QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

BIOLOGICAL CONTROL OF MYCOTOXIGENIC FUNGI AND ASSOCIATED MYCOTOXINS BY THE APPLICATION OF BACTERIAL AND YEAST ANTIFUNGAL COMPOUNDS

BY

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ABSTRACT

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Title: Biological Control of Mycotoxigenic Fungi and Associated Mycotoxins by The Application of Bacterial and Yeast Antifungal Compounds

Supervisor of Thesis: Prof. Samir Jaoua.

Mycotoxins are secondary metabolites of mycotoxigenic fungi and are considered as a serious threaten to the life of humans and animals, since some of these mycotoxins are carcinogenic compounds.

In this M.Sc research project, we demonstrated that the low fermenting yeast *L. thermotolerans 751* has a great antifungal potential through the synthesis of Volatile Organic Compounds (VOC) that are able to act against the mycotoxigenic fungi and the synthesis of their mycotoxins. *L. thermotolerans 751* has also shown a great adsorption potential to mycotoxins in *in-vitro* experiments. In addition, these VOCs were applied in *in-vitro* experiments to inhibit the fungal growth and spores' germination and protection of fungi inoculated tomato fruit, for more than one month.

On the other hand, our second biocontrol agent, the Qatari bacterial strain *Burkholderia cepacia* (*QBC03*) has shown a broad antifungal spectrum and a very efficient inhibition of the synthesis of mycotoxins.

DEDICATION

This work is dedicated to my father, Hisham Zedan.

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CHAPTER 1: INTRODUCTION

Mycotoxins are the secondary metabolites produced by the mycotoxigenic fungi that belong to three important genera, *Aspergillus, Fusarium* and *Penicillium* (Anfossi *et al.,* 2016). These fungi can grow on fruits and vegetables and pre- or post- to their harvest if the favorable conditions are present, such as humidity and moderate temperature. The growth of these fungi on the food and feed products can lead to their contamination with the mycotoxins. Mycotoxins contaminated food is a great threaten to the life of humans and animals, since some of these mycotoxins are grouped as carcinogenic compounds. The common mycotoxins that contaminate the food and feed are Aflatoxins, Ochratoxins, Deoxynivalenol, Zearalenone and fumonisins (Freire & Sant'Ana, 2018). There are many ways for the detoxification of these mycotoxigenic fungi and their mycotoxins. Physical, chemical and biological approaches are possible options for the detoxification of the mycotoxigenic fungi. However, the biological control methods applying mainly yeast and bacteria for the bioremediation of the mycotoxigenic fungi and their mycotoxins have been receiving attention (Recep *et al.,* 2009).

This M. Sc. Thesis project aims to explore and set up biological control methods based on microbial activities. In fact, we explored the biocontrol potentialities of two microbial strains, a low fermenting yeast *L. thermotolerans 751* and a local strain of *Burkholderia cepacia* (*QBC03*). The antifungal potentials of both *Lachancea* and *Burkholderia* to control the growth of mycotoxigenic fungal species of *Aspergillus*, *Fusarium* and *penicillium* and the synthesis of their mycotoxins were investigated.

1.1 Research objectives:

1. Investigation of the yeast (*Lachancea thermotolerans*) and yeast derivatives for the control of mycotoxigenic fungi and mycotoxins.

2. The investigation of the antifungal activities of a local isolate (*Qatari Burkholderia cepacia*) against mycotoxigenic fungi.

CHAPTER 2: LITERATURE REVIEW

2.1 Mycotoxins:

Mycotoxins are the secondary metabolites produced by mycotoxigenic fungi. Mycotoxins can contaminate variety of food commodities. The main studied and investigated mycotoxins are Ochratoxin A, deoxynivalenol, T2 and HT2 toxin, citrinin, patulin, fumonisins, zearalenone, aflatoxins and trichothecenes (Figure 1). These mycotoxins are mainly produced by the mycotoxigenic fungi belonging to the genera of *Aspergillus, Alternaria, Fusarium,* and *Penicillium* (Anfossi *et al.,* 2016).

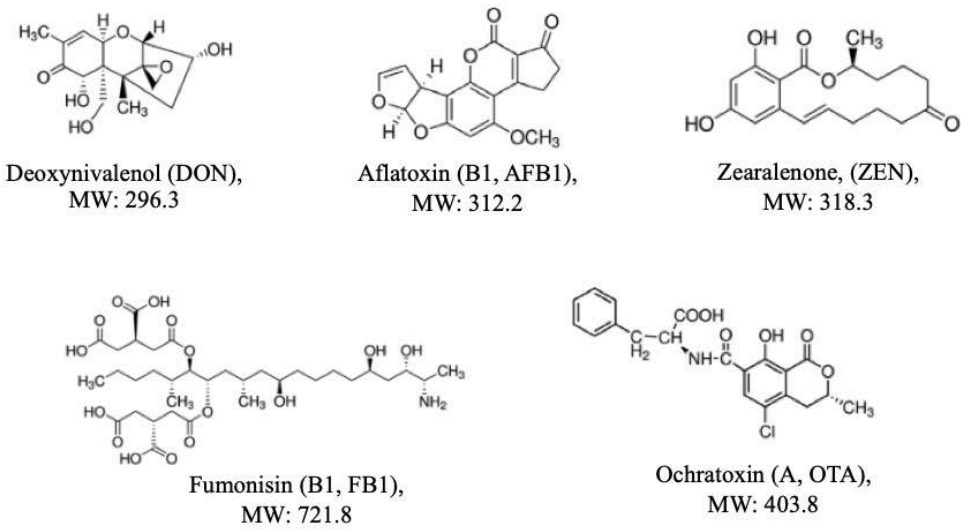


Figure 1: Chemical structure of mycotoxins contaminating food and feed products (Vila-Donat *et al.,* 2018).

One fungal species can produce more than one mycotoxin and a group of mycotoxigenic fungi might produce the same mycotoxin. Mycotoxins can be synthesized at any stage of the fungal growth; hence removal or inhibition of the fungal growth doesn't necessarily mean that the mycotoxin is not synthesized (Turner *et al.,*

2009; Yogendrarajah *et al.,* 2014).

The synthesis of the mycotoxins is affected by several environmental factors such as the nutrient availability, humidity, temperature, pH and pesticides application (Hameed *et al.,* 2013; Anfossi *et al.,* 2016). Therefore, mycotoxins contamination is more common in the subtropical and temperate countries compared to the tropical ones, where the climate doesn't support the synthesis of mycotoxins by the fungi (FAO, 2000). However, one factor of these environmental factors can affect the fungal growth and the synthesis of the mycotoxins differentially, hence, it is difficult to know the conditions that can foster the growth of the fungi and the production of the mycotoxins at the same pace (CAST, 2003; Garcia *et al.,* 2009; Freire & Sant'Ana, 2018). Mycotoxins can contaminate a variety of different food commodities, such as cereals, fruits and spices. There are other food commodities that have higher contamination chances with mycotoxins, such as chocolates, bread and beverages, that is due to the fact that they are made from raw material that might be originally contaminated with mycotoxins (Kabak, 2009; Turner *et al.,* 2009).

One of the most important factors affecting mycotoxins synthesis is the agricultural practices. Traditional manufacturing and agricultural technologies can lead to increased synthesis of the mycotoxins which can explain why the developing countries are subjected to more mycotoxin's exposure compared to the developed ones (Bhat *et al.,* 2010). The occurrence of the mycotoxins can cause several health issues upon the ingestion of the food that is contaminated with mycotoxins. Health implication such as carcinogenicity, immunosuppression, mutagenicity, teratogenicity and genotoxicity can be caused by the ingestion of food that is contaminated with mycotoxins (Zhu *et al.,* 2015). Table 1 represents the major health implications caused as a result to mycotoxins exposure. However, there are many factors that affect the body response to the mycotoxins' intake, such as the gender, body weight, intake dosage,

age, body weight and the type of the mycotoxin (Hussein & Brasel, 2001; Richard, 2007).

Table 1: Mycotoxins contaminating food and feed.

[Permissible concentrations of mycotoxins in food and feed and their dosage intake concentrations.]

