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MYCOTOXIGENIC FUNGI OF WINE GRAPE IN MEKNES VINIYARDS (MOROCCO)

Benaziz, Mohamed[1]; Hajji, Lhossin[2] ; Jadouali, Simohamed[1] ; Nait' Mbark, Hasna[2] ; Hajjaj, Hassan[2] ; Ainane, Ayoub[1] ; Ainane Tarik*[1]

[1]Superior School of Technology, University of Sultan Moulay Slimane, BP 170, Khenifra 54000 Morocco.

[2] Faculty of Sciences, Moulay Ismail University, Morocco

*t.ainane@usms.ma

Abstract

In the present study, a total of 119 isolates of fungi were isolated from forty nine (49) samples of eight grape varieties collected in two vinyards in Meknes area, Morocco. The identification of isolates of fingi was performed by macroscopic and microscopic methods. Selected *Penicillium* and *Aspergillus* strains were studied for their potential to produce mycotoxins especially Patulin, Citrinine and Aflatoxin. The results of distribution showed that penicillium and aspergillus genera, were present in all grape varieties samples, and the most infected grape varieties are *Chardonnay* and *Cabernet sauvignon*. The values were 22.6% and 15.1 %, respectively. Results of LC analysis indicated that 5 *Penicillium* isolates out of the 16 and 8 *Penicillium* isolates out of 38 produce patulin during veraison and maturity stage respectively. The high patulin quantity (41 ug ml^{-1}) is produced by *Penicillum expansium* isolated during veraison stage. In this study no citrinin was detected in extract derived from 119 isolates tested. 3 Aspergillus strains (two *A. flavus* and one *A. parasiticus*) out of the 25 and 2*Aspergillus flavus* out 40 were able to produce Aflatoxin during veraison and maturity stage respectively. In order to be able to limit the grape alterations and health security, it appears necessary to detect and identify the microorganisms responsible in order to limit their presence and their development in the grape.

Keywords: *Mycotoxin, Funji, Patulin, Citrinin, Aflatoxin, Grape.*

Introduction

Grapes and their derived products belong to most consumed products at world level [1]. The vineyard in Morocco occupies an area of 49.000 hectares with an annual production of 230.000 tons of grapes including 172.000 tons (38 200 Hectares) of table grapes and 58000 tons (10 800 hectares) of wine grapes. For the wine grapes, most of the vineyards are concentrated in the regions of El Hajeb, Meknes, which hold more than 60% of the total area [2]. The Food and Agriculture Organizaton (FAO) estmatedabout 25% of food crops in the world, including many staple foods are contaminated with mycotoxin [3]. Wine grapes like other food products can be contaminated by several fungi [4]. The latter cannot generate only economic losses of wine making products like alterating wine qualities, they canproduce secondary metabolites called mycotoxinswhich represents a risk to the health safety of the consumer. This contamination is due to several environmental factors as well as improper harvesting, storage and transformation processing [5]. Morocco enclosed by the Atlantic Ocean and Mediterranean Sea (4500 km of coasts), has a climate characterized by high temperature and high humidity that favor growth of fungi [6]. Some species of *Penicillium* and *Aspergillus*genera are known to be developed on wine grapes and derivatives and produce various mycotoxins like aflatoxin, patulin, ochratoxin [7]. This mycotoxin is produced by several species belonging to the genera Penicillium and Aspergillus. The aflatoxins, in principal B_1, are studied for their character strongly hepatotoxic. The *P. expansum* species is known to be the major responsible for the synthesis of patulin and citrinin on grapes [8-9]. The cited mycotoxins makeproblems to human health due to its teratogenic, mutagenic, nephrotoxic, immunotoxic and carcinogenic effects [10]. Ostry et al. (2018) [11] isolated microfungi of *Penicillium expansum* from grape and confirmed its ability to produce citrinin and high quantity of patulin. *Penicillum griseofulvum*, species producing patulin mycotoxin has also been discovered on vines [12-13].

In Morocco, several report have been made about the occurrence of different mycotoxins and toxigenic fungi in foods [14-16]. For grape wine and winemaking products consumed widely in Morocco, very few studies have been conducted to assess the degree of contamination of grapes by fungi producers of citrinin, patulin and aflatoxin. The aims of this work are to evaluate fungi quality of different grape varieties and to test their ability to produce patulin, citrinine and aflatoxin.

