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Research Article

Molecular characterization of aflatoxigenic and non-aflatoxigenic isolates of *Aspergillus flavus* for biological control of aflatoxins in Togo

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Abstract

The contamination of agricultural products and aflatoxins synthesis can be controlled and reduced by the application of a biological control product based on atoxinogenic *Aspergillus flavus* strains. The purpose of this work was to select atoxinogenic *Aspergillus flavus* strains that can be used in the biological control of aflatoxins. Thirty-four (34) strains of *Aspergillus flavus* were used and the Nor-1, Ver-1, Omt-A and AflR genes involved in the aflatoxin biosynthesis pathway were sought. The growth speed and ability of atoxigenic strains to inhibit the growth of toxigenic strains were also determined. The results showed that 50% of the strains have at least one of the genes sought and the percentages of inhibition (PI) of the atoxinogenic strains varied significantly from 16.98% to 62.50% at the threshold of 5% according to the Tukey test (p-value = 0.0000136). The mycelial growth speed (VCM) of the atoxinogenic strains varied from 7.5 to 10 mm/day with an average VCM of 8.4 ± 0.05 mm/day. Strains AKA-10; AGA-8; AMAN-35; ABA-28 and AGA-46 were found to be more effective in inhibiting mycelial growth of toxigenic strains.

Keywords

Aspergillus flavus; molecular characterization; antagonism; mycelial growth; biological control

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Introduction

Aflatoxins are secondary metabolites secreted by *Aspergillus flavus* and *Aspergillus parasiticus* (Mohamed et al. 2015; Diakite et al. 2017). They are toxic, carcinogenic, mutagenic and immunosuppressive compounds (Uzeh and Adebowale 2021). Among the four types of aflatoxins (AFB1, AFB2, AFG1, AFG2), AFB1 is the most dangerous and has been classified by the International Agency for Research on Cancer as a group 1 human carcinogen (IARC 2002; Ogara et al. 2017). Peanut is the preferred substrate for fungi, in particular *Aspergillus flavus* and *Aspergillus parasiticus*. It is rich in nutrients, especially proteins, fats, carbohydrates and minerals which promote fungal growth and the production of aflatoxins (Mutegi et al. 2012; Settaluri et al. 2012; Krnjaja et al. 2019).

Peanut seed contamination begins in the field and is favoured by growing conditions, recurrent insect attacks and droughts at the end of the cycle (Adjou and Aoumanou 2013). When conditions such as temperature (20-25°C), humidity (>7%), pH (3-8) are met, fungi can grow on food; which can have several consequences such as the alteration of organoleptic properties, the reduction of nutritional qualities, the appearance of diseases (allergies et mycosis), the production and accumulation of mycotoxins in food (Aliyu and Kutama 2010; Adjou and Aoumanou 2013; Rao et al. 2013). A study carried out in Togo by Tedihou et al. (2019) revealed the presence of aflatoxins in peanut products at very high levels ranging from 25.16-1010.09 $\mu g.kg^{-1}$.

The presence of aflatoxins in peanut indicates the presence of toxigenic fungi of the genus Aspergillus. According to Godet and Munaut (2010), fungi of the genus *Aspergillus* could be separated into two groups based on their impact on food and human health. The first group includes aflatoxigenic species such as *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*, while the second group includes non-aflatoxin producing species such as *Aspergillus tamarii*, *Aspergillus oryzae* and *Aspergillus sojae*. The contamination of food by aflatoxins represents a danger for consumers and constitutes a real public health problem. Among the control methods currently available, biological control is effective in preventing the contamination of food by aflatoxins (RELIEFWET 2010; Dieme et al. 2017). Indeed, this biological control is based on the use of non-toxigenic *Aspergillus* strains that can compete with the development of toxigenic Aspergillus strains in the fields. In recent years, molecular detection methods have been developed to identify aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* sp. All these methods are based on the detection of genes that code for the biosynthesis of key enzymes involved in the aflatoxins production (Uzeh and Adebowale 2021).