Mycotoxin	**Health effect**	**Permissible Dosage intake**	**Fungal strains producing mycotoxins**	**Reference**
Aflatoxins	Group 1 carcinogenic, liver cancer	10 µg/kg	*A. flavus, A. parasiticus*	WHO, (1998), JECFA, (2007); Gnonlonfin *et al.,* (2013)
Fumonisin	Group 2b possibly carcinogenic, nephrotoxicity	2 µg/kg bw/day	*F. verticilloid, F. proliferatum*	IARC, (2002); WHO, (2001), Vila-Donat *et al.,* (2018)

Mycotoxin	Health effect	Permissible Dosage intake	Fungal strains producing mycotoxins	Reference
Ochratoxin A	Acute toxicity at lower levels, carcinogenicity at long term effect	100 ng/kg bw/week	*A. ochraceus, P. verrucous, P. viridicatum*	Vila-Donat *et al.,* (2018)
Deoxynivalenol	Group 3, not classifiable as to its carcinogenicity to humans	1 µg/kg bw/day	*F. graminearum*	IARC, (1993); WHO, (2001a), Zeidan *et al.,* (2018)
Zearalenone	Group 3, not classifiable as to its carcinogenicity to humans	0.5 µg/kg bw/day	*F. graminearum*	IARC, (1993); WHO, (2000), Vila-Donat *et al.,* (2018)

2.2 Control techniques of mycotoxins contamination:

There are several ways for the detoxification of mycotoxins. Physical, chemical, and biological techniques are all possible approaches for the detoxification of

mycotoxins.

2.2.1 Physical Methods:

The physical methods are considered as the traditional ones. Washing of the grains, irradiation and adsorption or filtration are all considered physical methods. Washing of the grains can reduce the concentration of the mycotoxins by making them float on the surface of the water due to their light density, and then the floating fraction on the top can be removed (Fandohan *et al*., 2005).

Irradiation is another technique to reduce the concentration of the mycotoxins. Gamma and UV irradiations are used to eliminate mycotoxins contamination of AFB1 and OTA, respectively (Ameer Sumbal *et al.,* 2016). Filtration by binders or sorbents has also been studied and used for mycotoxins removal. Acid treated bentonite is an example on sorbents that can be used for the detoxification of mycotoxins, where the toxins can get attached to the pores of the bentonite (Jin *et al.,* 2017). The clay is also considered one of the sorbents used to reduce the concentration of the mycotoxins in soil, especially OTA (Jouany, 2007).

2.2.2 Chemical methods:

Application of some chemicals can reduce the concentration of mycotoxins. Organic acids are known to have the ability to detoxify mycotoxins contaminated products. As example, the organic acids are used in the detoxification process is the egg albumin, which is known to reduce OTA contamination (Quintela *et al.,* 2012). Oxidizing agents such ozone are also used in the detoxification process of many mycotoxins such as patulin and aflatoxins (Agriopoulou *et al.,* 2016). However, using bases such as ammonia can reduce the concentrations of the mycotoxins as wells as reduction in the growth of the mycotoxigenic fungi. However, applying this approach in the detoxification of the fungal mycotoxins in the food is not permitted by the European Community (Peraica *et al.,* 2002).

2.2.3 Biological techniques:

In the recent years, more researches have been dedicated for the biocontrol of mycotoxigenic fungi by using microorganisms like yeast, bacteria and fungi (Recep *et al.,* 2009). Mycotoxins can be transformed enzymatically into non-toxic metabolites by the enzymes synthesized by microorganisms such as yeast, bacteria and fungi. Biotransformation of the mycotoxins can happen through acetylation, deep oxidation, oxygenation, glucosylation, cleavage of the ring or side chain or isomerization (Wielogórska *et al.,* 2016). The biotransformation process of the mycotoxins into other metabolites is not applied widely and is limited. The reason is that not much studies have been done on the transformed metabolites and the toxicity of the derived compounds and their effect if found in the feed (Wielogórska *et al.,* 2016).

2.2.3.1 Yeast as a biocontrol agent:

Yeast strains have the potential to reduce the mycotoxins by producing antibiotics, enzymes and peptides that are considered antifungal compounds (Mari *et al.,* 2012*;* Zhang *et al.,* 2012; Liu *et al.,* 2013). Many yeast genera are able to synthesize such metabolites and possess many antifungal activities. As examples, one can cite strains of *Candida, Cryptococcus, Hansenula, Hanseniaspora, Kluyvermyces, Meyerozyma, Pichia, Rhodotorula, Saccharomyces and Tilletiopsis* (Hershkovitz *et al.,* 2013; Urquhart & Punja, 2002; Calvente *et al*., 2001).

Yeasts can produce volatiles that have the ability to act on the mycotoxigenic fungi. Volatile organic compounds are very light diffusible compounds that have the ability to diffuse in the atmosphere and soil due to their low molecular weight. These VOCs are produced in the primary or secondary metabolism and many of them are known to have a distinctive odor. Carbon is the main component of the VOCs that inter the gas phase at 20ºC under 0.01kPa (Morath *et al.,* 2012).

The function of these VOCs varies considerably, some of them can act against

the fungi and thus are considered antifungal VOCs. VOCs are being employed in many of the biotechnological applications. Example on that is the use of these VOCs in the biological control of some of the mycotoxigenic fungi in the agricultural and food industry and medicine field. The VOCs are capable of reducing the fungal growth even without any direct contact with the strain producing the VOCs and the mycotoxigenic fungal strain.

One of the mechanisms of how the VOCs affects the production of the mycotoxins by the mycotoxigenic fungi is by altering its gene expression. When the fungus is exposed to those VOCs, many genes are being differentially regulated as a result to that, and the synthesis of the mycotoxins pathway is being affected and therefore the inhibition of their synthesis is accomplished by these VOCs (Farbo *et al.,* 2018). One of the important and major yeast VOC is the 2-Phenylethanol. The potential of this antifungal compound has been evaluated and it's proved to inhibit the growth and spores' germination of the antifungal compounds, in addition to the inhibition of the mycotoxins' synthesis. 2-Phenylethanol has the potential to affect the gene expression of the mycotoxigenic fungi where it has been proved to down regulate the expression of aflatoxin synthesis genes to more than 10,000 folds (Hua *et al.,* 2014). Gil-Serna *et al.,* (2011) who studied the potential of yeast to reduce the biosynthesis of OTA, confirmed that the yeast is able to repress the expression of genes responsible of OTA biosynthesis when the yeast was co-cultured with *A. westerdijkiae.*

In addition to the fact that these VOCs have an antifungal activity, they can also promote the growth of the plants. Some VOCs lead to the upregulation of the plants' genes leading to the induction of the plant defense response against fungal infection (Morath *et al.,* 2012). VOCs have been used in *in-vitro* researches to control the growth of mycotoxigenic fungi as well as the synthesis of the mycotoxins from these fungi that contaminate the food and feed (Zeidan *et al.,* 2018). Fiori *et al.,* (2014) have used this

approach to control the growth of *Aspergillus carbonarius* that infects the grape and grape juice, where they studied the effect of the VOCs produced by different yeast strains on the growth of *A. carbonarius* inoculated on grape berries *in-vitro*. They found that three out of the four tested strains managed to significantly reduce the growth of the fungus on the berries, in addition to the reduction of the OTA synthesized by the mycotoxigenic fungi.

2.2.3.2 Bacteria as a biocontrol agent:

Bacteria have the potential to reduce the mycelial growth of mycotoxigenic fungi such as *Pseudomonas* strains, they are known to inhibit the growth of *B. cinerea* and *A. alternate* (Luo *et al.,* 2019; Zeidan *et al*., 2019, unpublished).

In the environment, there are many microorganisms that can promote and enhance the growth of the plants whether directly or indirectly, and these microorganisms are called plant growth promoting rhizobacteria (PGPR). They have the ability to promote the growth directly by the solubilization of essential elements that are not readily available to the plants, and indirectly, by promoting the plants growth by the antagonism against plants pathogens such as pathogenic fungi. However, the bacteria belong to these PGPR group (Bhattacharyya & Jha, 2012). Many bacteria from different genera have been identified to have antifungal activities. Some strains of those bacteria belong to the genera of *Pseudomonas, Bacillus, Streptomyces* and *Serratia* (Schmidt *et al.,* 2009). *Pseudomonas* species are very known to have antifungal activities against many pathogens. The strain *Pseudomonas fluorescens* is an example on the bacteria that can produce antifungal compounds and is used in the biological control of pathogenic fungi such *B. cinerea* and other *Fusarium* species (Kilani-Feki *et al.,* 2010).

- ***Burkholderia cepacia complexes:***

Burkholderia cepacia is a Gram negative, aerobic, non-fluorescent bacterium.

