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Early expression of the aflatoxin gene cluster in *Aspergillus nomiae* isolated from Brazil nut

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ABSTRACT

Aspergillus nomiae is the most important contaminant in Brazil nut due to its high incidence in these nuts and its strong production of carcinogenic metabolites: aflatoxins (AF). Aflatoxin biosynthesis pathway in *A. nomiae* is poorly studied. Thus, in present investigation, aflatoxin production and gene cluster (*aflC*, *aflQ*, *aflU*, and *aflX*) expression profile were evaluated on two strains of *A. nomiae* isolated from Brazil nut samples collected in the Amazon region, and cultivated on Brazil nut-based medium. The results showed that the expression of the aflatoxin gene cluster in *A. nomiae*, started at day 2 and occurred before the production of aflatoxins. Aflatoxin production (AFB_1 and AFG_1) was detected on day 3 on both strains. From day 4 onwards, all four toxins were detected and their production kept at similar proportions (AFG_1>AFB_1>AFG_2>AFB_2). Thus, the anticipated information obtained through early expression profile results of *aflC*, *aflQ*, *aflU*, and *aflX* gene cluster in *A. nomiae* may foresee aflatoxin production before its detection in Brazil nuts.

1. Introduction

Brazil nut tree (*Bertholletia excelsa*) is native to the Amazon region. Its nuts are a great source of unsaturated fatty acids, phytosterols, tocopherols, squalene, phenolics, flavonoids, and micronutrients, particularly selenium, all associated with several potential health benefits (United States Department of Agriculture USDA, 2019). The rich composition of micronutrients allows Brazil nuts to help prevent heart disease and cancer.

The total production of Brazil nut worldwide in 2019 was 70 thousand tons, and the most producing countries was Brazil with 33 thousand tons (FAO, 2019). Due to economic importance of this commodity, fungal contamination in Brazil nuts has been studied since the last century (Mori and Prance, 1990). Climatic conditions (high humidity and warm temperatures) in the Amazon rainforest are directly correlated with *Aspergillus* species contamination and aflatoxin production (Olsen et al., 2008).

Aspergillus genus contains about 446 species, currently divided into six subgenera, subdivided into 27 sections of correlated species (Houbraken et al., 2020). *Aspergillus* section *Flavi* comprises a closely related group of fungi, which are dispersed worldwide, principally in soil, air, organic matter, and plants (Houbraken et al., 2020). Three species of this section have received major attention because of their capability to produce aflatoxins (AF) in Brazil nut: *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomiae* (Baquião et al., 2013; Reis et al., 2012, 2014).

A. nomiae (*A. nomius*) belongs to series *Nomiarum*, including also *Aspergillus pseudonomiae* and *Aspergillus luteovirescens* (Houbraken et al., 2020). Despite the phylogenetic distinction, *A. nomiae* are morphologically similar to *A. flavus*, however they are able to produce AFB and AFG as *A. parasiticus* (Frisvad et al., 2019) which belong to another sister large series (*Flavi*). Several reports showed that *A. nomiae*is a significant Brazil nuts contaminant due to its high occurrence and more importantly because it is capable of producing high levels of aflatoxins (Olsen et al., 2008; Reis et al., 2012).

Aflatoxins, a group of polyketide-derived furanocoumarins, are the most toxic and carcinogenic compounds among the known mycotoxins (Bennett and Klich, 2003). Aflatoxins (including AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1) were classified by the International Agency of Research on Cancer as Group 1 carcinogen to humans, with high risks for hepatocellular carcinoma in individuals exposed to them (International Agency for Research on Cancer IARC, 2012; European Food Safety Authority EFSA, 2020).

Aflatoxin biosynthesis occurs through a series of highly organized

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oxidation-reduction reactions from acetyl-CoA and malonyl-CoA (Roze et al., 2015). The complete aflatoxin cluster consists of 30 genes, which are coordinately regulated (Caceres et al., 2020). The code "*afl*" and a letter in alphabetical order from "*A*" to "*Y*" is used according to the position of the genes in the biosynthetic pathway (Yu et al., 2004).