Materials and Methods

Samples collection

49 grape samples (Table 1) belonging to different soils, different grape varieties and two stages of maturity (veraison and maturity stages) were collected for analysis in the laboratory. The samples were then stored in the refrigerator at 4°C before being analyzed.

Fungal isolaton

The strains have been isolated from grapes,the surface of grapes were disinfected with 0.2% sodium hypochlorite solution for 2 min and cleanedthree times with sterile distilled water. Fivegrape berryof each sample were randomly selected and then plated in Petri plates (90-mm diameter, 10 grains/dish) containing CZapek Agar (CZA) medium. Petri plates were incubated at 25°C for 7-10 days. Fungi developing in each grape berry were re-isolated on CZA medium, were purified after 2 or 3 successive subcultures in order to obtain pure strains that will subsequently be stored at 4 °C for subsequent analyzes [17].

Identifcaton of fungi

Aspergillus and *penicillums* strains were identified using macroscopic and microscopic morphological criteria in accordance with appropriate keys [18].

Patulin, citrinin and aflatoxin extraction

The broth fermentation is obtained after 7 days of incubating the isolates in the static liquids mediums (YES). The liquids cultures were filtered through a 0.45µm nitrocellulose membrane. The 15 mL of the filtrate was acidified with 100 µL concentrated HCl and mixed with 15 mL of chloroform. The organic phase was collected and concentrated to dryness in a rotary evaporator at 50 °C and the extract is taken up with 1 mL of methanol for the subsequent analysis [19].

Mycotoxins analysis

TLC analysis

The chloformic extracts were analysed using silicagel with a mobile phase for patulin–citrinin: chloroforme/methanol/water (65/25/4, v/v/v) at 365 and 254 nm respectively. For aflatoxin: (chloroforme/Acetate), (97/3, v/v) at 254 nm. The different mycotoxins were identified by comparison with appropriate reference standards [17].

HPLC analysis of patulin and citrinin

HPLC analysis of patulin was performed with a Waters 2695pump, auto sampler and Waters 2998 photodiode-array detector, with Spectra Manager sofware and Empower 3 sofware data registraton.Patulin is analysed at 276 nm wave lonth, the separation was achieved using a Waters Spherisorb ODS_2 (5 µm, 250×4 mm) column. The system was used in isocratc mode with a mobile phase consisting of a mixture of water and acetonitrile (95:5, v/v) at a flow rate of 1 $mL.min^{-1}$for patulin.H_3PO_4 (0.33M)/acetonitrile/propanol-2 volume (60/40/5) for citrinin, at a flow rate of 1 ml/min. The injection volume of the standard and sample extract was 50 µl. All assays were performed at room temperature conditions. The quantification of patulin was performed by the measurement of the peak area at Pa retenton time and the comparison with the relevant calibraton curve (patulin and citrinin standard) [17].

Results and discussion

The occurrence of fungi

The results of the mycological quality study of grape samples collected from the vinyards (companions 2019) are shown in Table 2. Macroscopic and microscopic evaluation of the isolates led us to identify the penicillium and aspergillus genera. We noted that the analysed samples are contaminated with fungus and show a large fluctuation in the contamination level from one variety to another and from one stage to another within samples of the same origin. This fluctuation is probably associated with several parameters including humidity, temperature, rainfall, etc. These conditions were recorded favorable to the proliferation of flamentous fungi [17]. The main fungal genera isolated from the grapes are *Penicillium* and *Aspergillus* known by their potential for mycotoxin production [20-22]. These two fungi are among those mycotoxin-producing molds and responsible for spoilage of the harvest and which represent the contaminants most present on grape berries and which can be the cause of losses significant economic and health problems for the consumer [23]. A genus Aspergillus was the most frequent isolated in grape. According to the results obtained in this study regarding penicillium and aspergillus genera, was present in all grape varieties samples. The most infected grape varieties being, *Chardonnay* and *Cabernet sauvignon*. The values were22.6%and15.1 %, respectively (Table 2). The levels of *Aspergillus* (33%) and *Penicillium* (31%) in the samples collected at the mature stage are two time higherthan that of the veraison stage (21%) and (13%) respectively (Figure 3). At the veraison stage, in the *Merlot*, *Arinarnoa* and *Chrdonnay* grapes, we noticed a predominance of *Aspergillus* genus with a rate of 20%, 16% and 16% of total *Aspergillus*, respectively. We also noticed a predominance of the *Penicillium* genus contaminating both grape *Chardonnay* (31%) and *Arinarnoa* (12%).