It belongs to ß subgroup of the proteobacteria. It was described as *Pseudomonas cepacia* by Walter Burkholder in 1950, as it was noticed to cause sour skin in onion (Burkholder, 1950). In 1992, the bacterial genus name was changed and given a new one and it was started to be called *Burkholderia cepacia*.

Burkholderia has more than 50 described species which are very diverse in the environment and occupy different niches such as water, soil, pests or fungi. There are 17 phenotypically heterogeneous groups for *Burkholderia* which have been classified, and these groups contain the species *B. cepacia, B. multivorans, B. cenocepacia, B. stabilis, B. vietnamiensis, B. dolosa, B. ambifaria, B. anthina, B. pyrrocina, B. ubonesis, B. latens, B. diffusa, B. arboris, B. seminalis, B. metallica, B. lata, B. contaminas* (Schmidt *et al.,* 2009).

B. cepacia has an antagonistic activity against a wide range of the mycotoxigenic fungi that are pathogenic to some crops (Recep *et al.,* 2009). *B. cepacia* are considered among the bacteria that have the potential to enhance the plants' growth. It is capable of doing nitrogen fixation and is able to adapt to many environmental stresses (Compant *et al.,* 2008). Such bacteria are known to produce compounds with antagonistic activity against fungi infecting plants. *B. cepacia* produces iron chelating metabolites that are called siderophores, and these metabolites help the plants resists against pathogens (Pandey *et al*., 2005). Many studies regarding the antifungal antagonistic activity have been established using *B. cepacia.* Kilani-Feki *et al.,* (2011) studied the potential of *B. cepacia* culture extract to inhibit the growth of mycotoxigenic fungi such as *A. alternata, A. niger, F. culmorum, F. graminearum, F. oxysporum and R. solani*. According to their study, *B. cepacia* culture extract has an important antifungal activity. When *B. cepacia* extracts are applied *in-vitro* against many pathogenic fungi, they could inhibit the fungal mycelial growth, induce malformations in the mycelial shape as well as inhibition of spores' germination. However, *B. cepacia*

can promote the plant growth when it colonizes the plants roots, thus it protects the plants from the pathogenic fungi. The protection of the plants comes from a full colonization of *B. cepacia* to the plant's roots, where the bacteria either produces sufficient antifungal compounds to protect the plant, or it can induce the defense mechanism in the plant against pests (Compant *et al.,* 2005; Kilani-Feki *et al.*, 2010). The antifungal compounds produced by some strains of *B. cepacia* are characterized by being thermostable, that their antifungal activity against some mycotoxigenic fungi doesn't vanish even at very high temperatures. Table 2 shows some antifungal compounds produced by *B. cepacia.*

Table 2: Metabolites produced by *B. cepacia*.

Antagonistic molecules	**Reference**
Pyrrolnitrin	Vial *et al.,* (2007)
Phenzazines	Vial *et al.,* (2007)
2,4-diacethylephloroglucinol	Vial *et al.,* (2007)
Lipopeptides	Vial *et al.,* (2007)
Cepaciacheline	Compant *et al*., (2008)
Cepabactine	Compant *et al*., (2008)
1-amino- cyclopropane-1-carboxylate	Pandey *et al*., (2005)
Cepaciamide A	Vial *et al.,* (2007)
Didecyl-phthalate	Kilani-Feki *et al.,* (2011)
Alkyl-quinolone	Kilani-Feki *et al.,* (2011)
Chitosanase	Kilani-Feki *et al.,* (2012)
Phenazine	Ayyadurai *et al.,* (2006)
Pyoluteorin	Ayyadurai *et al.,* (2006)
HCN	Ayyadurai *et al.,* (2006)

3.1 Materials

3.1.1 Strains

Table 3: List of microorganisms used in this project

Reference Code	Strain
Bacterial strain	
QBC03	*Burkholderia cepacia*
Fungal strain	
AC82	*A. carbonarius*
CECT 2687	*A. flavus*
Af14	*A. fumigatus*
AN8	*A. niger*
CECT 2948	*A. ochraceus*
AF82	*A. parasiticus*
AW82	*A. westerdijkiae*
FAn01	*F. anthophilum*
FCh01	*F. chlamodosporum*
FCu11	*F. culmorum*
FGr14	*F. graminearum*
Fox9	*F. oxysporum*
FS05	*F. solani*
FSuF12	*F. subglutinus*
FP08	*F. prolifiratum*
FV04	*F. verticilliod*
PC44	*P. camemberti*

Reference Code	Strain
PE82	*P. expansum*
PD43	*P. digitatum*
PI48	*P. italicum*
TF11	*P. verrucosum*
Yeast strain	
751	*Lachancea thermotolerans*

3.1.2 Media preparations:

NBY broth (Nutrient Broth Yeast Extract):

3 g/L meat extract (Mikrobiologie, Darmstadt, Germany), 5 g/L peptone (Acumedia, Heywood, UK), 5 g/L yeast extract (HIMEDIA, Mumbai, India), 10 g/L mannitol (BDH, England), 1 mM/L $ZnSO_4.7H_2O$ (BDH, England), 1 mM/L $(NH_4)6Mo_7O_{24}.4H_2O$ (Analar, England).

Nutrient Agar:

1.3% nutrient broth (HIMEDIA, Mumbai, India) corresponds to peptic digest of animal tissue (5 g/L), Sodium chloride (5 g/L), beef extract (1.5 g/L) yeast extract (1.5 g/L), and 1.5% agar (HIMEDIA, Mumbai, India). All components were suspended 100 mL of sterile dH_2O.

PDA (Potato Dextrose Agar):

potato dextrose (FORMEDIUM, Hunstanton, England) which contains (200 g/L) potato infusion from dextrose (20 g/L). 41 g of potato dextrose agar were suspended in 1 L of dH_2O.

Soft PDA:

20.5 g of potato dextrose agar suspended in 1 L of dH_2O.

YPDA (Yeast Potato Dextrose Agar):

20 g/L D(+)Glucose (SCOTT SCIENCE UK, Headcorn, England), 20 g/L peptone (Acumedia, Heywood, UK), 10 g/L yeast extract (HIMEDIA, Mumbai, India), 15 g/L agar (HIMEDIA, Mumbai, India), all components were suspended in 1 L of dH_2O

All media were autoclaved at 121C, 15 psi for 15 minutes.

All media were poured in equal volumes in all the experiments:

50 mL volume of media were poured in 150 mm x 15 mm Petri dishes

20 mL volume of media were poured in 90 mm X 15 mm Petri dishes

12 mL volume of media were poured in 60 mm X 15 mm Petri dishes

3.1.3 Solutions and Buffers

Aflatoxins B1 extraction solvent:

1 mL of formic acid was dissolved in 99 mL of Methanol-dichloromethane-ethylacetate (1:2:3). Methanol (SIGMA-ALDRICH, UK), dichloromethane (SIGMA-ALDRICH), ethylacetate (Analar, England), ethylacetate (Analar, England).

Artificial mycotoxins:

5 µg/mL total aflatoxin B1, B2, G1, G2 (4:1:4:1) in 10 mL acetonitrile

100 µg/mL deoxynivalenol in 10 mL methanol

10 µg/mL ochratoxin A in 5 mL methanol

Buffer pH 5 (acetate):

13.608 g of sodium acetate 3-hydrate (M.wt= 136.08) were dissolved in 900 ml dH_2O and the pH was adjusted to 3 using acetic acid. The volume was then brought up to 1 L by adding dH_2O to make a final concentration of 0.1 mol/L

Buffer pH 7 (phosphate):

A 0.1 mol/L were prepared from sodium di-hydrogen phosphate 2-hydrate by dissolving 15.601 g of $NaH_2PO_4–2H_2O$ (M.wt= 156.01) in 1 L of dH_2O. A solution of di-sodium hydrogen phosphate 12-hydrate having 0.1 mol/L concentration was

prepared by dissolving Na_2HPO_4–$12H_2O$ (M.wt= 358.14) in 1 L of dH_2O. The pH of Na_2HPO_4 solution was adjusted to 7 by adding the solution of NaH_2PO_4 to it.

Chloramphenicol (100 mg/mL):

1 g of chloramphenicol powder (SIGMA-ALDRICH, UK) was suspended in 10 mL absolute ethanol. The solution was filtered using 0.2 µm syringe filter (Acrodisc, USA) and kept at -20ºC.