The purpose of this work was to characterize aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus* strains from peanut sold in northern Togo. Specifically, it involved detecting, using the molecular tool, the genes involved in the aflatoxin biosynthesis pathway and testing the antagonistic effect of the atoxinogenic strains against the toxigenic strains identified in this study.

Materials and Methods

Fungal material. Thirty-four strains of *Aspergillus flavus* (ACI-3, AKE-4, AKA-6, ACI-7, AGA-8, AKA-10, AKO-22, AMAG-23, ABAG-24, AKO-26, AKA-27, ABA-28, AMA-30, ANA-31, ATC-32, ANAM-33, ANA-34, AMAN-35, ANA-36, ANAM-37, ANAM-38, AMAN-39, AKO-40, ANA-41, AKA-42, ABAN-44, AGA-46, AKE-47, ACI-48, ADA-49, ANA-50, AMAN-51, AKA-52, AMA-53) were isolated and used for this study. Toxigenic (GHG040-1) and non-toxigenic (GHM217-8) *Aspergillus flavus* reference strains were provided by IITA (International Institute of Tropical Agriculture) Ibadan based in Nigeria and were used respectively as positive controls and negative controls.

Isolation and identification of fungi. The isolation of fungi was carried out on mouldy peanut seeds from samples collected in northern Togo respectively in the Kara region and the Savannah region. These seeds were disinfected with calcium hypochlorite for 10min under a vertical laminar flow hood; rinsed three times with sterile distilled water before being placed in a Petri dish containing Potato Dextrose Agar (PDA) culture medium (Mohammed and Chala; 2014; Dongmo et al. 2017). Incubation was carried out at 25 °C for 5 to 7 days (Ogara et al. 2017; Ben Bordi and Oubiri 2020). Fungi from this culture were subcultured onto Czapek Yeast Agar (CYA) medium (Rao et al.

2013; Matmoura et al. 2019). The identification of the fungi was carried out on the basis of their macroscopic and microscopic aspects (Chabasse et al. 2002; Tsuneo 2002).

Detection of aflatoxin production genes

DNA extraction. The Cethy Trimethyl Ammonium Bromide (CTAB) DNA extraction method used is adapted from that of Campos (2018). Mycelium and five days old conidia were collected in 2ml Eppendorf tubes containing three glass beads (3mm) under aseptic conditions. A volume of 500µL of CTAB extraction buffer (0.1 M Tris-HCL pH 8; 1.4 M NaCl; 20 mM EDTA; 20 $g.l^{-1}$ CTAB) was added and the suspension was vigorously homogenized using an automatic grinder (MP Biomedicals, FastPrep-24) for 2min, then incubated at 65°C for 15min in a water bath. The suspension was homogenized again for 1min and incubated at 65°C for 15min in a water bath. After incubation, 500µL of a chloroform: isoamyl alcohol solution (24:1, v/v) was added to the suspension and centrifuged for 5min at 17000xg (Sigma 3-30K centrifuge). The supernatant was collected and placed in a new 1.5 ml Eppendorf tube. A volume of 64µL of sodium acetate (3M) and 233µL of isopropanol were added to the supernatant and centrifuged at 21,000xg for 5min. The pellet was washed by adding 500µL of 70% ice-cold ethanol. After centrifugation at 21,000xg for 5min, the supernatant was discarded and the pellet was dried under a vertical laminar flow hood for 3 hours. The dry pellet was dissolved in 50µL of TBE buffer (Tris Borate-Ethylenediamine-tetra acetic acid, EDTA).

