxin-producing *A. flavus* strains (Afsharmanesh et al., 2014; Gong et al., 2014; Moyne et al., 2001). Among the different lipopeptide families (iturins, fengycins and surfactins), iturin A and bacillomycin D (both of them belonging to the iturin family) demonstrated to be the most effective, although they exhibited lower surface activity when compared with surfactin (Afsharmanesh et al., 2014; Gong et al., 2014). In the case of bacillomycin D, growth inhibition achieved 85% at a concentration of 200 mg/L (Gong et al., 2014). Several studies demonstrated that these biosurfactants alter the integrity of the fungal cell wall and the permeability of the cytoplasmic membrane of hyphae and spores of *A. flavus* (Afsharmanesh et al., 2014; Gong et al., 2014). According to Moyne et al. (2001), ergosterol and cholesterol could be the target of bacillomycin D. Ergosterol was also identified as the target for the antifungal compounds present in the cell-free supernatants of cultures of *Bacillus amyloliquefaciens* UTB2 and *B. subtilis* UTB3 (probably lipopeptide biosurfactants), which reduced the growth of the aflatoxigenic fungus *Aspergillus parasiticus* NRRL 2999 by 90% (Siahmoshteh et al., 2018). RLs display a lower inhibitory activity against *A. flavus* when compared with lipopeptide biosurfactants, which can be due to their different mechanisms of action resulting from their different structures.

3.2. Effect of RLs on aflatoxins production by Aspergillus flavus MUM 17.14

The effect of RLs on the production of aflatoxins by *A. flavus* MUM 17.14 was studied using the RL mixture and the purified mono- and di-RL congeners at different concentrations. Analyzing Table 3 it can be observed that the RL mixture almost completely inhibited AFB_1 production (93.9–99.5%) at all the concentrations tested. On the other hand, although at the highest concentration tested (1500 mg/L) the mono- and di-RLs inhibited AFB_1 production by 98–99%, their inhibitory activity at low concentrations was significantly lower ($p < 0.05$) comparing with the RL mixture. Furthermore, at concentrations between 190 and 1500 mg/L, di-RLs exhibited a statistically significant higher inhibition ($p < 0.05$) than mono-RLs. The high inhibitions observed for the RL mixture at concentrations up to 190 mg/L when compared with the purified mono- and di-RL congeners can be due to a synergistic effect resulting from their combination. Similar synergistic effects were previously reported for lipopeptide biosurfactants. Surfactin, despite its outstanding surface activity, exhibits a weak antifungal activity against *A. parasiticus*. However, it considerably increases the antifungal activity of other lipopeptide biosurfactants such as iturin A and fengycins (González Pereyra et al., 2018).

Similarly to the results herein obtained, previous works demonstrated an inhibition of aflatoxins production not directly related to the inhibition of fungal growth. Since aflatoxins are secondary metabolites, their production is not necessary for fungal growth. The cell-free supernatants of *Lactobacillus plantarum* UM55 cultures inhibited the growth of *A. flavus* MUM 17.14 by 32%, whereas the production of aflatoxins was reduced by 95%. This inhibitory effect was due to the presence of different organic acids that can act as specific inhibitors of aflatoxins production, being phenyllactic acid the most relevant (Guimarães et al., 2018).