Normal saline:

0.89 of NaCl (BDH, England) were dissolved in 100 mL sterile dH_2O

3.1.4 Kits:

ELISA Kit of Aflatoxin (B1), DON and OTA (RIDASCREEN, Darmstadt, Germany): components are 96 microtiter plate, standards (1.3 mL), wash buffer salt tween, conjugate (6 mL), antibody (6 mL), substrate/chromogen (10 mL), stop solution (14 mL), washing buffer pH 7.4 (10 mM phosphate buffer in 0.025% Tween 20). The concentration of all standards of mycotoxins are listed in table 4.

Table 4: Mycotoxins concentrations in ELISA kit's standards

Standard no.	**AFB_1 (µg/L)**	**DON (µg/L)**	**OTA (ng/L)**
1	0	0	0
2	1	3.7	50
3	5	11.1	100
4	10	33.3	300
5	20	100	900
6	50	-	1800

3.1.5 Equipment and machines

- Autoclave (P SELECTA, Barcelona, Spain)
- Centrifuge: Thermo SCIENTIFIC (Germany)
- ELISA plate reader: (Multiskan FC, Thermo Scientific, Waltham, MA, USA)
- Fridge: SANYO (Japan)
- Incubator: BINDER (Germany)
- Laminar: LABCONCO (USA)
- Microscopes: Light Compound microscope (Leica, China) and (Zeiss, Germany), Inverted Light microscope (OPTIKA, Italy)
- Oven: (Friedberg, Germany)
- Shaking incubator: (SHEL LAB, USA)
- Sonication machine: (BANDELIN SONOREX, W. Germany)

3.2 Methods

3.2.1 Investigation of the effect of yeast cells' VOCs on the inhibition of mycotoxigenic fungal growth

3.2.1.1 The exploration of the effect of yeast's VOCs on the growth of point inoculated fungi on PDA

A preserved yeast strain was taken out from -80ºC and was streaked on YPDA plate. A yeast preculture was prepared by transferring a single colony to 10 mL YPDB in a 50 mL conical tube using an inoculating loop, and the tube was incubated at 26ºC/140 rpm for 8 hours. A 100 µL of the preculture was transferred to 10 mL YPDB tube which was incubated at 26ºC/140 rpm for 24 hours. A suspension of yeast cells having a concentration of 10^7 cells/mL was prepared from the 24 h yeast broth culture by transferring 10 µL from the yeast culture to 990 µL of 0.9% saline. Yeast cells (10^7/mL) were counted using a hemocytometer chamber under a light microscope at 40X lens. From that yeast suspension, 100 µL of the yeast cells suspension were

transferred to the YPDA plates and were spread on the media using sterile disposable plastic spreaders, and the plates were incubated at 26ºC for 48 h. Fungus spores' suspensions of *A. parasiticus, P. verrucosum* and *F. graminearum* were prepared from 7 days old fungi PDA plates by scratching the surface of the plate using an inoculating needle to transfer the inoculum to Eppendorf tubes each containing 1 mL 0.9% saline. The fungal spores' suspensions for the three fungi were counted in the same way as the yeast's and the concentration of spores was adjusted to be 10^6 spore/mL. From the spore's suspension; an inoculum of 10 µL was transferred to the center of the PDA plates and those plates were sealed to the 48 h yeast YPDA plates using two layers of parafilm and transparent tape (Fiori *et al.*, 2014). Figure 2 illustrates how the sealing process was done. The sealed plates where incubated at 26ºC for 10 days, and the diameters of the fungal colonies sealed to the yeast plates were measured on the 3rd, 5th and 7th day of incubation. The diameters of the fungal colonies exposed to yeast's VOCs were compared to those in the control plates which were sealed to plates of YPDA media only.

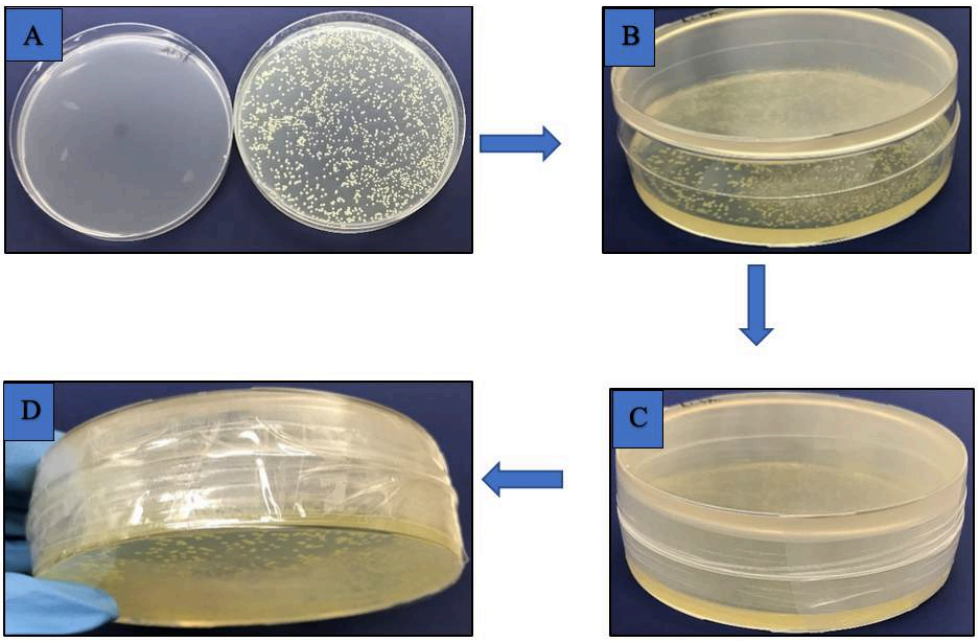


Figure 2: Steps of the sealing process for the exploration of the yeast's VOCs effect on mycotoxigenic fungi.

[A: fungal plate and yeast prior to sealing; B: plates were placed top-to-top; C: plates were sealed with two layers of parafilm; D: plates sealed with transparent tape.]

3.2.1.2 Effect of yeast's VOCs on individual fungal growth on PDA

Yeast's cells suspension was prepared (10^7 cells/mL) and 100 µL of that suspension was spread on the YPDA plates, and they were later incubated for 48 h to be sealed with the PDA fungus plates. Fungal spores' suspensions for *A. parasiticus, P. verrucosum* and *F. graminearum* were prepared to be 10^6 spores/mL and 1 µL of sterile Tween80 was added to the spores' suspension to prevent spores' coagulation (Cole, 1994). Spores suspension was serially diluted to 10^{-3} spores/mL and 100 µL of the spores' suspension was spread on the PDA plates using sterile plastic spreaders. Both fungus and yeast plates were sealed together and incubated at 26ºC for 7 days. The diameters of single fungal colonies exposed to yeast VOCs were measured and compared to the ones in the control plates that were not exposed to the VOCs.

3.2.1.3 Investigation of the effect of yeast's VOCs in different yeast's concentrations on the growth of F. graminearum

Two concentrations of the yeast cells were prepared to investigate the effect of yeast's cells concentration on the growth of *F. graminearum*. From a 24 h yeast culture, a yeast cells stock solution was prepared in a concentration of 10^7 cell/mL. A 10 folds serial dilution was conducted from the stock solution and the concentration of 10^{-1} and 10^{-3} cell/mL were selected to correspond to higher and lower yeast cells concentration, respectively. From each of the two concentration, 100 µL was transferred and spread on YPDA plates which were incubated at 26ºC for 48 hours. From a spore stock solution of *F. graminearum* having a concentration of 10^6 spore/mL, 10 folds dilution were made, and 10^{-4} spore/mL was chosen to work with. 1µL/mL of tween 80 was added to the 10^{-4} spore/mL suspension to prevent spores' aggregation and 100 µL from that concentration were spread on PDA plates. Afterward, Those PDA plates having the fungal spores spread on them were sealed to the YPDA plates of high and low yeast concentrations. The sealed plates were then incubated at 26ºC and the radial diameters of the fungal colonies were measured.