Mycotoxicologique Analysis

The isolated *Penicillium* and *Aspergillus* strains were placed in liquid culture on YES medium, and the extracts obtained were analysed. The isolates that were found positive and showed a patulin visible band under UV light at 246 nm (Figure 3A) were analysed by HPLC, the figure 2A shows an example of the patulin chromatogram profile. For aflatoxin we are limited to TLC analyses. Figure 3B illustrates an example of aflatoxin migration profile by the TLC technique under UV at 365 nm. The ability of isolated strains to produce patulin, cirinin and aflatoxin are presented in table 3. Not all the species of a genus are able to produce mycotoxins and their production level is different (Table 3), for Patulin is a $C_7H_6O_4$ lactone (Figure 4) which has been tested as an antibiotic against mushrooms. But given its carcinogenic properties in animals, it cannot therefore be used from a pharmaceutical point of view. Patulin inhibits the fermentation yeast Saccharomyces cerevisiae but it is partly

degraded by moleculs of SO_2 (sulfur dioxide) and totally during the alcoholic fermentation [24]. Its presence in wines is therefore unlikely. Five *Penicillium* isolates out of the 16 and 8 *Penicillium* isolates out of 38 were able to produce patulin during veraison and maturity stage respectively. The patulin HPLC analysis shows that the patulin amounts varies from one isolate to another. The high patulin quantity (41 ug ml^{-1}) is produced by penicillum genus isolated during veraison stage (table 3). These results are similar to the results of other works which report that this mycotoxin is produced by several species belonging to the genera Penicillium. The *P. expansum* species is known to be responsible for the synthesis of the patulin on grapes [25]. *Penicillum griseofulvum*, a species producing patulin has also been found on grapes [26]. For the Citrinin with the formula $C_{13}H_{14}O_5$ (Figure 4) was isolated for the first time from *Penicillium citrinium*. Citrinin is produced mainly by species belonging to the genera Penicillium spp. and Aspergillus spp. (*A. terreus, A. niveus*). *Penicillium citrinum*, the main producer of citrinin has been isolated from grapes [27]. The Citrinin is not degraded during the alcoholic fermentation and can be weakly present in wines. In this study No citrinin was detected in extract derived from 119 isolates tested(Figure 2B and Table 3). The aflatoxins, are studied for their character strongly hepatotoxic. The structure of the $C_{17}H_{12}O_6$ molecule is given in figure 4. Some species of the genus Aspergillus and in particular *A. flavus* are producers of this mycotoxin. In this study, three *Aspergillus* strains (two *A. flavus* and one *A. parasiticus*) out of the 25 and two *Aspergillus flavus* out 40 were able to produce Aflatoxin during veraison and maturity stage respectively. In Portugal, 27 strains producers were isolated on grapes [28]; in Lebanon these strains can reach 43.1% of strains isolated from grapes [29-] and 23% in Tunisia [30].

Conclusion

Grapes are considered a food that is exposed to fungal contamination and mycotoxin producton. This study shows that the Aspergillus and Penicillium species isolated from grapes are a source of the mycotoxins, patulin and Aflatoxin. All the results related to the study of the morphological characters of the isolates indicate that they are dominated by the mycotoxin-producing species Penicillium expansium and Aspergillus flavus. The isolation of Penicillumand Aspergillus genera ingrape requires a lot of vigilance. The rapid and specific detection of Penicillum and Aspergillus is important to ensure the microbiological safety of food.It needs to use grapes under good condition so as to reduce the risk of contamination with mycotoxigenic fungi and subsequent mycotoxin occurrence in wine.