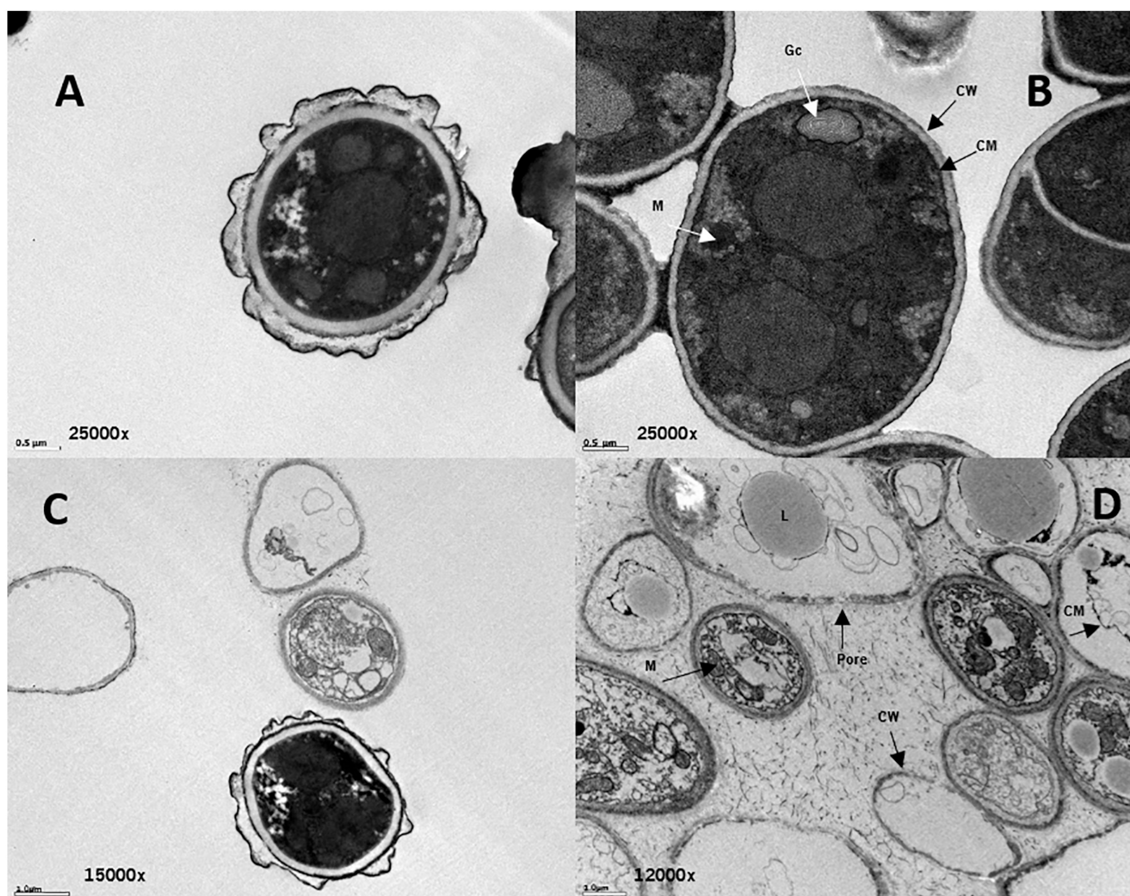

Fig. 3. Transmission electron microscopy sections of spores and hyphae of *Aspergillus flavus* MUM 17.14 grown in MEA plates for 5 days at 25 °C. A, B: MEA. C, D: MEA supplemented with 1500 mg rhamnolipid mixture/L. CW: cell wall; CM: cell membrane; Gc: Golgi's complex; M: mitochondria; L: lipid content.

Table 3
Aflatoxin B_1 (AFB_1) concentration and percentage of AFB_1 reduction (relative to the control) in cultures of *Aspergillus flavus* MUM 17.14 grown in the presence of the rhamnolipid mixture and the purified mono- and di-rhamnolipids at different concentrations. The results represent the average of three independent experiments ± standard deviation.

	AFB_1 concentration (ng/mL) (AFB_1 reduction (%))		
[RL] mg/L	RL mixture	Mono-RL	Di-RL
0	3393 ± 122^{a}	3393 ± 122^{a}	3393 ± 122^{a}
45	205 ± 19^{bC} (93.9 ± 0.5)	1297 ± 130^{bcB} (61.8 ± 0.4)	1850 ± 268^{bA} (45.5 ± 7.9)
90	218 ± 23^{bB} (93.6 ± 0.6)	1372 ± 189^{bA} (59.6 ± 6.6)	1658 ± 74^{bA} (51.1 ± 2.2)
190	212 ± 56^{bC} (93.7 ± 1.6)	1013 ± 132^{cdA} (70.1 ± 3.9)	565 ± 86^{cB} (83.3 ± 2.5)
375	74 ± 21^{bcB} (97.8 ± 0.6)	816 ± 160^{deA} (75.9 ± 4.7)	116 ± 50^{dB} (96.6 ± 1.4)
750	43 ± 18^{cB} (98.7 ± 0.5)	559 ± 65^{eA} (83.5 ± 1.9)	58 ± 1^{dB} (98.3 ± 0.0)
1500	16 ± 1^{cB} (99.5 ± 0.0)	72 ± 2^{fA} (97.9 ± 0.0)	22 ± 2^{dB} (99.3 ± 0.0)

Different lower case letters within the same column and different capital letters within the same row indicate statistically significant differences ($p < 0.05$).