The core biosynthetic gene *aflC* encodes an enzyme, polyketide synthase (PKS) that synthesizes dodecaketide (polyketide skeleton) from hexanoyl-CoA (Yu et al., 2004). In addition, PKS is involved in conversion of the polyketide structure into a precursor of the first stable metabolite norsolonic acid (NOR) and norsolorinic acid anthrone (NAA) (Caceres et al., 2020). Next the biosynthetic cascade continues, forming several intermediate metabolites, until at the end of the pathway, when the *aflQ* gene encodes an oxidoredutase enzyme, converting O-methylsterigmatocystin (OMST) to AFB_1 (Frisvad et al., 2007; Yu et al., 2004) and dihydro-O-methylsterigmatocystin (DHOMST) to AFB_2, (Carbone et al., 2007; Yu, 2012). Similarly, *aflU*, involved in AFG production, encodes also a monooxygenase enzyme, which converts OMST to AFG_1, and DHOMST to AFG_2 (Caceres et al., 2020; Carbone et al., 2007; Yu, 2012).

In regard to *aflX*, located near the proximal end of the cluster, Cary et al. (2006) observed that its disruption in an aflatoxigenic *A. flavus* strain reduced AFB_1 production and increased the accumulation of versicolorin A (VA). Thus, they concluded that the *aflX* gene encodes an enzyme involved in the conversion of VA to demethylsterigmatocystin (DMST) in the aflatoxin biosynthetic pathway.

As reported by Liao et al. (2020) in a recent review, aflatoxin biosynthetic pathway was widely studied in several aflatoxigenic species, but poorly studied in *A. nomiae*, although this species is the most important aflatoxin producer in Brazil nuts and a strong aflatoxin producer (Reis et al., 2012). Previous studies showed that *A. nomiae* was the most frequent species found in Brazil nut samples collected from Brazilian Amazon region (56%), following *A. flavus* (24%), and *A. parasiticus* (2.8%) (Reis et al., 2012).

Thus, the evaluation of the genes involved in the *A. nomiae* aflatoxin biosynthetic pathway can predict possible health risks related to the imminent aflatoxin contamination, based on the early detection of this aflatoxigenic species, it may be better understood through the early expression of the aflatoxin gene cluster. For that, we aimed to carry out a transcription analysis of the *aflC*, *aflQ*, *aflU*, and *aflX* genes, and correlate with AFB_1, AFB_2, AFG_1, and AFG_2 production in *A. nomiae* strains isolated from Brazil nut samples.

2. Materials and methods

2.1. Fungal strains

Two strains of *A. nomiae* (AN56 and AN101) previously isolated from Brazil nuts samples from Acre State, Amazon region were used in this study. The molecular identification of the species was done by sequencing of β-tubulin (Glass and Donaldson, 1995) and calmodulin genes (Carbone and Kohn, 1999). The strains are being maintained at the Department of Microbiology at the University of São Paulo. For inoculum preparation, the *A. nomiae* strains were grown on Potato Dextrose Agar for 7 days at 25 °C.

2.2. Culture conditions

To conduct the aflatoxin production and gene expression analysis we first analyzed the growth rate of AN56 and AN101 *in vitro* under the same conditions. Conidia were resuspended in aqueous solution containing Tween 80 (0.05%), and the final concentration was adjusted to 1×10^6 conidia/mL using a Neubauer counting chamber. Ten microliters of each suspension were inoculated into the center of Petri plates (90 × 15 mm) containing Brazil nut-based medium: 4% ground Brazil nut, 2% bacteriological agar (Gallo et al., 2016). The water activity of the medium was 0.99. The Brazil nut sample used as the medium substrate was collected from Manicoré, Amazonas, Brazil.

2.3. Fungal growth assessment

The cultures were incubated for 7 days at 25 °C in the dark. The diameter of the colonies was measured in three directions perpendicular to each other every 24 h in quadruplicate. The growth rate (cm/day) of each strain was calculate during the period of seven days.

2.4. Aflatoxigenic potential of A. nomiae

The aflatoxigenic potential of the strains was evaluated according to the method described by Reis et al. (2012), as the limits of detection (LOD) and limits of quantification (LOQ), recovery tests, calibration curve, and coefficients of calibration curves. Briefly: daily, the whole content of each plate was removed and mixed with chloroform (30 mL per 10 g of culture medium). After shaking, the solution was filtered through Whatman No. 1 filter paper, and the content was evaporated to residue under nitrogen stream. For clean-up step, a Strata C18-E cartridge (500 mg/3 mL) (Phenomenex, Santa Clara, CA, USA) was used. Then, the residues were derivatized with trifluoroacetic acid (TFA) and hexane and injected into Shimadzu Prominence HPLC system (Kyoto, Japan) equipped with an RF 10AXL fluorescence detector (excitation: 365 nm, emission: 450 nm) and an autosampler system. The analytical column (Shimadzu, Shim-Pack VP ODS, 150 × 4.6 mm) was coupled to a pre-column cartridge (Shim-Pack GVP-ODS, 10 mm × 4.6 mm) maintained at 40 °C in an oven. The isocratic mobile phase consisted of acetonitrile:methanol:water (1.5:1.5:8, v/v/v)+0.1% TFA, at a flow rate of 1 mL/min.