3.2.1.4 Investigation of the effect of the nutrients' availability on the yeast's VOCs production against F. graminearum

The exploration of the effect of the nutrients' concentration on the yeast potential to synthesize VOCs was examined through diluting the components of the YPDA media into two concentrations which were ½ and $^1/_{10}$ the original concentrations of the YPDA components, keeping the agar concentrations the same as the original (1.5%). Stock yeast cells solution of 10^8 cells/mL was 10 folds diluted to 10^{-6} cell/mL and dilutions from 10^{-1} to 10^{-5} cell/mL were chosen to be spread on the diluted YPDB media. 100 µL from these dilutions were transferred and spread on the ½ and $^1/_{10}$ diluted YPDB media and on normal undiluted YPDA as well. The plates were incubated for

48 hours at 26ºC and yeast CFUs were counted on the three concentrations of YPDA media. The plates were sealed to PDA plates after which they were point inoculated in the center with 10 µL of *F. graminearum* spores' suspension having a concentration of 10^6 spore/mL. Sealed plates were incubated at 26ºC for 7 days and the diameters of the fungal colonies were measured and recorded alternatively for 3 days.

3.2.1.5 Exploration of the effect of increasing yeast's CFUs on the growth kinetics of F. graminearum

To explore the kinetics of *F. graminearum* growth in response to VOCs exposure from increasing yeast cells numbers; a stock yeast solution of 10^8 cell/mL was prepared and was 10 folds diluted to 10^{-1} in 1 mL Eppendorf tubes. Those tubes of dilutions were also 10 times serially diluted and 100 µL from all dilutions were transferred and spread on ½ YPDA plates. The plates were incubated at 26 ºC for 48 h. Fungal spores of *F. graminearum* were prepared in a concentration of 10^6/ml and a point inoculum of 10 µL was transferred to PDA plates. Fungal PDA plates were sealed to the yeast plates and were incubated at 26 ºC for 5 days. Radial diameters of the fungal colonies treated with yeast's VOCs were measured on daily bases for 5 days and were compared to colonies which were not exposed to the yeast's VOCs (Zeidan *et al.*, 2018).

3.2.2 Exploration of the yeast's VOCs effect on the synthesis of mycotoxins

3.2.2.1 Detection of Aflatoxin concentrations in A. parasiticus

Yeast's VOCs effect on the synthesis of aflatoxin B_1 was tested from the point inoculated and individual colonies. Using a cork borer of 7 mm diameter, three plugs were cut from the treated and control point inoculated colonies of *A. parasiticus.* The plugs were taken from three consecutive spots; one from the center of the colony, the second was further away from the center and the third was closer to the margin of the colony. For the individual fungal colonies; plugs were cut using a sterile scalpel, and all plugs were weighed and transferred to 2 mL amber glass tubes. Figure 3 demonstrate

how the colonies were cut from point inoculated and individual fungus colony. Aflatoxin B_1 extraction was conducted according to what was done by Smedsgaard., (1997) with a slight modification. 1 mL of the aflatoxin extraction solvent; methanol-dichloromethane-ethylacetate (1:2:3) having 1% formic acid, was poured on the weighed cut colonies and the tubes were sonicated after that for 60 minutes. After that, 500 µL of the extract were transferred to clean Eppendorf tubes and the extract was dried using liquid nitrogen. When the tubes were completely dried out, the residues were resuspended with 500 µL of methanol: water (35:65) and the tubes were vortexed vigorously. The concentration of aflatoxin B_1 in the colonies was determined using ELISA kit. In a 96 microtiter plates that is coated with antibodies specific to bind to anti-aflatoxin antibodies; 50 µL of the standards and the samples were loaded into the wells. 50 µL of the enzyme conjugate were loaded on the same wells and were followed by other 50 µL of the anti-aflatoxin antibody solution. The plate was gently shaken and incubated at room temperature for 20 minutes. Thereafter, the wells were emptied by pouring out the liquid to the sink and they were filled again with 250 µL of the washing buffer (PBS-Tween buffer) solution which was directly removed by poured out the liquid to the sink. The washing step was done twice, and 100 µL of the substrate/chromogen solution were added to the wells. The plate was gently shaken and incubated in the dark for 25 min. The reaction was stopped by adding 100 µL of the stop solution (1 N sulfuric acid) to all wells. The absorbance was measured directly at 450 nm using an ELISA plate reader that was installed with Skanlt software. The concentrations of AFB_1 was determined through comparing the absorbance values in the sample with a standard curve which was generated from the 5 standards absorption values. The absorbance values are inversely proportional to AFB_1 concentrations in the samples.

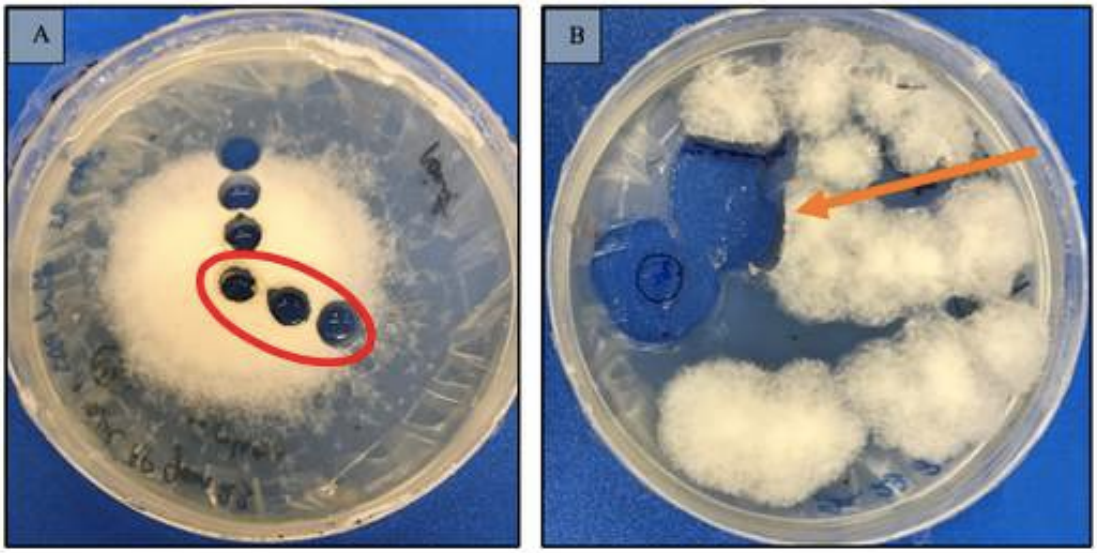


Figure 3: Plugs cut from *A. parasiticus* colonies for the estimation of mycotoxins concentration.

[A: three plugs removed by a cork borer from a point inoculated colony as the eleptic circle shows; B: individual fungal colony cut by a scalpel as the arrow points]

3.2.2.2 Determination of Deoxynivalenol concentration by F. graminearum as an effect of increasing yeast's CFUs

DON concentrations were measured in *F. graminearum* colonies which were sealed to different yeast cells numbers on ½ YPDA media. Samples were cut from *F. graminearum* using a sterile scalpel as shown in figure 4. Three cuts were taken out from each of *F. graminearum* colonies which were sealed to yeast cells from different CFUs. The weight of the plugs cut sample was measured and they were transferred to brown glass vials. The cuts were soaked with 1 mL methanol (70%) for DON's extraction and the tubes were sonicated for 60 min. 500 µL of the sonicated extract were transferred to clean Eppendorf tubes and were dried with liquid nitrogen. The residues were resuspended with 1 mL deionized H_2O and the tubes were vortexed well.

DON's concentration was determined in the extract using ELISA Kit. Concentration of DON in the extract of the treated colonies was referred to those of the control which were not sealed to yeast cells. Concentrations of DON in the fungal colonies sealed to yeast cells were compared to those which were not.

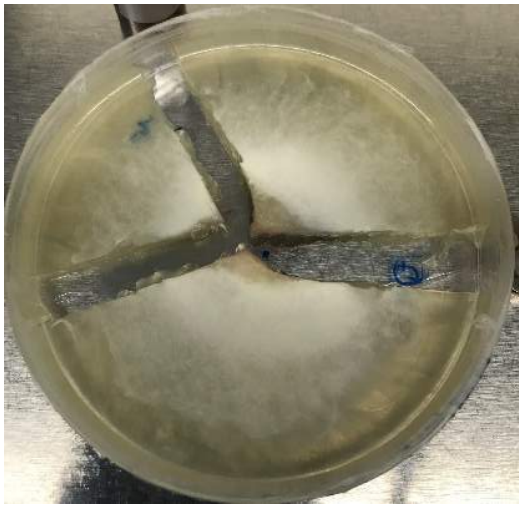


Figure 4: A demonstration of how samples were cut from *F. graminearum* colonies

3.2.3 Exploration of the yeast cells adsorption properties to mycotoxins

3.2.3.1 Preparation of living yeast cells

Yeast culture was prepared from a 24 h yeast culture streaked YPDA by transferring one colony to 100 mL Erlenmeyer flask containing 20 mL YPDB. The flask was incubated at 26ºC/140 rpm for 16 h and from that preculture; 100 µL was used to seed 20 mL of YPDB in a 100 mL flask. The culture was incubated at 26ºC/140 rpm for 24 h. From this flask, a stock yeast cells suspension having a concentration of 10^8 cell/mL was used for the adsorption assay experiment.