References

1. Hasan, M., & Bae, H. (2017). An overview of stress-induced resveratrol synthesis in grapes: perspectives for resveratrol-enriched grape products. Molecules, 22(2), 294.
2. Afechtal, M. (2021). First report of grapevine yellow speckle viroid 1 infecting grapevine (Vitis vinifera L.) in Morocco. Journal of Plant Pathology, 103(1), 383-383.
3. Eskola, M., Kos, G., Elliott, C. T., Hajšlová, J., Mayar, S., & Krska, R. (2020). Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate'of 25%. Critical reviews in food science and nutrition, 60(16), 2773-2789.
4. Ayofemi Olalekan Adeyeye, S. (2020). Aflatoxigenic fungi and mycotoxins in food: a review. Critical reviews in food science and nutrition, 60(5), 709-721.
5. Neme, K., & Mohammed, A. (2017). Mycotoxin occurrence in grains and the role of postharvest management as a mitigation strategies. A review. Food Control, 78, 412-425.
6. Tantaoui-Elaraki, A., Riba, A., Oueslati, S., & Zinedine, A. (2018). Toxigenic fungi and mycotoxin occurrence and prevention in food and feed in northern Africa–a review. World Mycotoxin Journal, 11(3), 385-400.
7. Kioroglou, D., Kraeva-Deloire, E., Schmidtke, L. M., Mas, A., & Portillo, M. C. (2019). Geographical origin has a greater impact on grape berry fungal community than grape variety and maturation state. Microorganisms, 7(12), 669.

8. Li, B., Chen, Y., Zhang, Z., Qin, G., Chen, T., & Tian, S. (2020). Molecular basis and regulation of pathogenicity and patulin biosynthesis in Penicillium expansum. Comprehensive Reviews in Food Science and Food Safety, 19(6), 3416-3438.
9. Sajid, M., Mehmood, S., Yuan, Y., & Yue, T. (2019). Mycotoxin patulin in food matrices: occurrence and its biological degradation strategies. Drug metabolism reviews, 51(1), 105-120.
10. Taheur, F. B., Kouidhi, B., Al Qurashi, Y. M. A., Salah-Abbès, J. B., & Chaieb, K. (2019). Biotechnology of mycotoxins detoxification using microorganisms and enzymes. Toxicon, 160, 12-22.
11. Ostry, V., Malir, F., Cumova, M., Kyrova, V., Toman, J., Grosse, Y., ... & Ruprich, J. (2018). Investigation of patulin and citrinin in grape must and wine from grapes naturally contaminated by strains of Penicillium expansum. Food and Chemical Toxicology, 118, 805-811.
12. Felšöciová, S., & Kačániová, M. (2021). Microfungi of grapes from Small Carpathian region in Slovakia. Journal of Microbiology, Biotechnology and Food Sciences, 2021, 478-482.
13. Valente, S., Cometto, A., Piombo, E., Meloni, G. R., Ballester, A. R., González-Candelas, L., & Spadaro, D. (2020). Elaborated regulation of griseofulvin biosynthesis in Penicillium griseofulvum and its role on conidiation and virulence. International Journal of Food Microbiology, 328, 108687.
14. El Jai, A., Juan, C., Juan-García, A., Mañes, J., & Zinedine, A. (2021). Multi-mycotoxin contamination of green tea infusion and dietary exposure assessment in Moroccan population. Food Research International, 140, 109958.
15. Cherkani-Hassani, A., Ghanname, I., Zinedine, A., Sefrioui, H., Qmichou, Z., & Mouane, N. (2020). Ochratoxin a in breast milk in Morocco: the affecting dietary habits of the lactating mothers and the degree of exposure of newborns "CONTAMILK study". Drug and Chemical Toxicology, 1-7.
16. Bouafifssa, Y., Manyes, L., Rahouti, M., Mañes, J., Berrada, H., Zinedine, A., & Fernández-Franzón, M. (2018). Multi-occurrence of twenty mycotoxinsin pasta and a risk assessment in the Moroccan population. Toxins, 10(11), 432.
17. Mansouri, A., Hafidi, M., Mazouz, H., Zouhair, R., El Karbane, M., & Hajjaj, H. (2014). Mycoflora and patulin-producing strains of cereals in North-Western Morocco. South Asian J. Exp. Biol, 4(5), 276-282.
18. Sacco, C., Donato, R., Zanella, B., Pini, G., Pettini, L., Marino, M. F., ... & Marvasi, M. (2020). Mycotoxins and flours: Effect of type of crop, organic production, packaging type on the recovery of fungal genus and mycotoxins. International Journal of Food Microbiology, 334, 108808.
19. Cheng, H., Yang, Y., Chen, Y., Chen, X., Cai, Z., & Du, A. (2018). Novel monoclonal antibody-based immunochromatographic strip for detecting citrinin in fruit from Zhejiang province, China. PloS one, 13(5), e0197179.
20. Alasmar, R., Ul-Hassan, Z., Zeidan, R., Al-Thani, R., Al-Shamary, N., Alnaimi, H., Jaoua, S. (2020). Isolation of a novel Kluyveromyces marxianus strain QKM-4 and evidence of its volatilome production and binding potentialities in the biocontrol of toxigenic fungi and their mycotoxins. ACS omega, 5(28), 17637-17645.
21. Felšöciová, S., & Kačániová, M. (2019). Mycotoxin-producing Penicillium spp. and other fungi isolated from grapes for wine production in Small Carpathians wine region. Potravinarstvo, 13(1).
22. Perrone, G., Logrieco, A. F., & Frisvad, J. C. (2017). Comments on "Screening and Identification of Novel Ochratoxin A-Producing Fungi from Grapes. Toxins 2016, 8, 333"—In Reporting Ochratoxin A Production from Strains of Aspergillus, Penicillium and Talaromyces. Toxins, 9(2), 65.
23. Gava, A., Emer, C. D., Ficagna, E., Fernandes de Andrade, S., & Fuentefria, A. M. (2021). Occurrence and impact of fungicides