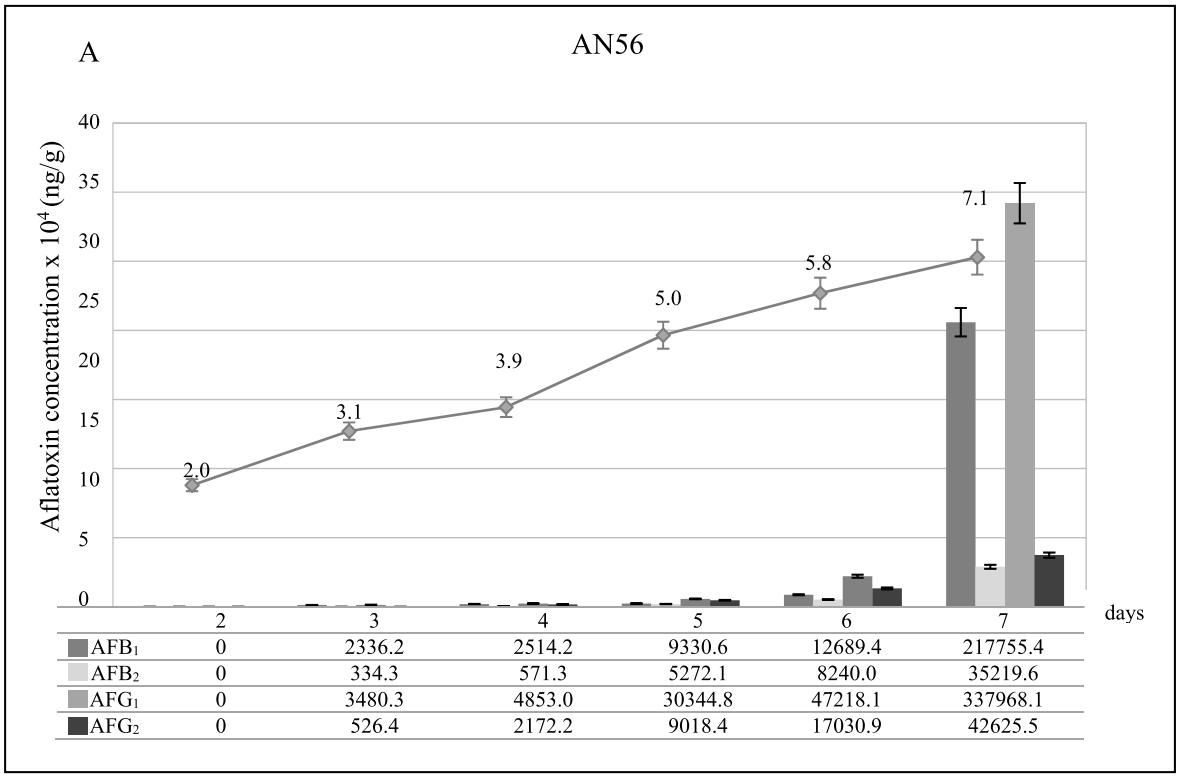
The LOD and LOQ were 0.75 and 0.5 μg/kg for each aflatoxin, respectively. The coefficients of the calibration curves were 0.999 for all aflatoxins. The recovery tests were 80% for AFB_1, 83% for AFB_2, 116% for AFG_1, and 84% for AFG_2, and the coefficients of variation of recovery were 6.2%, 3.7%, 4.9%, and 5.7%, for AFB_1, AFB_2, AFG_1, and AFG_2, respectively.