Detection of aflatoxin biosynthesis genes. Four primer sets were used according to the method described by Mahror et al. (2019) for detection of genes involved in aflatoxin biosynthetic pathway as follow Nor-1F 5′ -ACC GCT ACG CCG GCA CTC TCG GCAC-3’ and Nor-1R 5′-GTT GGC CGC CAG CTT CGA CAC TCC G-3’, Ver-1F 5′-ATG TCG GAT AAT CAC CGT TTA GAT GGC-3’ and Ver-1R 5′-CGA AAA GCG CCA CCA TCC ACC CCA ATG-3’, Omt-1F 5′-GGC CCG GTT CCT TGG CTC CTA AGC-3′ and Omt-1R 5′-CGC CCC AGT GAG ACC CTT CCT CG-3′ and AflR-F 5′-TAT CTC CCC CCG GGC ATC TCC CGG-3′ and AflR-R5′-CCG TCA GAC AGC CAC TGG ACA CGG-3′. The 400, 895, 1232 and 1032 bp fragments were amplified respectively. The PCR was performed in 25µL containing: 12.5µL of one taq Quick load, 2X master Mix with standard buffer, 0.5µL of each primer (Inquaba Biotec West Africa Ltd), 4µL of extracted DNA as template and 7.5µL of sterile distilled water. Gene amplification was performed using a thermocycler (Applied Biosystems 2720). A total of 35 cycles was started by heating at 95°C for 1min and continued by denaturation 1min at 95°C, annealing 1min at 67°C for Nor-1, Ver-1, AflR gene and 61°C for Omt-1 gene, elongation 1min at 72°C and a final extension 5min at 72°C. The amplified products were visualized by UV illumination after electrophoresis on 1% agarose gel and ethidium bromide staining.

Evaluation of the antagonistic effect of isolates. The demonstration of the antagonistic effect of the strains of *Aspergillus flavus* was carried out *in vitro* according to the technique of double confrontation as described by Soumaya et al. (2013) and Mokhtari et al. (2017). The technique consisted in transplanting, on the one hand, into the same Petri dish, containing the PDA medium, 5µL of a fungal suspension of the pathogen (toxigenic strain) and 5µL of a fungal suspension of the antagonist strain (atoxigenic strain) at test and on the other hand, in another petri dish serving as a control, 5µL of the fungal suspension of the pathogen and 5µL of sterile distilled water.

The spores of the young cultures (3 days) of each antagonistic and pathogenic strain are recovered in a volume of 500µL of sterile distilled water. The determination of the optical density (OD) of each fungal suspension was carried out using a spectrophotometer (Jenway) at a wavelength of 630nm in order to standardize the spore suspension at 10^9 spores.mL^{-1}. It is estimated that an OD of 0.04 corresponds to a concentration of 10^7 spores.mL^{-1} (Touaibia 2015). Each test was repeated three times and incubation was performed in the dark at 25°C. Measurements of the radial growth, R1 and R2 of the pathogen, began after 48h of incubation and continued over fourteen days at two-day intervals (48h). The percentage inhibition (PI) of radial growth of non-aflatoxigenic *Aspergillus flavus* strains was determined according to the formula used by Qjidaa et al. (2018):

$$PI = (R2-R1)/R2 \times 100 \quad (1)$$

R1 - radius of mycelia growth of pathogenic fungus in the presence of antagonist

R2 - radius of mycelia growth of pathogenic fungus in control plates

The inhibition mode (IM) deployed by each antagonist strain was also evaluated according to a scale ranging from 1 to 4 where 1 - the mycelial growth of the pathogenic fungus ceases following the proliferation of the antagonist, 2 - partial inhibition of the mycelial growth of pathogen and antagonist but both grow slowly on top of each other, 3 - mutual inhibition at distance less than 2mm, 4 - pathogen growth is inhibited at greater distance at 2mm (Soumaya et al. 2013).

Statistical analysis. Data entry was carried out in Excel and R version 4.0.5 software was used for the comparison of the average percentage inhibitions and growth speeds of the fungi tested according to the Tukey test at the 5% threshold.

Results

Detection of aflatoxin biosynthesis genes. *Aspergillus flavus* strains isolated from peanut were used to detect the presence of genes involved in the aflatoxin biosynthetic pathway. The results obtained after visualization of the DNA fragments of the Nor-1, Ver-1, Omt-A and AflR genes at 400, 895, 1032 and 1232 bp respectively, indicate the presence of these genes in some strains analysed. Fig. 1 and Fig. 2 show the results of the electrophoresis of some strains. Seventeen fungal strains (50%) have at least one of the four genes sought. Table 1 shows that five strains (AKA-6; ANA-31; ANAM-33; ABAN-44; AMAN-51) each have three genes; four strains (AMA-30; ANA-34; ADA-49; ANA-50) have two genes and eight strains (AMAG-23; ABAG-24; AKO-40; AKA-42; AKE-47; ACI-48; AKA-52; AMA-53) each have one gene.