residues on fermentation during wine production–A review. Food Additives & Contaminants: Part A, 1-19.
24. Zegota, H., & Pietka, M. (1992). Action of γ-irradiated patulin on Saccharomyces cerevisiae. Zeitschrift für Lebensmittel-Untersuchung und Forschung, 194(5), 465-468.
25. Bragulat, M. R., Abarca, M. L., & Cabañes, F. J. (2008). Low occurrence of patulin-and citrinin-producing species isolated from grapes. Letters in Applied Microbiology, 47(4), 286-289.
26. Welke, J. E., Hoeltz, M., Dottori, H. A., & Noll, I. B. (2011). Patulin accumulation in apples during storage by Penicillium expansum and Penicillium griseofulvum strains. Brazilian Journal of Microbiology, 42, 172-180.
27. Ostry, V., Malir, F., & Ruprich, J. (2013). Producers and important dietary sources of ochratoxin A and citrinin. Toxins, 5(9), 1574-1586.
28. Rodrigues, P., Venâncio, A., Kozakiewicz, Z., & Lima, N. (2009). A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of Aspergillus section Flavi isolated from Portuguese almonds. International journal of food microbiology, 129(2), 187-193.
29. Makhlouf, J., Carvajal-Campos, A., Querin, A., Tadrist, S., Puel, O., Lorber, S., Bailly, S. (2019). Morphologic, molecular and metabolic characterization of Aspergillus section Flavi in spices marketed in Lebanon. Scientific reports, 9(1), 1-11.
30. Fredj, S. M. B., Chebil, S., & Mliki, A. (2009). Isolation and characterization of ochratoxin A and aflatoxin B1 producing fungi infecting grapevines cultivated in Tunisia. African Journal of Microbiology Research, 3(9), 523-527.

Table 1. Grape sampling in various vineyards.

Vineyard	Grape variety	Type of grape	Samples number
DVB	*Syrah*	R	**7**
DVB	*Arinarnoa*	R	**7**
DVS	*Merlot*	R	**7**
DVB	*Cabernet sauvignon*	R	**7**
DVS	*Cabernet franc*	R	**7**
DVB	*Marcellon*	R	**7**
DVB	*Chardonnay*	W	**7**
DVS	*Sauvignon Blanc*	W	**7**

DVB: Boufkran vinyard; DVB: Sais viniyard; R: Red grape; W: with grape.

Table 2. *Aspergillus* and *Penicillium* strains contaminating grape varietises during veraison and maturity stage.