2.5. Gene expression analysis

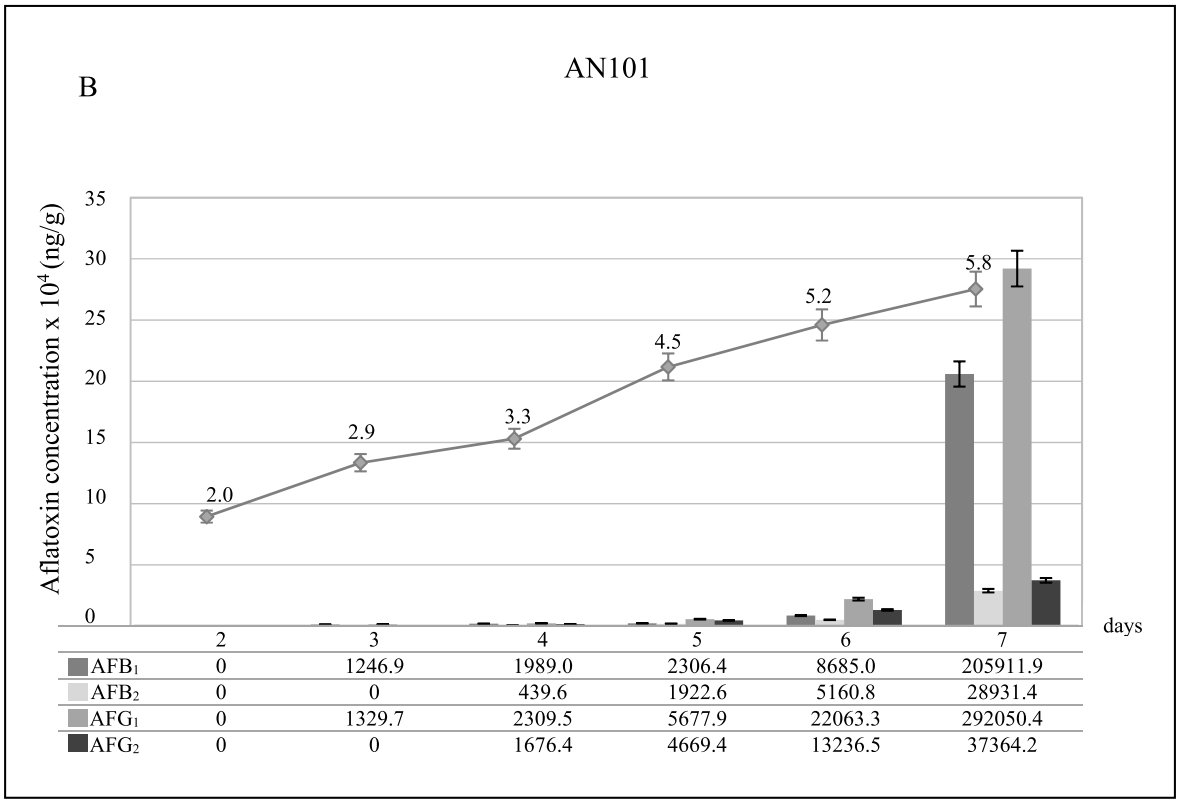
The fold change gene expression was evaluated by the comparation between the two data points of collection: between the two strains (AN101 *versus* AN56), and between the two days (day 2 *versus* day 7).

For gene expression the fungal strains were grown in triplicates. The RNA was extracted from liquid nitrogen powdered mycelium of two and seven days old fungal cultures using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. We selected two days old fungal cultures because it was when the fungal growth was first noted, and seven days because is the sufficient time to produce large amounts of aflatoxin. The extracted RNA was treated with RNAse-free DNAse-l Kit (Promega, Madison, WI, USA), and its concentration and purity were determined in a NanoDrop 2000c™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The samples were diluted to 10 ng/μL and were reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems): 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C, according to the manufacturer's instructions of the kit. The cDNA samples were stored at −20 °C until use.

The expression profile of the genes *aflC*, *aflQ*, *aflU*, and *aflX* (Suppl. Fig. 1) and *β-TUB* gene (used as reference) were performed by quantitative real-time polymerase chain reaction (qRT-PCR) in a StepOne Plus RT-PCR system (Applied Biosystems). Primers were designed based on previously characterized sequences deposited in NCBI GenBank (accession AY510454.1) using the Primer3plus software (Table 1). The reactions were prepared in triplicate using the Power SYBR Green PCR Master Mix (Invitrogen, Thermo Fisher, Carlsbad, CA, USA), according to the manufacturer's protocol. The concentration of each primer was



	2	3	4	5	6	7
AFB_1	0	2336.2	2514.2	9330.6	12689.4	217755.4
AFB_2	0	334.3	571.3	5272.1	8240.0	35219.6
AFG_1	0	3480.3	4853.0	30344.8	47218.1	337968.1
AFG_2	0	526.4	2172.2	9018.4	17030.9	42625.5



	2	3	4	5	6	7
AFB_1	0	1246.9	1989.0	2306.4	8685.0	205911.9
AFB_2	0	0	439.6	1922.6	5160.8	28931.4
AFG_1	0	1329.7	2309.5	5677.9	22063.3	292050.4
AFG_2	0	0	1676.4	4669.4	13236.5	37364.2

Fig. 1. Graphic representation of aflatoxins production (ng/g) (triplicate), and line graph represents growth measurement (cm) (quadruplicate) of *A. nomiae* strains AN56 (Fig. 1A) and AN101 (Fig. 1B) cultivated in Brazil nut-based medium for 7 days.