Evaluation of the antagonistic effect of strains. The double confrontation technique was performed to identify atoxinogenic (antagonist) *Aspergillus flavus* strains able of inhibiting the growth of toxigenic (pathogenic) *Aspergillus flavus* strains. These antagonist strains, once identified, can be used in the biological control process against aflatoxins. The results showed (Fig. 3) that the mycelial growth of the control strains is greater compared to that obtained in confrontation plate; which proves that the antagonistic *Aspergillus flavus* strains inhibited the mycelial growth of the toxigenic *Aspergillus flavus* strains tested in this study. The inhibitions percentage varied from 16.98% to 62.50% and was obtained respectively with strains AGA-46 and ABA-28. The comparison of the PI of the mycelial growth of the antagonist strains according to the Korsten scale shows that 2.90% of the atoxinogenic strains have a PI between 0 and 25%; 81.74% of the strains have a PI between 25 and 50%, 15% strains have a PI between 50 and 75% and finally 0% of the strains have a PI between 75 and 100%. Inhibition Mode (IM) 4 was observed for all of the antagonist strains which ensured inhibition of the mycelial growth of the pathogen at a distance greater than 2mm.

Table 2 show the mean of the different percentages of inhibitions obtained by an antagonist strain against all the pathogenic strains. The results show also that the PI of the toxigenic strains varied depending on each toxigenic isolate. The comparison of the means of the PI according to the Tukey test at the 5% threshold indicates a significant difference in the PI ($p = 0.0000136$) depending on the non-toxigenic strains tested (Table 1). The mean mycelial growth speed (VCM) of the antagonist strains was also measured during this study. The results showed that the mean VCM of the strains is 8.4 ± 0.05 mm.d^{-1}. The comparison of the mean of the VCM of the antagonist strains with the threshold of 5% according to the Tukey test, indicates a significant difference (p-value = 0.00996) in the speed of growth from one strain to another.

However, the AMAN-35 and AKA-10 strains obtained the highest speeds (10 mm.d^{-1}). An antagonist strain must have not only a high PI but also a high mycelial growth speed before to be selected as a good strain for biological control. The comparison of the PI and the VCM made it possible to select five (05) interesting antagonistic strains having at least a PI greater than 50% and a VCM greater than 8.40 mm.d^{-1}. These are in order of effectiveness the strain AKA-10; AGA-8; AMAN-35; ABA-28 and AGA-46.

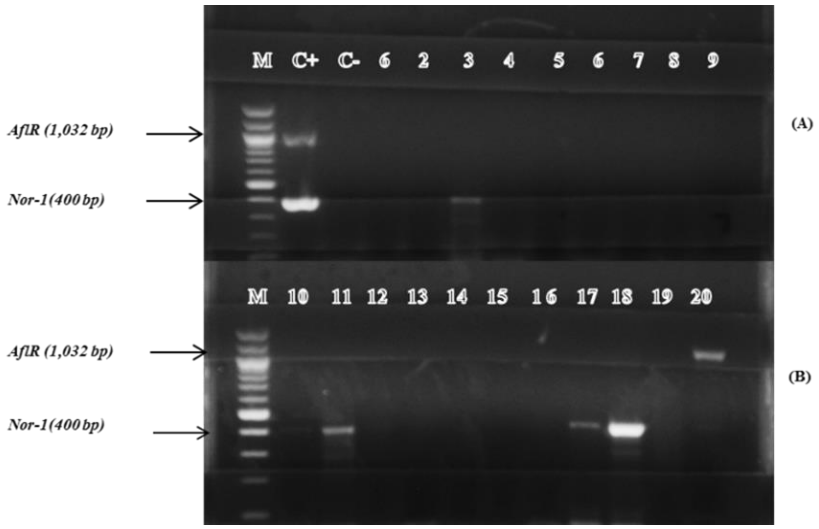

Figure 1. Detection of AflR and Nor-1 gene

(M: 100bp DNA ladder; C+: positive control (Aspergillus flavus GHG 040-1); C-: negative control (Aspergillus flavus GHM 217-8); lane 20: AflR positive strains; lane 3, 11, 17, 18: Nor-1 positive strains).