The inhibitory activity of RLs on AFB_1 production was similar to those reported for lipopeptide biosurfactants in previous works. The production of AFB_1 by *A. flavus* A12 was reduced by 99.8% when it was grown in co-culture with two different *Bacillus* sp. strains due to the production of iturin A and surfactin (Veras et al., 2016). Likewise, when *A. parasiticus* NRRL 2999 was grown in co-culture with *Bacillus mojavensis* RC1A (which produced surfactin, iturin A and fengycin), the AFB_1 production was reduced by 97.5% (González Pereyra et al., 2018).

Although *Pseudomonas* species (different from *P. aeruginosa*) have been reported to inhibit the growth of aflatoxigenic fungi, as well as aflatoxins production, that inhibitory activity has never been related to the production of RLs (Mannaa et al., 2017; Yang et al., 2017).

3.3. Effect of RLs in the expression of genes involved in sporulation in Aspergillus flavus MUM 17.14

From the TEM images, it was possible to observe that RLs have a detrimental effect in the hyphal development, including lack and disorganization of the organelles (Fig. 3). This could lead to nutrient deprivation which is the primary signal inducing sporulation in many fungi (Wu et al., 2017). Furthermore, it was also demonstrated that spores production increased 2.8 times in the presence of RLs (Section 3.1). For that reason, the effect of RLs in the expression of regulatory genes involved in the sporulation of *A. flavus* MUM 17.14 was studied. The genes *brlA*, *abaA* and *wetA* encode essential transcription factors which constitute the central regulatory pathway that modulates the expression of the genes involved in the different stages of conidiophore development and sporogenesis in *A. flavus*. BrlA activates the expression of the gene *abaA*, and AbaA activates the expression of the gene *wetA* (Wu et al., 2017). It has been reported that a higher expression of the genes *brlA* and *abaA* is associated with a higher production of spores in *A. flavus* (Han et al., 2016).

As it can be seen in Fig. 4, at the RLs concentration tested (1500 mg/L), the genes *brlA* and *abaA* were down-regulated (2.4-fold and 1.7-fold,

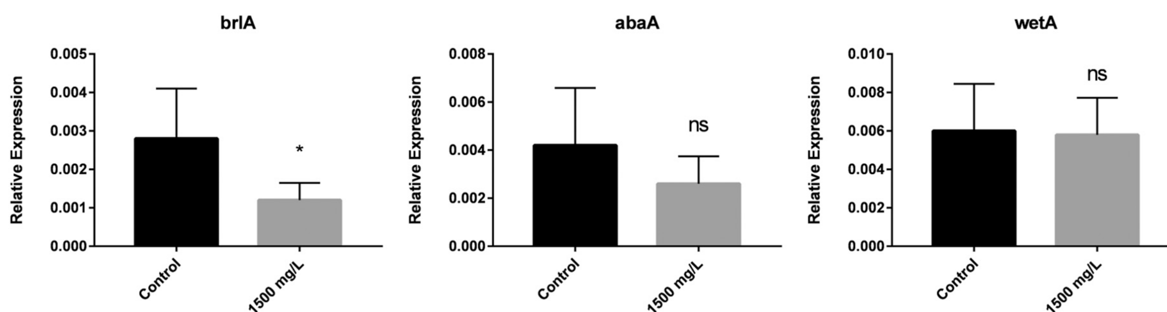

Fig. 4. Relative expression of the genes *brlA*, *abaA* and *wetA* in *Aspergillus flavus* MUM 17.14 grown in the presence of rhamnolipids (1500 mg RL mixture/L). The results correspond to four independent cultures analyzed in triplicate. The β-*tubulin* gene was used as internal control to normalize the expression data. ns: no significant differences ($p > 0.05$); * significant differences ($p < 0.05$) (determined by the Student's *t*-test).

respectively), although significant differences ($p < 0.05$) were only observed for the first one. Regarding the gene *wetA*, its expression was not affected by RLs. Consequently, the higher sporulation observed in the presence of RLs could not be explained by the changes in the expression of the genes studied, suggesting that RLs may have another mechanism of action. Defective expression of the genes *brlA*, *abaA* and *wetA* can result in the formation of aberrant conidiophores and spores with defective cell walls (Chang et al., 2012a; Wu et al., 2017), which was not observed in the TEM images of spores exposed to RLs (Fig. 3C) and agrees with the results obtained for the expression of these genes.