100 nM. Water was added to the reaction as negative control for all runs. All amplifications were carried out in MicroAmp Optical 96-Well Reaction Plates sealed with MicroAmp Optical Adhesive Covers (Applied Biosystems). The amplification conditions were an initial step at 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s, and a final cycle at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Amplification efficiency (E) was evaluated by constructing a standard curve with cDNA from one sample with 6 serial dilutions (RNA: 50, 25,

Table 1
List of target genes and nucleotide sequences of primers used in qRT-PCR analysis.

Gene[a]	Old gen symbol[b]	Direction	Nucleotide sequences (5′- 3′)
aflC	*pksA*	F	ACGGGGCCAACTTATAATCC
		R	TGGATTGTTCGCTACACAGC
aflQ	*ordA*	F	ATGGATGTGGCCTATGGGTA
		R	CGGAGAGAAGATGTGGGAAA
aflU	*cypA*	F	TCCCTCTTTTCCCAGAGGTT
		R	CTCAAGGCCCATGACGTATT
aflX	*ordB*	F	CTCTTATCGTCCGCGTTAGC
		R	GTGCAACGGGGTCATATTCT
β-TUB		F	AAGTACAACCCGCGTACACC
		R	GATCGGAGGAGCCATTGTAA

[a] Gene names were adopted on International Convention of Fungal Nomenclature (2004).
[b] Old gene symbols were given when the gene was first cloned.

12.5, 6.25, 3.12, 1.56 ng/μL) for each gene in quintuplicate using the formula: $E = 10^{(-1/slope)} - 1$. Thus, the efficiency was calculated from each linear regression of the standard curves. The values were satisfactory for all genes (90–110%).

2.6. Data analysis

The nucleotide sequences (β-tubulin and calmodulin) were aligned with those corresponding to closest matches from GenBank using Multiple Sequence Alignment software included in the Molecular Evolutionary Genetics Analysis 7.0.26 package. Aligned data sets were analyzed using the maximum likelihood method with the Kimura 2-parameter model, the bootstrap analysis used 1000 replicates (Yilmaz et al., 2014). The phylogenetic tree was done based on concatenation and coalescence methods. We included reference fungal strains: *A. nomiae* (NRRL 3161), *A. pseudonomiae* (CBS 119388), *A. parasiticus* (CBS 117618), and *A. flavus* (CBS 100927). *Aspergillus nidulans* (DTO 320-C2) was used as an outgroup.

The relative gene expression was determined using the comparative ΔΔCT method (Livak and Schmittgen, 2001; Pfaffl, 2001) using the RT^2 qPCR Array software (Qiagen). The ΔCT was calculated between the gene of interest and the reference gene (β-TUB), followed by ΔΔCT calculations (ΔCT (condition 1)- ΔCT (condition 2)). Fold-Change (2^(-ΔΔCT)) was calculate as the ratio of the normalized gene expression between (2^(-ΔCT)) conditions: day 2 *versus* day 7 of fungal growth for the two strains and AN 56 *versus* AN 101 strains. The p-values were calculated based on the Student's t-test with a 5% significance level.

3. Results

3.1. Analysis of molecular data

The sequences of amplicons from β-tubulin, and calmodulin were highly similar (100%) to the sequences of *A. nomiae* deposited in GenBank® (Table 2). The phylogenetic tree was based on concatenation and coalescence methods (β-tubulin and calmodulin genes). The maximum likelihood was the method used to build the tree. (Suppl. Fig. 2).

Table 2
GenBank accession number of *Aspergillus nomiae* strains.

Isolate number	GenBank accession number	
	β-tubulin	Calmodulin
AN56	MT764841	MT764843
AN101	MT764842	MT764844

3.2. Growth assessment

On day 2 both strains showed equal growth rates (2 cm of diameter). However, by day 7, AN56 showed a higher mycelial growth (7.1 cm) compared to AN101 (5.8 cm). During the seven days period of analysis the average growth rate (cm/day) was of 1.02 for AN56 (Figs. 1A) and 0.76 for AN101 (Fig. 1B). No significant differences were found between the two species.