3.2.3.2 Preparation of inactive yeast cells

Inactive yeast cells were prepared from a living yeast cells culture. The live yeast culture was prepared in 50 mL conical. After the incubation period; the tube was centrifuged at 5000g/4ºC/5 min and the pellet was obtained and washed twice with 10 mL of 0.9% saline. The tube was centrifuged, and the pellet was obtained again. The tube was transferred to the oven and was incubated at 80ºC overnight. The crusty dry yeast pellet was ground using the mortar and pestle, and the fine yeast powder was collected in an Eppendorf tube (Bzducha-Wróbel *et al*., 2014).

3.2.3.3 Preparation of mycotoxins doses

To test the adsorption of AFs, DON and OTA to the yeast's cell wall; two doses for each of the three mycotoxins were prepared to be in the detectable limit of the ELISA kit. Therefore, a higher and lower concentration of each of the mycotoxins were prepared by diluting mycotoxins in dH_2O to obtain the concentration shown in table 5.

Table 5: Mycotoxins concentrations prepared for the adsorption assay

Mycotoxin	**High dose (µg/L)**	**Low dose (µg/L)**
AFs	0.2	0.4
DON	40	80
OTA	0.9	1.8

3.2.3.4 Adsorption to viable yeast' cell wall

The live yeast cells stock which was prepared in section 3.2.3.1 was used to test the potential of AFs, DON and OTA to adsorb to the yeast's cell wall. The adsorption was conducted in two buffer solutions having pH 5 (acetate) and 7 (phosphate). From the buffer solutions, 970 µL were transferred to Eppendorf tubes which were seeded with 20 µL of the yeast cells stock solution. The tubes were vortexed and were divided into two sets; where the first was contaminated with 10 µL of the higher mycotoxin dose, and the second was contaminated with 10 µL of the lower mycotoxin dose. The tubes were shake incubated end-to-end at 37ºC/140 rpm for 30 min and were directly centrifuged after that at 9200g/4ºC for 10 min. The supernatant was separated from the pellet and was transferred to Eppendorf tubes. The pellet was resuspended in 10% methanol, dH_2O or 0.13 M sodium hydrogen carbonate solution for the detection of AFs, DON and OTA.

3.2.3.5 Adsorption to inactive yeast cell wall.

The adsorption procedure of the inactive yeast cells to mycotoxin was done similarly to that of living yeast cells, with the change that the living yeast cells were exchanged with 5 mg of the ground yeast cells powder and were added to 990 µL of the buffer solutions. The tubes were contaminated with 10 µL of mycotoxins and the rest of the adsorption procedure was completed as done in 3.2.3.4. section for living yeast cells

3.2.3.6 Determination of yeast's adsorption potential using ELISA kits

ELISA Kit was used to determine the adsorption potential of the mycotoxins to cells in the pellet of the viable and non-viable yeast incubated in buffer 5 pH and 7 pH. 50 µL were transferred to the wells of ELISA from the tubes of the pellet and supernatant of all three mycotoxins and the percentage of the removal of the mycotoxin from the buffers were determined for the three mycotoxins in both buffer of pH 5 and 7.

3.2.4 Exploration of the *In-Vitro* effect of yeast's VOCs on *F. oxysporum* contaminating tomato fruit

Yeast's VOCs effect on *F. oxysporum* growth was tested in an *in-vitro* experiment on the tomato fruits surface. The tomato washed with dH_2O was contaminated on the surface with 5 µL of spores' suspension having a concentration of 10^4 spore/mL. Ten tomato fruits having almost the same size (~0.8 g) were contaminated with *F. oxysporum* spores where five of them were added to an autoclaved glass box (Tupperware) having a 48 h streaked yeast plate, and the other five contaminated tomato were added to another glass box having a plate of YPDA only. The Petri dish's size was small (60 mm X 15 mm) in order to fit inside the glass boxes. The Petri dishes were covered with a sterile lid of a bigger Petri dish to serve as the base of which the tomato will be placed on. Small pieces of polystyrene were used to partially elevate the lid from the Petri dish. Small desiccator bags were placed in each box and the boxes were then covered with their tight lid (Figure 5). The experiment was performed in aseptic conditions and the boxes were then incubated at 26ºC for 33 days.

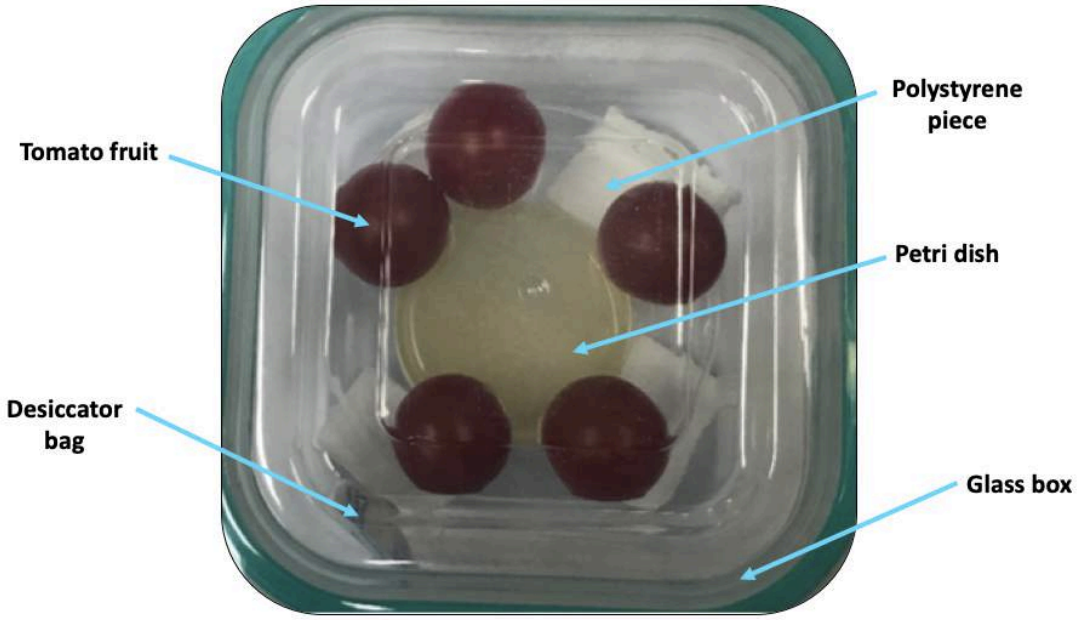


Figure 5: Tomato fruits and yeast sorted in the glass sealed box

3.2.5 Investigation of the antifungal activities of a local isolate *QBC03* against mycotoxigenic fungi contaminating food

3.2.6 *QBC03's* growth conditions and extract sterilization

Preserved *QBC03* was taken out from -80ºC and streaked on a fresh plate of nutrient agar for 24 h at 30ºC. Thereafter, one colony was transferred to 50 mL conical tubes having 10 mL nutrient broth and 10 mL NBY broth (5) which was prepared according to Kilani-Feki & Jaoua., (2011). From these pre-cultures, 100 µL were transferred to 10 mL nutrient broth and NBY, and the tubes were incubated at 30ºC for 48 h. The tubes were centrifuged at 5500g for 20 minutes and the supernatant was transferred to other tubes. To obtain a sterile *QBC03*'s supernatant; two ways of sterilization were used which were: using syringe filters of 0.2 µm and 0.45 µm for extract filtration, and sterilization by UV for 20 minutes. 100 µL from *QBC03*'s sterilized extract were added to wells drilled in PDA plates having 100 µL *P.*

verrucosum spores inoculum spread on them from a stock suspension of 10^6 spores/mL. The sterilized extract was also added to wells in PDA media having 100 µg/L chloramphenicol and all plates were incubated at 26ºC for three days.