Grape variety	Veraison		Maturity		Total n(%)
	Aspergillus **n(%)***	***Penicilliums*** **n(%)**	***Aspergillus*** **n(%)**	***Penicilliums*** **n(%)**	
Syrah	2(8)	0(0)	3(7.5)	1(2.5)	**6(5)**
Arinarnoa	4(16)	2(12.5)	4(10)	5(13.2)	**15(12.6)**
Merlot	5(20)	1(6.2)	4(10)	6(15.8)	**16(13.4)**
Cabernet sauvignon	1(4)	4(25)	6(15)	7(18.4)	**18(15.1)**
Cabernet franc	4(16)	1(6.2)	5(12.5)	3(7.9)	**13(10.9)**
Marcellon	3(12)	2(12.5)	5(12.5)	3(7.9)	**13(10.9)**
Chardonnay	4(16)	5(31.2)	8(20)	10(26.3)	**27(22.6)**
Sauvignon Blanc	2(8)	1(6.2)	5(12.5)	3(7.9)	**11(9.2)**
Total number of strains	**25**	**16**	**40**	**38**	**119**

n(%) : number of strains (percentage of strains)

Table 3. Patulin (Pa) and citrinin (Ci) and Aflatoxin (Af) producing ability of *Aspergillus* spp. and *Penicillium* spp. strains isolated from grapevins.

Step	Strains	Total strain	Number of positif Strain (CCM)			Range mycotoxin ug.ml^{-1} (HPLC)	
			Pa	Ci	AF	Pa	Ci
Veraison	***Aspergillus spp***	25	0	0	3		
	Aspergillus flavus 1		-	-	+	--	--
	Aspergillus flavus 2		-	-	+	--	--
	Aspergillus parasiticus 1		-	-	+	--	--
	Penicilliums spp	16	5	0	0		
	Penicilliums expancium 1		+	-	-	0.18	N.d
	Penicilliums expancium2		+	-	-	0.14	N.d
	Penicilliums expancium3		+	-	-	18	N.d
	Penicilliums expancium4		+	-	-	41	N.d
	Penicilliums expancium5		+	-	-	7	N.d
Maturity	***Aspergillus spp***	40	0	0	2		
	Aspergillus flavus 1		-	-	+	--	--
	Aspergillus flavus 2		-	-	+	--	--
	Penicilliums spp	38	8	0	0		
	Penicilliums expancium 1		+	-	-	10	N.d
	Penicilliums expancium 2		+	-	-	0.2	N.d
	Penicilliums expancium 3		+	-	-	0.15	N.d
	Penicilliums expancium 4		+	-	-	26.40	N.d
	Penicilliums expancium 5		+	-	-	12.7	N.d
	Penicilliums expancium 6		+	-	-	0.11	N.d
	Penicilliums expancium 7		+	-	-	1.5	N.d
	Penicilliums expancium 8		+	-	-	0.25	N.d

Nd : no detected

Figure 1. Distributon of *Penicilliums* and *Aspergillus* genuses isolated from grape during veraison and maturity stage.