3.4. Effect of RLs in the expression of genes involved in aflatoxins biosynthesis in Aspergillus flavus MUM 17.14

In the previous sections, it was demonstrated that RLs (RL mixture, mono- and di-RLs) inhibited almost completely the production of aflatoxins in *A. flavus* MUM 17.14. Accordingly, the effect of RLs in the expression of six genes involved in aflatoxins production was studied: two regulatory genes (*aflR* and *aflS*), and four structural genes that participate in different stages of their biosynthesis (*aflC*, *aflE*, *aflP* and *aflQ*) (Fig. 5). These studies were performed growing *A. flavus* MUM 17.14 in liquid MEA medium, and it was confirmed that the RL mixture (1500 mg/L) completely inhibited AFB_1 production under those conditions.

At least 23 enzymatic reactions and 27 genes clustered in an 80 Kb operon have been identified in the aflatoxins biosynthetic pathway in *A. flavus* and *A. parasiticus* (Ren et al., 2020). The genes *aflR* and *aflS* are the most important regulators of the pathway. AflR and AflS interact to form a transcriptional activation complex that recognizes a consensus sequence in the promoter region of the structural genes from the pathway (Kong et al., 2010; Zhao et al., 2020). Regarding the structural genes studied, the *aflC* gene encodes a polyketide synthase, involved in the synthesis of the first stable aflatoxin precursor (norsolorinic acid (NOR)). The gene *aflE* encodes a short-chain aryl alcohol dehydrogenase that participates in the conversion of NOR into averantin (AVN). The *O*-methyltransferase encoded by the gene *aflP* catalyzes the conversion of sterigmatocystin (ST) and dihydrosterigmatocystin (DHST) into *O*-methylsterigmatocystin (OMST) and dihydro-*O*-methylsterigmatocystin (DHOMST), respectively. The P-450 monooxygenase encoded by the gene *aflQ* participates in the conversion of OMST and DHOMST into AFB_1/AFG_1 and AFB_2/AFG_2, respectively (Fig. 5) (Chang et al., 2012a; Moon et al., 2018; Wang et al., 2017).

As it can be seen in Fig. 6, the treatment with RLs significantly decreased the expression of the genes *aflC* (6.4-fold), *aflE* (44.3-fold), *aflP* (38.1-fold) and *aflQ* (2.0-fold), which is in accordance with the complete inhibition of AFB_1 production. However, the expression of the regulatory genes studied (*aflR* and *aflS*) was almost unaffected.

A similar relationship between gene expression and aflatoxins production in *A. flavus* was previously reported. Inhibition of aflatoxins production by different biocontrol agents (*e.g. Bacillus megaterium, Eurotium cristatum*, *Pichia anomala* or *Streptomyces alboflavus*) was associated with a down-regulation of the genes from the aflatoxins