3.3. Aflatoxigenic production of A. nomiae

We investigated the aflatoxin production potential of *A. nomiae* on Brazil nut-based medium. Both strains produced all four aflatoxins investigated in the current study (AFB_1, AFB_2, AFG_1, and AFG_2). On day 2 no aflatoxin was detected. On day 3, AFB_1 and AFG_1 were detected on both strains, however AFB_2 and AFG_2 were detected only in AN56. From day 4 onwards, all four toxins were detected on AN56 and AN101. Interestingly, on day 7 of fungal growth we found similar levels of aflatoxins between the strains. For example, AN56 produced 217,755.4 × 10^4 ng/g of AFB_1 while AN101 produced 205,911.9 × 10^4 ng/g. Although, our results AN56 produced more aflatoxins than AN101, no significant differences were found between the two species. For both strains the similar aflatoxin profile production was found (AFG_1->AFB_1>AFG_2>AFB_2) (Fig. 1A and B).

3.4. Transcriptional analysis by real time qRT-PCR

The expression profile of four genes (*aflC*, *aflQ*, *aflU*, and *aflX*) within the aflatoxin gene cluster at different time points using two *A. nomiae* strains were analyzed. Primers were designed and their validation results of qRT-PCR method are shown in Table 3. The efficiency of the qRT-PCR assay ranged from 93.6% (*aflC*) to 100.3% (*aflQ*), the slope ranged from −3.48 (*aflC*) to −3.31 (*aflQ*), and the correlation coefficient of the standard curve ranged from 0.97 (*aflQ* and *aflU*) to 0.99 (*aflX*). These results were considered satisfactory to continue the analysis.

The results of the fold change gene expression between the two data points of collection, day 7 *versus* day 2 for each strain (Fig. 2A) and between the two strains AN56 *versus* AN101 for each point. We found that all four genes analyzed were already being expressed on day 2. However, the transcription profile between the strains differed. On day 2 of growth, the AN56 strain expressed significant less *aflU*, *aflQ* and *aflX* compared to AN101 (p-value < 0.05). Although no statistical significance was found for the core gene *aflC*, the data showed the gene followed a similar trend. On day 7 of fungal growth, no significant intraspecies difference was found (Fig. 2B).

When examining the relative gene expression differences between day 7 *versus* day 2, we found that on day 7, the gene a*flU* was significantly up regulated (1.50 times more expressed) in AN101 while, the gene *aflX* was downregulated (0.87 times less expressed) in AN56. Yet is important to note that in the strain AN56 all genes showed an up regulation trend on day 7 while AN101 showed the opposite profile on the same data point compared to day 2 of fungal growth.

4. Discussion

The Recommended Dietary Allowance (RDA) for selenium in the United States is 55 μg/day for adult males and females, and in the United Kingdom it is 75 μg/day for men and 60 μg/day for women (Fisinin et al., 2009). The range in the Se concentration in Brazil nuts are considerably wide, one single Brazil nut can provide 11–288% of daily Se requirement (Silva Junior et al., 2017). An acceptable threshold for Se is 850–900 μg/day, acute Se intoxication can cause hair loss, muscle tenderness, tremors, lightheadedness, facial flushing, severe gastrointestinal and neurological symptoms, respiratory syndrome, cardiac and kidney failure, and, in rare cases, death (Sunde, 2006).