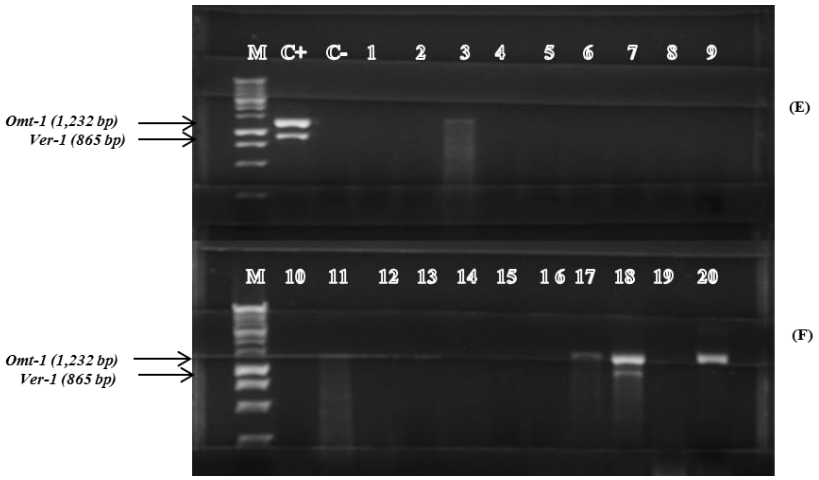

Figure 2. Detection of Omt-1 and Ver-1 gene

(M: 100bp DNA ladder; C+: positive control (*Aspergillus flavus* GHG 040-1); C-: negative control (*Aspergillus flavus* GHM 217-8); lane 3, 17, 18, 20: Omt-1 positive strains; lane 18: Ver-1 positive strains)

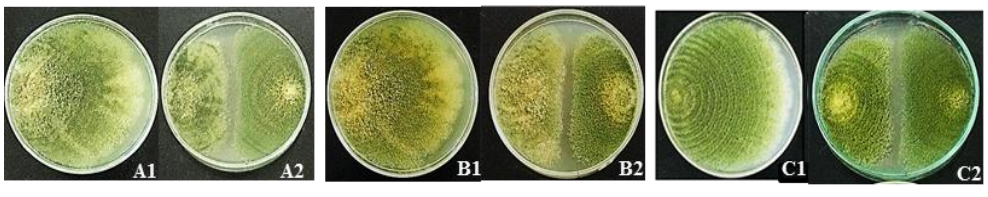

Figure 3. Inhibition of mycelial growth of some toxigenic *Aspergillus flavus* strains by non-toxigenic strains.

(**1)** - ANA-41: 52.85%; (**2) -** AKE-27: 40%;

(**3)** - ANAM-39: 25%. (A1, B1, C1 control plate; A2, B2, C2 confrontation plate).

Table 1. PCR results for all strains

Samples		PCR result				Aflatoxin
Strains code	**Visualization code**	**Nor-1**	**Ver-1**	**Omt-1**	**AflR**	**production**
GHG 040-1	C+	+	+	+	+	+
AKA-6	S3	+	-	+	+	+
AMAG-23	S10	+	-	-	-	+
ABAG-24	S11	+	-	-	-	+
AMA-30	S17	+	-	+	-	+
ANA-31	S18	+	+	+	-	+
ANAM-33	S20	+	-	+	+	+
ANA-34	S21	+	+	-	-	+
AKO-40	S27	+	-	-	-	+
AKA-42	S29	-	-	+	-	+
ABAN-44	S30	+	+	+	-	+
AKE-47	S33	-	-	-	+	+
ACI-48	S34	-	-	-	+	+
ADA-49	S35	+	-	-	+	+
ANA-50	S36	+	-	-	+	+
AMAN-51	S37	+	-	+	+	+
AKA-52	S38	+	-	-	-	+
AMA-53	S39	+	-	-	-	+