3.2.7 Estimation of the antifungal activity of *QBC03* strain by overlaying assay

QBC03's antifungal spectrum was tested against 20 fungal species belonging to the genera of *Aspergillus, Fusarium and Penicillium* (Table 1). The dual-culture overlaying assay method was used to determine the inhibition zone of *QBC03* against the fungal strains (Santos *et al*., 2004). *QBC03* was streaked on a fresh plate of nutrient agar and was incubated at 30ºC for 24 h. The streaked plate was used to transfer *QBC03* to the center of NA plates using a sterile toothpick and the plates were kept at 30ºC for 48 h. Spores' suspension was prepared from 7 days old fungal colonies by transferring a scratch from those plates with an inoculating needle to 1 mL 0.9% saline in Eppendorf tubes. Spores suspension prepared for the 20 species were counted using a hemocytometer and their concentration was adjusted to 10^6/mL. Each of the spores' suspension was resuspended in 10 mL soft PDA in 50 mL conical tubes. 2 mL from those tubes were assayed very closely around the 48 h *QBC03* colonies and the tubes were vortexed vigorously each time the spores were assayed. The plates were incubated at 26ºC and diameters of zones around *QBC03* were measured after 3 days of incubation.

3.2.8 Evaluation of *QBC03's* antifungal compounds by well-diffusion method

The activity of the antifungal compounds of *QBC03* supernatant was evaluated on PDA using the wells-diffusion method that is modified from Eloff (1998). *QBC03* was cultured in 10 mL NBY (5) in 50 mL conical tubes and was incubated in the shaker at 30ºC/140 rpm for 24, 48 and 72 h. After that, the extract was collected from those

cultures by centrifuging the tubes at 5500g for 20 min and transferring the supernatant to other tubes. The supernatant of the 24, 48 and 72 h culture was obtained and diluted in saline to 1%, 20%, 60% and 80%. PDA media having 100 µg/L chloramphenicol was prepared and poured in 150 X 15 mm Petri dishes. Spores suspension for *A. carbonarius, F. culmorum* and *P. verrucosum* were prepared (10^6/mL) and 200 µL were transferred and spread on the PDA plates. A cork borer (7mm) was used to drill wells on the PDA media, and 100 µL of 1%, 20%, 60%, 80% and 100% extract were loaded to the wells. The plates were incubated at 26ºC for 3 days and diameters for zones around the wells were recorded.

3.2.9 Evaluation of *QBC03's* antifungal compounds by incorporation of the supernatant with PDA

A preculture of *QBC03* was prepared and 100 µL were transferred to 10 mL NBY (5) broth which was shake incubated at 30ºC/180 rpm for 48 h. The extract was obtained by centrifuging the tubes at 5500g for 20 min. PDA media with 100 µg/L chloramphenicol was prepared in 500 ml flasks and different volumes of the bacterial extract were incorporated to correspond to the percentages of 2.5%, 3.5%, 4.5%, 5.5%, 6.5%, 7.5%, 8.5%, 9.5%, 10.5%, 11.5% & 12.5%, 13%, 14% and 15%. The control plates were prepared in the same way, but the bacterial extract was exchanged with NBY (5) broth instead. Fungal spores' suspensions for *A. carbonarius, F. culmorum* and *P. verrucosum* were prepared (10^6 spore/mL), and 3 µL were loaded to the center of the PDA plates of the control and the treatment. The plates were incubated at 26ºC for 7 days and the diameters of the fungal colonies were measured and referred to the control and the inhibition ratio was calculated using the below formula (Kilani-Feki *et al*., 2011).

$$Inhibition\ ratio = \frac{C-T}{C} * 100$$

C: diameter of colony in the control

T: diameter of colony in the treatment

3.2.10 Evaluation of *QBC03's* antifungal compounds effect on fungal mycelial biomass

To explore the effect of *QBC03's* antifungal compounds on the fungal mycelium biomass; a 6 h preculture of *QBC03* was prepared in 10 mL NBY (5) in 50 mL conical tube out of which 100 µL were transferred to other 10 mL NBY (5). The culture was shake-incubated at 30ºC/180 rpm for 48 h. The bacterial extract was obtained through centrifugation at 5500g for 20 min and was shifted to other tubes. The extract was serially diluted (1% to 6%) in 100 mL flasks having 20 mL PDB and 500 µg/L chloramphenicol. The control contained only PDB+500 µg/l chloramphenicol. 10 µL inoculum of fungal spores' suspension (*A. carbonarius* and *P. verrucosum*) having a concentration of 10^6 spores/ml was transferred to all flasks which were shake incubated at 26ºC/140 rpm for 72 h. After the incubation period, the fungal growth was estimated by measuring the fungal dry mass through filtration. Nitrocellulose filter papers were kept in the oven at 60ºC for 24 h prior to the filtration and their initial weight was measured using an analytical balance. The mixture of fungi and PDB was filtered through nitrocellulose filter paper in Buchner funnel which was connected to a vacuum pump (Figure 6). The dry biomass was calculated in the treatment and the control as described in the formula below, and the filtrate of each treatment was collected separately in Eppendorf tubes for the analysis of mycotoxin concentration. Microscopic morphological alterations of the mycelia were examined under the microscope.

$$Dry\ biomass = weight\ of\ empty\ filter\ paper - weight\ of\ filter\ paper\ after\ filtrtion$$

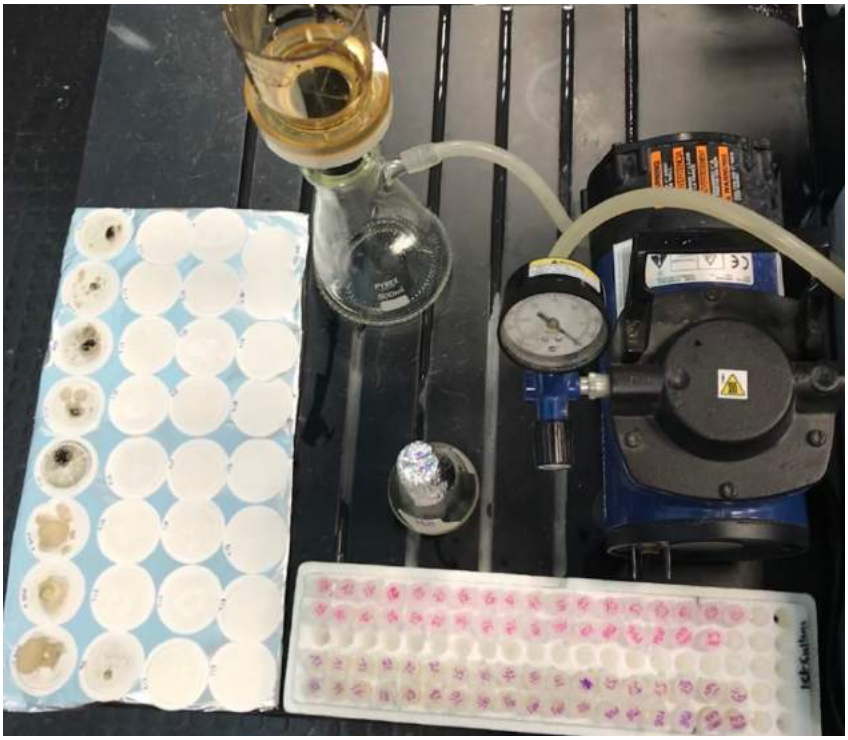


Figure 6: Fungal biomass filtration system

3.2.11 Estimation of the effect of temperature on the stability of *QBC03*'s antifungal compounds

QBC03's supernatant treated with different temperatures was tested against *A. carbonarious, F. culmorum and P. verricusom* in well-diffusion method. From a 7 hr preculture of *QBC03,* 100 µL were transferred to 10 ml NBY broth which was incubated at 30ºC/180 rpm for 48 h. The extract was obtained by centrifuging the tube at 5500g/20 min. 1 mL of the supernatant was transferred to Eppendorf tubes where each was incubated at different temperatures (-80º, -20º, 4º, 26º, 30º, 40º, 60º, 80º & 100ºC) for 30 mins. PDA having 100 µg/mL chloramphenicol was poured in plates (150 mm X 15 mm) and 200 µL from fungal spores' suspension (10^6/mL) were spread on the media surface. Using a cork borer (7 mm), wells were drilled in the PDA plates and a 100 µL of the treated extract

were loaded each in each well. The plates were incubated at 26ºC for 72 h and zones around the wells were measured.