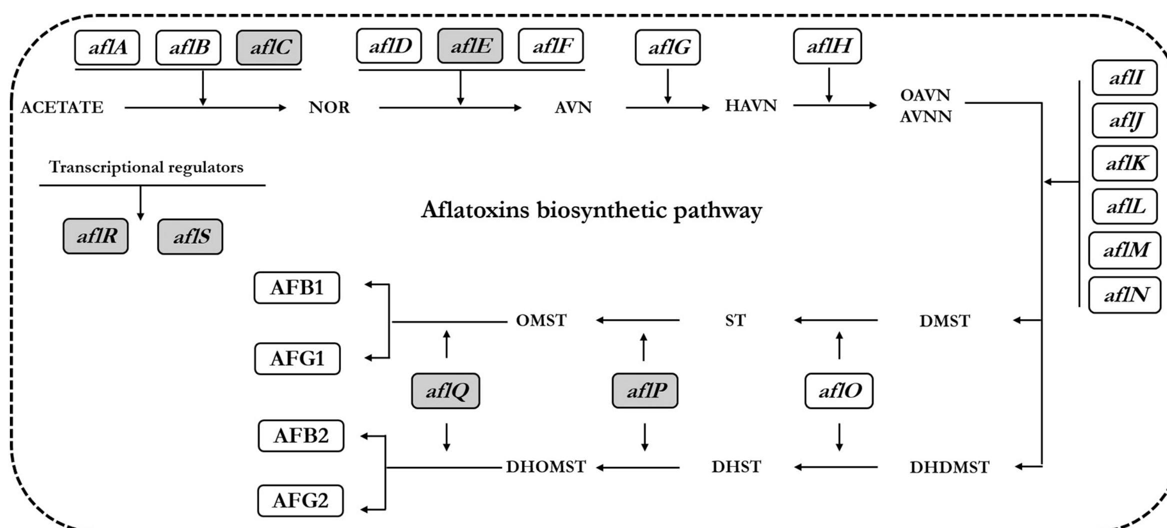

Fig. 5. Schematic representation of the aflatoxins biosynthetic pathway (adapted from Yu et al. (2004)) showing the studied genes. AVN: averantin; AVNN: averufanin; DHDMST: dihydrodemethylsterigmatocystin; DHOMST: dihydro-*O*-methylsterigmatocystin; DHST: dihydrosterigmatocystin; DMST: demethylsterigmatocystin; HAVN: 5′-hydroxyaverantin; NOR: norsolorinic acid; OAVN: oxoaverantin. OMST: *O*-methylsterigmatocystin; ST: sterigmatocystin.

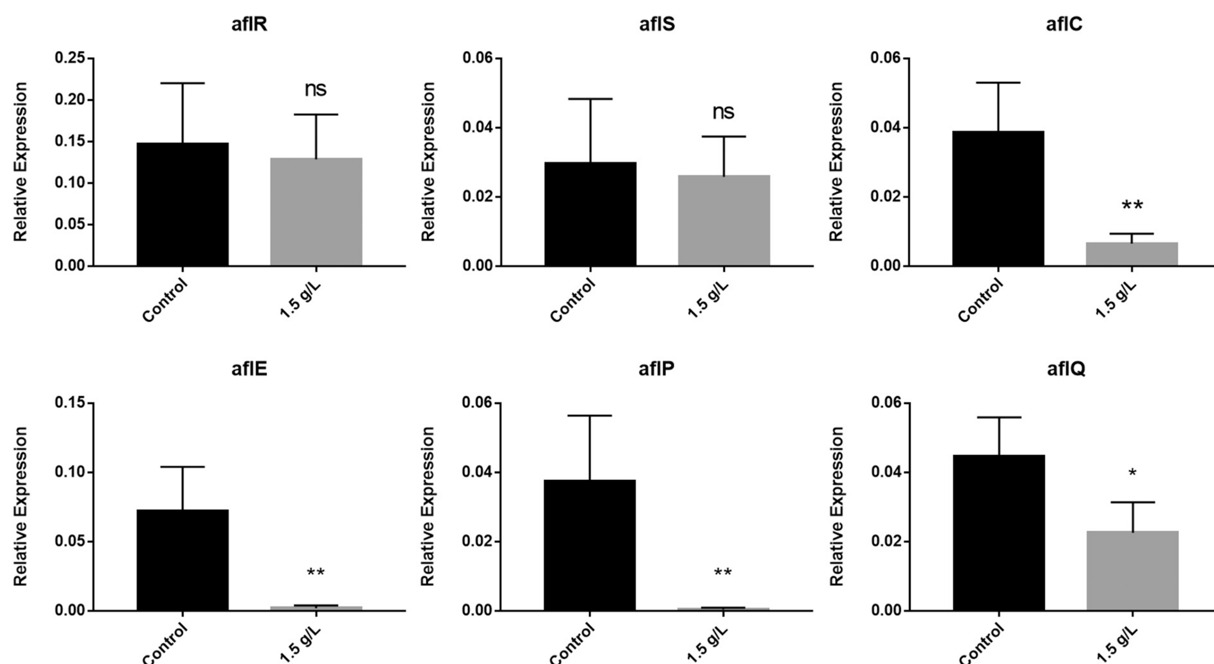

Fig. 6. Relative expression of the genes *aflR*, *aflS*, *aflC*, *aflE*, *aflP* and *aflQ* in *Aspergillus flavus* MUM 17.14 grown in liquid MEA medium in the presence of 1500 mg RL mixture/L. The results correspond to four independent cultures analyzed in triplicate. The β-*tubulin* gene was used as an internal control to normalize the expression data. ns: no significant differences ($p > 0.05$); *: significant differences ($p < 0.05$); **: significant differences ($p < 0.01$) (determined by the Student's *t*-test).