Table 2. PI and VCM mean of antagonistic strains

Antagonist strains code	Means	
	PI	**VCM, mm**
ACI-7	52.93 ± 0.97^{cd}	8.00 ± 0.03^{b}
AGA-8	54.40 ± 0.94^{d}	8.50 ± 0.03^{d}
AKA-10	54.40 ± 0.94^{d}	10.00 ± 0.17^{f}
AKO-22	48.51 ± 1.07^{ab}	8.50 ± 0.0^{cd}
AKO-26	47.04 ± 1.10^{a}	8.50 ± 0.13^{c}
AKE-27	49.98 ± 1.04^{ac}	7.50 ± 0.03^{a}
ABA-28	51.46 ± 1.00^{bcd}	8.50 ± 0.00^{cd}
ATC-32	47.79 ± 0.04^{ab}	9.00 ± 0.04^{e}
AMAN-35	52.93 ± 0.97^{cd}	10.00 ± 0.19^{f}
ANA-36	48.51 ± 1.07^{ab}	7.50 ± 0.01^{a}
ANAM-37	51.46 ± 1.00^{bcd}	8.00 ± 0.02^{b}
ANAM-38	48.98 ± 1.04^{ac}	8.00 ± 0.02^{b}
ANAM-39	51.46 ± 1.00^{bcd}	8.80 ± 0.02^{b}
ANA-41	52.93 ± 0.97^{cd}	8.00 ± 0.00^{b}
AGA-46	51.46 ± 1.00^{bcd}	8.50 ± 0.06^{cd}

Discussion

Agricultural products in tropical Africa are very often contaminated by fungi because of the climate and environmental conditions that are very favourable to their growth. The food preservation techniques used by most producers are still traditional and prove to be ineffective in the fight against the contamination of their foodstuffs by fungi. Some of these fungi can then develop and produce toxins such as aflatoxins which are very dangerous for humans and animals. In this study, thirty-four strains of *Aspergillus flavus* isolated from peanut seeds were used in order to determine, on the one hand, their aflatoxigenic potential and on the other hand, the ability of atoxinogenic strains to inhibit growth of toxigenic strains. Molecular analysis was based on the detection of genes coding for intermediate enzymes involved in the aflatoxin biosynthetic pathway. These are the Nor-1 gene coding for norsolorinic acid reductase, the Ver-1 gene coding for versicolorina dehydrogenase, the Omt-1 gene coding for sterigmatocystin 0-methyl transferase, and the AflR regulatory gene.