The nut sample used to formulate the Brazil nut-based medium was

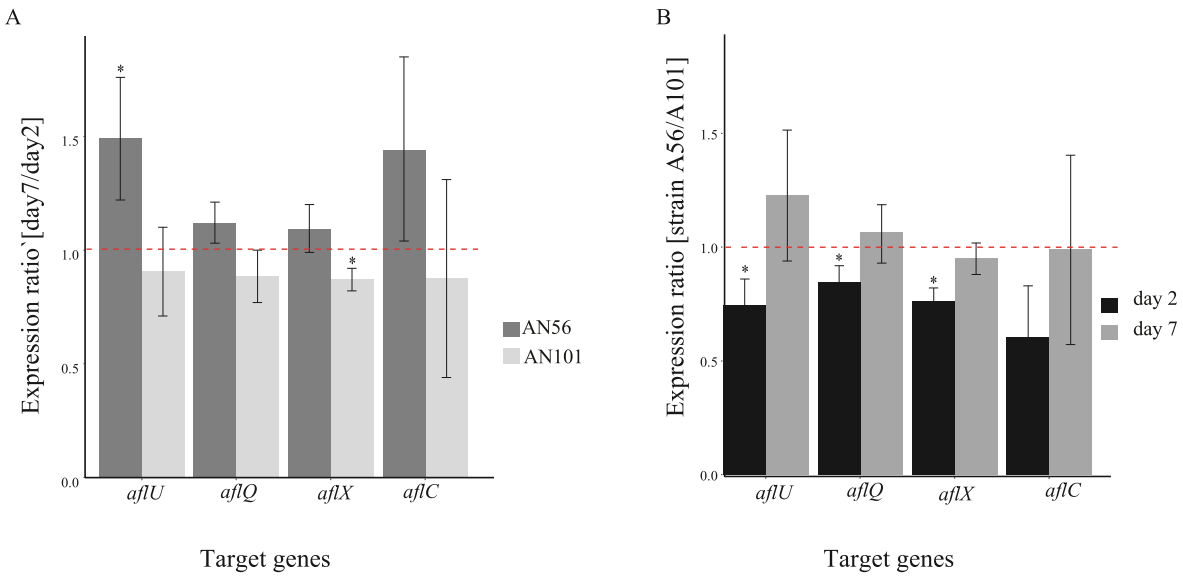


Fig. 2. Gene expression ratio during two days of growth (day 7 and 2, Fig. 2A) and within fungal isolates (strain A56 and A101, Fig. 2B) of the aflatoxin biosynthetic cluster.

Table 3
Validation of $\Delta\Delta C_T$ method parameters results: PCR efficiency (%), Coefficient correlation (r^2), Slope, Melting temperature (°C).

Gene	PCR efficiency (%)	Coefficient correlation (r^2)	Slope	Melting temperature (°C)
aflC	93.6	0.98	−3.48	85.6
aflQ	100.3	0.97	−3.31	83.2
aflU	98.1	0.97	−3.37	82.5
aflX	95.4	0.99	−3.44	83.8
TUB	100.5	0.95	−3.31	81.4

originated from Manicoré, this municipality is located in the state of Amazonas. We found 6.61 mg/kg of Se in Brazil nut sample, this value is according to prior studies, due to its geographic origin, Western Amazon region (Pacheco and Scussel, 2007). Comparison of Brazil nut samples between Amazon regions showed Se levels ranged from 0.03 to 31.7 and 1.25–512 mg/kg for Western and Central areas, respectively (Chang et al., 1995), and ranged from 8.5 to 35.1 and 20.7–69.7 mg/kg for Western and Eastern Amazon region, respectively (Pacheco and Scussel, 2007), and varied from 2.07 (state of Mato Grosso) to 68.15 mg/kg (state of Amazon) (Silva Junior et al., 2017). Se is absorbed by Brazil nut trees from the soil depending on Se concentration in the soil and the soil acidity (Silva Junior et al., 2017).

According to some authors, nutritional composition of substrates seems to affect the growth of fungi more than the environmental conditions, since a wide range of water activity and temperature allow the development of *A. flavus* and *A. parasiticus,* species belonging to *Aspergillus* Section *Flavi* (Bernáldez et al., 2018; Gallo et al., 2016). For this reason, we emphasized the importance of using a culture medium based on the original substrate of *A. nomiae.*

In our study, we analyzed growth rate, aflatoxin production and gene expression of two strains of *A. nomiae* to better understand the aflatoxin biosynthesis of this important species of contaminant of Brazil nuts. Cultivation conditions applied to our study (temperature: 25 °C, water activity of the medium: 0.99) were chosen to simulate the equatorial climate of the Amazon rainforest, which presents annual mean temperature ranging between 24 and 26 °C and total annual rainfall is considerable, at least 1900 mm (Alvares et al., 2013). The relationship between high relative humidity of the air and high-water activity of the medium (0.99) can be established.

Under the conditions of the present study, mycelial diameter reached 5.8 (AN101) and 7.1 cm (AN56) in 7 days. Similar results were previously reported for *A. nomiae* at the same temperature (25 °C) and time (7 days) with different culture media, on Czapek agar the growth was 4–7 cm of diameter (Kurtzman et al., 1987), and on Blakelee's malt agar the growth was 4.5–6.5 cm of diameter (Peterson et al., 2001).