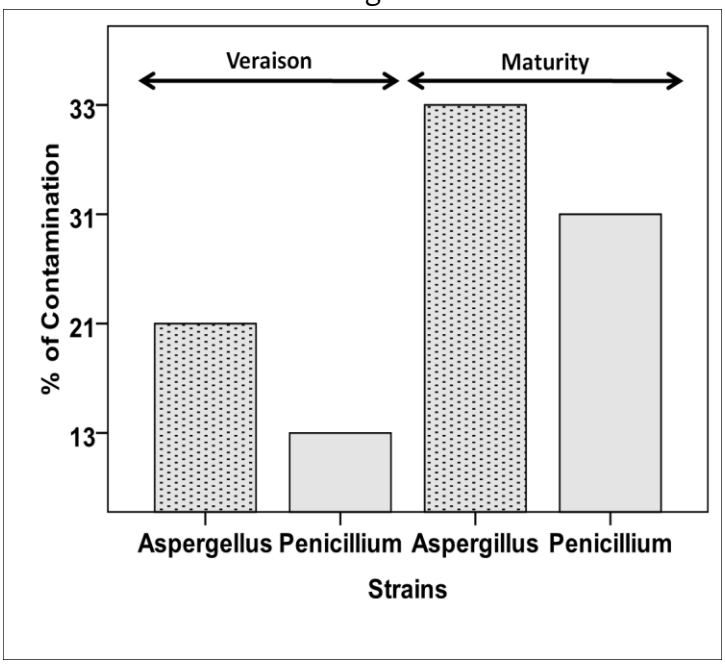
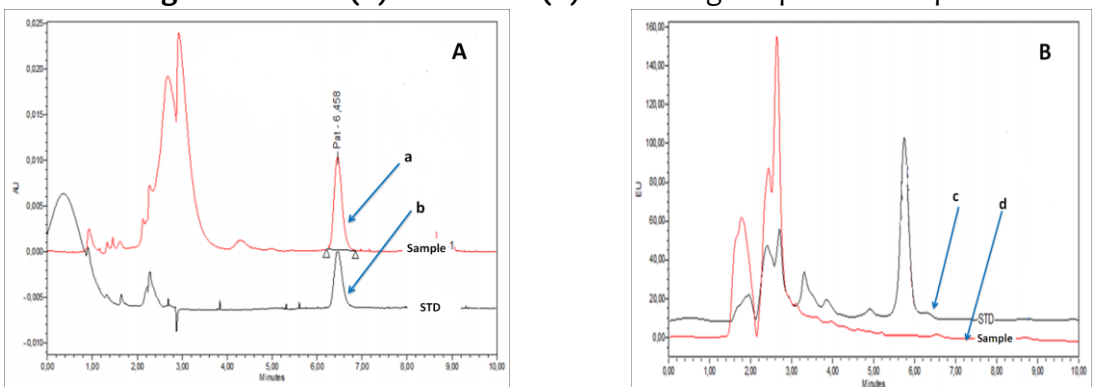


Figure 2. Patulin (**A**) and Citrinin (**B**) chromatogram profile example.



a: Patulin extract chromatogram; **b**: Patulin standard chromatogram; **c**: Citrinin extract chromatogram; **d**: Citrinin extract chromatogram ;

Figure 3. Thin layer chromatographie (TLC) Migration profile of patulin **(A)** and aflatoxin **(B)**.

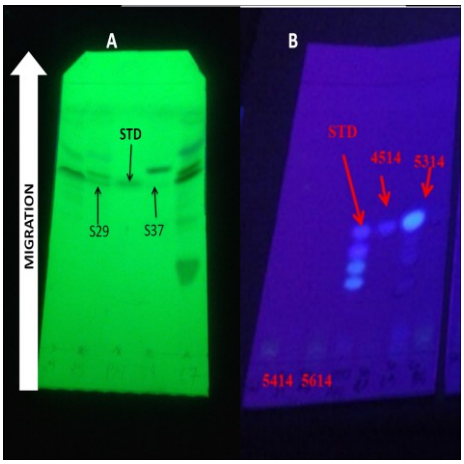
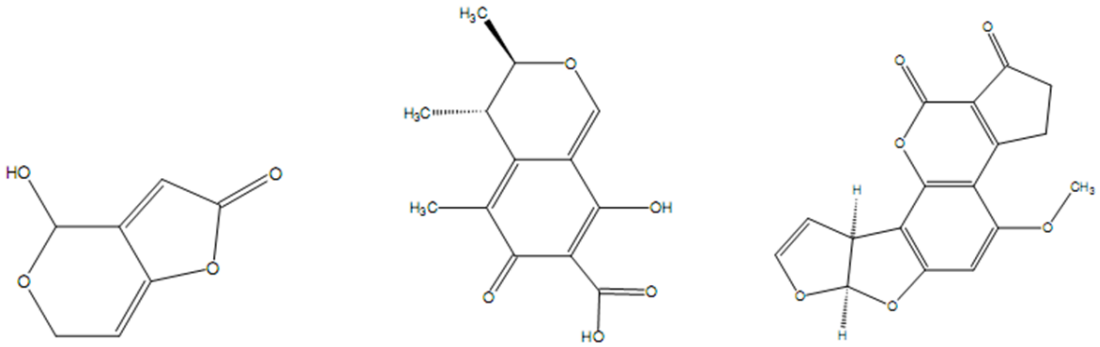


Figure 4. Chemical structures of three mycotoxins.



Patul Citrinine Aflatoxine B_1