The results showed that seventeen fungal strains (50%) have at least one of the four genes sought. One strain (positive control) has the four genes; five strains each have three genes; four strains have two genes and eight strains each have one gene. These results are similar to those obtained by Uzeh and Adebowale (2021) in his study on aflatoxigenic fungi and aflatoxins in locally processed peanut butter in Lagos Nigeria where two *Aspergillus flavus* strains had all four aflatoxin genes (Nor-1, Ver-1, Omt-1 and AflR), one strain had 3 genes except AflR while two strains each of *Aspergillus flavus* and *Aspergillus parasiticus* only had the Nor -1 gene. The presence of these four types of genes indicates both the toxicity of these strains but also their ability to produce aflatoxins B1 and aflatoxins B2 in peanut seeds when conditions are favourable. A fungal strain can however be toxic and not produce aflatoxins even if all the genes were present. Indeed, the conditions allowing gene expression in aflatoxin-producing species depend on physiological and environmental conditions. (El Mahgubi 2013; Mahror et al. 2019). A study by Abdel-Hadi and Magan (2010) demonstrated a significant difference in Nor-1 gene expression at three levels of water activity (a_w) in which higher expression was observed at 0.90 a_w versus 0.95 a_w, and no expression occurred at 0.85 a_w. However, the absence of genes observed in some strains indicates their inability to produce aflatoxins. This incapacity could be explained by a mutation in the genes involved in the signalling pathway or having a regulatory role in the activation of the aflatoxin biosynthesis pathway (Chang and Dorner 2005). Biological control methods currently use this inability of some strains of *Aspergillus flavus* to fight against the contamination of foodstuffs by aflatoxins. The Biological control method is based on the use of non-toxic strains to inhibit the growth of toxic strains and thus prevent the production and accumulation of aflatoxins in foods. According to Zouhair et al. (2014), the application of fungicides can stimulate the production of mycotoxins in response of fungi to stress. Biological control is an effective alternative to control aflatoxins. Therefore, the inhibitory potency (PI) of the non-toxic strains of *Aspergillus flavus* identified in this study was determined by the double confrontation technique. The results proved that the non-toxic strains of *Aspergillus flavus* significantly reduced *in vitro* the growth of the toxic strain with a better efficiency recorded with the ABA-28 strain. Indeed, the PI of the antagonist strains varied according to each pathogen. The comparison of the means of the PI indicated a significant difference ($p = 0.0000136$) of the PI from one antagonist strain to another. These results are similar to those obtained by Soumaya et al. (2016) which showed that the percentages of inhibition of strains of *Aspergillus* spp species varied according to the pathogen tested. A decrease in the mycelial growth of the pathogenic strains is observed from 16.98% to 62.50% with a mean percentage inhibition of 50.88±1.92%. These results are different from those obtained by Kenza and Moufida (2015) which obtained 54.02% inhibition percentage of *Aspergillus flavus*. The difference in the PI obtained in this study could be explained by the ability of the pathogenic strain to oppose resistance to the antagonistic strain. The cell wall is essential for fungal growth and for the resistance of a fungus to external attacks and its alteration is linked to the virulence of the pathogen. Inhibition mode 4 (MI = 4) was used by all the antagonist fungi which ensured an inhibition of the mycelial growth of the pathogen at a distance greater than 2 mm. This result is identical to that obtained by Soumaya et al. (2016) where the inhibition mode 4 was also used by *Aspergillus*

flavus strains. Despite the absence of direct contact between the antagonistic strains and the pathogenic strains, the latter succeeded in exerting an inhibitory activity on the development of the toxigenic strains. This was due to the ability of antagonistic strains to produce volatiles or spores that caused lysis of the mycelium or arrest of pathogen growth. It is for this reason that in biological control mechanisms using non-toxic fungal strains, growth speed is an important factor to consider. According to Bandyopadhyay et al. (2016) the speed of growth allows non-toxic strains to invade and disperse quickly compared to aflatoxin-producing strains initially present in the field.

The growth speed results of the non-toxic strains detected in this study showed that the growth speed ranged from 7.5 to 10mm per day with an average growth speed of 8.4±0.05 $mm.d^{-1}$. These growth speeds are similar to those obtained by (Qjidaa et al. 2018). However, according to Tukey's test at the 5% threshold, a significant difference ($p = 0.00996$) was observed in the growth speed of the antagonist strains tested. The comparison of the percentage inhibition of mycelial growth and the growth speed made it possible to select atoxinogenic strains having both a PI and a VCM above the average (PI > 50%; VCM > 0.84 $mm.d^{-1}$). These atoxinogenic strains (AKA-10; AGA-8; AMAN-35; ABA-28; AGA-46) had the best inhibition percentages and growth speeds and were effective in inhibiting the growth of the toxigenic strains identified in this study.

Conclusion

Food contamination by aflatoxins is everyone's business. This contamination begins in the field and continues during conservation or storage when conditions are favourable to the growth of fungi of the genus *Aspergillus*. The fight against these fungi requires not only good agricultural practices (GAP) but also the use of strains of the non-toxic *Aspergillus flavus* fungus to inhibit the growth of toxic strains. This work has made it possible to detect the presence of genes involved in the aflatoxin biosynthesis pathway in some strains of *Aspergillus flavus.* Among the strains of *Aspergillus flavus* having none of the four genes, five proved to be effective in inhibiting the mycelial growth of aflatoxigenic strains by more than 50%. In-depth tests based on a larger number of fungal strains and on field tests would make it possible either to detect other more interesting strains or to definitively enhance the inhibitory potential of these strains in the fight against aflatoxins.

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