Both strains (AN56 and AN101) produced high levels of aflatoxins in Brazil nut-based medium, keeping the same ratio: AFG_1>AFB_1>AFG_2>AFB_2. In the literature there is no consensus on the production ratio between AFB and AFG. Reis et al. (2014) selected coconut agar to evaluate aflatoxin potential in *A. nomiae* strains from organic Brazil nut samples, and found higher AFG concentration than AFB. Olsen et al. (2008) reported a near 1:1 ratio between aflatoxin B_1 and G_1 in-shell Brazil nut samples. Differently, some investigations reported higher levels of AFB than AFG in *A. nomiae* strains from Brazil nut samples cultivated in coconut agar (Baquião et al., 2013; Reis et al., 2012; Yunes et al., 2019). In turn, Doster et al. (2009) observed differences in aflatoxin production by *A. nomiae* strains in three different media, they produced higher AFG than AFB when inoculated directly in pistachio nut. Although there is no uniformity among culture medium and incubation time in the investigations presented, *A. nomiae* is undoubtedly an important aflatoxin producer.

Aflatoxin biosynthesis pathway has been widely studied in *A. flavus* and *A. parasiticus* (Caceres et al., 2020). Yet, in *A. nomiae* information regarding the gene cluster and their expression are still scarce. For this investigation, four genes were chosen: *aflC*, *aflQ*, *aflU*, and *aflX*. The *aflC* gene encodes PKS that synthesizes the polyketide skeleton from hexanoyl-CoA and is implied in conversion of the polyketide structure into a NOR and NAA (Caceres et al., 2020; Yu et al., 2004). At the end of the pathway, the *aflQ* gene encodes an oxidoredutase enzyme, converting OMST to AFB_1 and DHOMST to AFB_2, (Frisvad et al., 2007; Yu, 2012; Yu et al., 2004). Similarly, *aflU*, involved in AFG production, encodes a monooxygenase enzyme, converting OMST to AFG_1, and DHOMST to AFG_2 (Caceres et al., 2020; Yu et al., 2004; Yu 2012). In this turn, the *aflX* gene encodes an enzyme involved in the conversion of VA to DMST in the aflatoxin biosynthetic pathway. In our study, all four genes analyzed were already being expressed on day 2. The genes (*aflQ*, *aflU*, and *aflX*) of AN56 strain expressed significant less on day 2 than strain AN101. Interesting on day 7 this difference was absent and the genes of AN56 began to express more, but there was no statistical difference, indicating that they were expressing similar profile.

Unlike *A. nomiae*, gene expression profile and aflatoxin production

have been widely reported to *A. flavus* strains. In relation to regulatory genes (*aflR* and *aflS*), authors observed that both genes were highly expressed in stress conditions, although very low amounts of aflatoxin were produced (O'Brian et al., 2007). Schmidt-Heydt et al. (2009) reported diverse gene expression profile and aflatoxin accumulation matching different temperature and water activity levels. In *A. flavus* and *A. parasiticus* strains, authors observed a positive correlation between *aflQ* expression and aflatoxin production (Mahmoud, 2015; Jamali et al., 2013; Rodrigues et al., 2009). However, other studies did not find any correlation between the aflatoxigenic profile of *A. flavus* strains and *aflQ* gene expression (Accinelli et al., 2008). Aldars-Garcia et al. (2018) studied different temperature scenarios, and observed intraspecies variability in *A. flavus* strains for AFB_1 production, which started its production only after 7 days. Yunes et al. (2019) reported *A. nomiae* strains cultivated on coconut agar were able to produce aflatoxins under three temperatures (25, 30 and 35 °C), after 7 days, being the highest toxin levels at 25 °C. In addition, no association was found between aflatoxin production and *aflR*, *aflD*, and *aflQ* genes expression.

5. Conclusion

This study showed the early expression of some genes involved in the aflatoxin biosynthetic pathway of *A. nomiae*. All the genes studied in this investigation were expressed on the second day after cultivation of *A. nomiae* on Brazil nut-based medium, and aflatoxin production started its production on third day. Our results showed that the molecular data studied in this investigation were able to foresee the production of aflatoxin in this species of *Aspergillus*, which is very common in Brazil nuts, through the early expression of the studied genes.

Credit author statement

Tatiana Alves Reis: Conceptualization, Methodology, Writing – original draft preparation. **SabinaMoser Tralamazza**: Software, Data curation, Writing- Reviewing and Editing. **Ednei Coelho**: Data curation, Writing. **Patricia Zorzete**: Methodology, Writing. **Déborah Inês Teixeira Fávaro**: Methodology. **Benedito Corrêa**: Supervision

Ethical Statement for Solid State Ionics

Hereby, I, Tatiana Alves dos Reis, consciously assure that for the manuscript "Early expression of the aflatoxin gene cluster in *Aspergillus nomiae* isolated from Brazil nut" the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.
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Date: July 30 th, 2021

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2022.01.008.

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