biosynthetic pathway. In most cases, both structural and regulatory genes were down-regulated (Hua et al., 2014; Kong et al., 2010; Moon et al., 2018; Yang et al., 2019). In other cases, only the expression of one regulatory gene was affected (Zhao et al., 2020). On the contrary, an increase in aflatoxins production was associated with an up-regulation of the gene *aflS* (Wang et al., 2017). The fact that the expression of some structural genes from the aflatoxins biosynthetic pathway was not correlated to the expression of the regulatory genes *aflR* and *aflS* can be due to the existence of a more complex regulatory system, as previously suggested by other researchers. Gallo et al. (2016) studied the effect of temperature and water activity on AFB_1 production by *A. flavus* ITEM7828. A direct relationship between AFB_1 production and the expression of the structural genes *aflD* and *aflO* was found. However, the same did not happen with the regulatory genes *aflR* and *aflS*, which were highly expressed at conditions that resulted in the production of high and low levels of AFB_1 (Gallo et al., 2016). Methyl jasmonate (400 μL/L) inhibited AFB_1 production in *A. flavus* NRRL3357 by 47% (Li et al., 2021). Under those conditions, 22 of the structural genes from the aflatoxins biosynthetic pathway were significantly downregulated. However, the expression of *aflR* and *aflS* was not significantly reduced, thus suggesting that other transcription factors may regulate the expression of the structural genes (Li et al., 2021). Gallic acid (8 g/L) completely inhibited AFB_1 production in *A. flavus* NRRL3357 (Zhao et al., 2018). Accordingly, almost all the structural genes from the aflatoxins biosynthetic pathway were significantly downregulated; however, the same was not observed for *aflR* and *aflS*. The carbon repression regulator CreA, which can bind directly to the promoter region of at least 14 genes of the aflatoxins biosynthetic pathway, seems to be involved in their regulation, which was further demonstrated by deleting the gene *creA* in *A. flavus* NRRL3357 (Zhao et al., 2018).

As natural products synthesized by microorganisms, RLs have been extensively studied for application in cosmetics and pharmaceutics, as well as in agriculture and the food industry, due to their low toxicity and high biodegradability (Hruzová et al., 2020; Robineau et al., 2020; Rodrigues et al., 2017). The applicability of RLs in agriculture, as an alternative to the chemical surfactants, to improve the foliar uptake of herbicides by modifying the wetting leaf surface and enhancing penetration across the cuticular membrane has been demonstrated (Liu et al., 2016). The commercial biofungicide Zonix™ (NOP Supply LLC., USA), which includes RLs in its formulation, has been approved by the Food and Drug Administration (FDA) to be directly used on vegetables, legumes, and fruit crops to prevent contamination by pathogenic fungi, due to its low mammalian toxicity and non-mutagenicity (Rodrigues et al., 2017). Furthermore, several studies demonstrated that RLs can also stimulate the plant immune system, improving plant protection against fungal and bacterial pathogens (Borah et al., 2015; Crouzet et al., 2021; Robineau et al., 2020). Accordingly, RLs could be used in combination with other fungicides to manage aflatoxins contamination in crops.

4. Conclusions

In this work, the inhibitory effect of RLs on the growth and aflatoxins production by *A. flavus* was reported for the first time. Although growth inhibition did not surpass 50%, aflatoxins production was reduced by 99%. As expected, RLs inhibited the fungal growth by damaging the cell membrane and the cell wall, which caused significant injuries in the hyphal cells. Additionally, they also down-regulated the expression of some genes from the aflatoxins biosynthetic pathway, which almost completely inhibited their production. Therefore, the results gathered in this study suggest that RLs can be considered a promising environmentally-friendly solution that may help in the mitigation of aflatoxins contamination. Nonetheless, more *in vitro* and field studies are needed to fully understand their anti-aflatoxigenic activity.

CRediT authorship contribution statement

Ana I. Rodrigues: Conceptualization, Investigation, Writing – original draft. **Eduardo J. Gudiña:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Luis Abrunhosa:** Investigation, Writing – review & editing. **Ana R. Malheiro:** Investigation. **Rui Fernandes:** Investigation. **José A. Teixeira:**

Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Lígia R. Rodrigues:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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