3.2.12 Evaluation of the effect of *QBC03's* antifungal compounds on the germination of fungal spores.

The influence of *QBC03*'s antifungal compounds on the germination of fungal spores was explored by culturing the spores with the extract in a 96-microtiter tray as done by Joo *et al*., (2015). The fungal spores were transferred from 7 days old colonies on PDA to 1 mL dH_2O Eppendorf tubes using a sterile needle. The tubes were centrifuged at 5000 rpm for 5 min and the pellet was washed with 1 mL dH_2O, and the process was repeated twice. The fungal spores (*A. carbonarius, A. westerdijkiae, F. oxysporum, F. culmorum* and *P. verrucosum*) were counted and adjusted to 10^6 spore/mL. The bacterial supernatant of *QBC03* was obtained by centrifugation at 5500g for 20 min and 100 µL of the extract were added to wells in 96 microtiter plate containing 900 µL PDB and 500 µg/L of chloramphenicol. In the negative control wells; the supernatant was replaced with 100 µL of NBY broth and 2 µL of spores were inoculated in all wells. The effect of the antifungal compounds from the supernatant on the spores' germination was detected using an inverted light microscope after 24 h of incubation.

CHAPTER 4: THE INHIBITION OF MYCOTOXIGENIC FUNGAL GROWTH AND SYNTHESIS OF THEIR MYCOTOXINS BY YEAST'S VOCS

Introduction

Mycotoxins are secondary metabolites produced by toxigenic fungal species and are produced by three main genera that are *Aspergillus, Fusarium* and *Penicillium*. Such mycotoxigenic fungi can contaminate different crops and cereal either pre or post the harvesting (Milani, 2013). Mycotoxins are very stable compounds and they can't be degraded in routine cooking processes, which makes them problematic due to the health issues they can cause (Abbas *et al.*, 2009). Due to their toxigenic profile; AFA B_1, DON, OTA, fumonisins and zearalenone are considered on the top of the list of more than 400 identified mycotoxins (Bhat *et al*., 2010). Biocontrol agents like yeast are used to control the mycotoxigenic fungal growth as well as their mycotoxins. The biocontrol approach is considered a promising strategy due to the potential it has in decreasing economical losses caused by the contamination of the mycotoxigenic fungi. Yeast can antagonize the mycotoxigenic fungi by several mechanisms; like the production of antifungal compounds, formation of a biofilm or competition on the nutrients and space, and many other mechanisms (Pfliegler *et al.*, 2015). Yeast produce volatiles that can inhibit the fungal growth of mycotoxigenic fungi (Farbo *et al*., 2018). There are more than 30 yeast strain that have been proved to have antifungal activities against mycotoxigenic fungi. However, the use of yeast can be problematic due to the production of alcoholic compounds by the yeast in the fermentation process (Liu *et al*., 2014; Fiori *et al*., 2014). To meet with the Islamic standards which prevent the presence of alcoholic substance in food and beverages; the use of a low- or non-fermenting yeast can resolve the problem (Fiori *et al*., 2014).

In this work, *L. thermotolerans 751* strain has been used as a biocontrol agent against the mycotoxigenic fungal strains *A. parasiticus AF82, F. graminearum FGr14*

and *P. verrucosum TF11*. Besides the VOCs inhibition of the mycotoxigenic fungal growth; this work has also focused on the reduction of the associated mycotoxins synthesized by fungal species. A study of the effect of *L. thermotolerans 751* VOCs on the inhibition of point inoculated and individual fungal colonies was conducted. The synthesis of AFB_1, DON and OTA were measured from the fungal colonies exposed to the yeast's VOCs was done in this project. In addition, we study, the effect of nutrients availability to the yeast cells on their biocontrol potential against *F. graminearum FGr14*.

4.1 Investigation of the effect of yeast's VOCs on the growth of point inoculated fungi

4.1.1 Growth inhibitory effect of yeast's VOCs on *A. parasiticus AF82*

The diameters of an inoculum of *A. parasiticus AF82* that was transferred to PDA and sealed to yeast cells were measured and compared to those which were sealed to empty YPDA plate. It was noticeable that the yeast's VOCs were able to reduce the growth of the *A. parasiticus AF82* compared to the control colonies in the 3rd, 5th and 7th day, where the inhibition percentage reached 25%,15% and 6%, respectively (Figure 7). However, the decrement in the diameter on the 7th day was not much different than colony diameter in the control (control: 45 mm, treated: 48 mm). At the 7th day and after, the colony size of the treated *A. parasiticus AF82* started increasing reaching to that of the control. The radial growth of the treated fungal colonies wasn't the only thing affected by the yeast's VOCs where the pigmentation of the fungus was also affected too (Figure 8). The coloration of the fungal colonies which were treated was completely different than the control which was not exposed to the VOCs. The treated colonies had a white cottony shape, unlike the colonies in the control plates which retained the yellow brown pigmentation in the center of their colony. Figure 8 shows how exposure to yeast VOCs has resulted in undefined margins in the fungal colony.

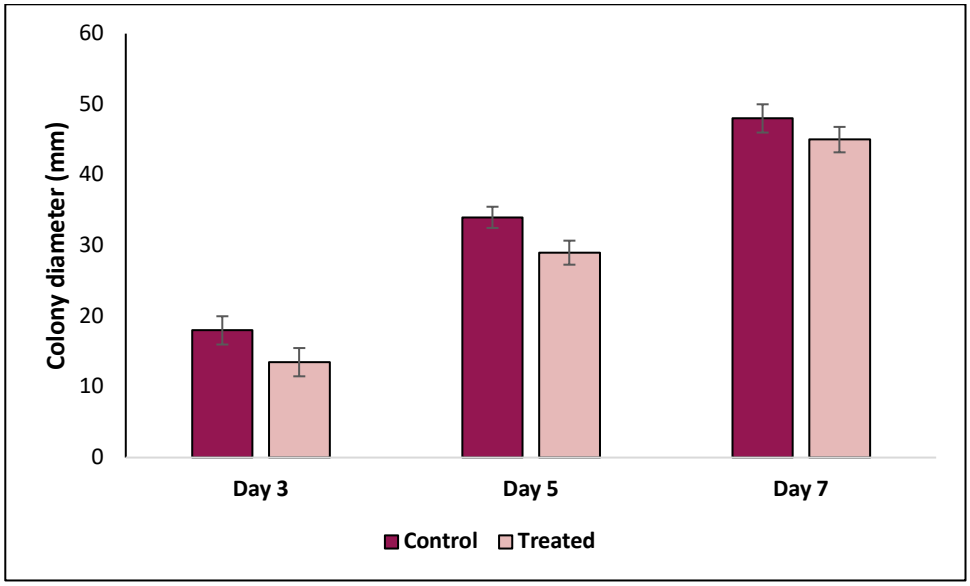


Figure 7: Effect of VOCs on the size of colonies of point inoculated *A. parasiticus AF82.*

[The sizes of the *A. parasiticus AF82* colonies were measured after 3, 5 and 7 days of sealing]

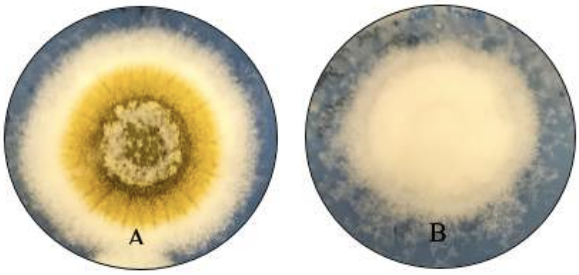


Figure 8: Effect of yeast VOCs on the characteristics of *A. parasiticus AF82.*

[A: control fungal colony without VOCs; B: fungal colony treated with VOCs]

4.1.2 Effect of yeast's VOCs on *F. graminearum FGr14* growth

From the diameters measured for the treated and untreated colonies of *F. graminearum FGr14* with VOCs, there was a clear decrease in the radial dimeter and

growth of the colonies (16 mm, 34 mm, 50 mm) on the 3rd, 5th and 7th day, respectively (Figure 9). Yeast VOCs were able to inhibit the growth of the point inoculated *F. graminearum FGr14* colonies when sealed to yeast cells, compared to the control. More inhibition has happened to the fungal growth as per to the measured diameters of the colonies on the 3rd day, where after that, the fungus was growing gradually and inhibition zones were recorded as 46%, 31% and 23% for the three days, respectively. A loss in the pigmentation was also observed for the treated colonies which had completely lost the pink color in the center compared to the control colonies that retained it. Undefined margins were seen in colonies exposed to VOCs as shown in figure 10.

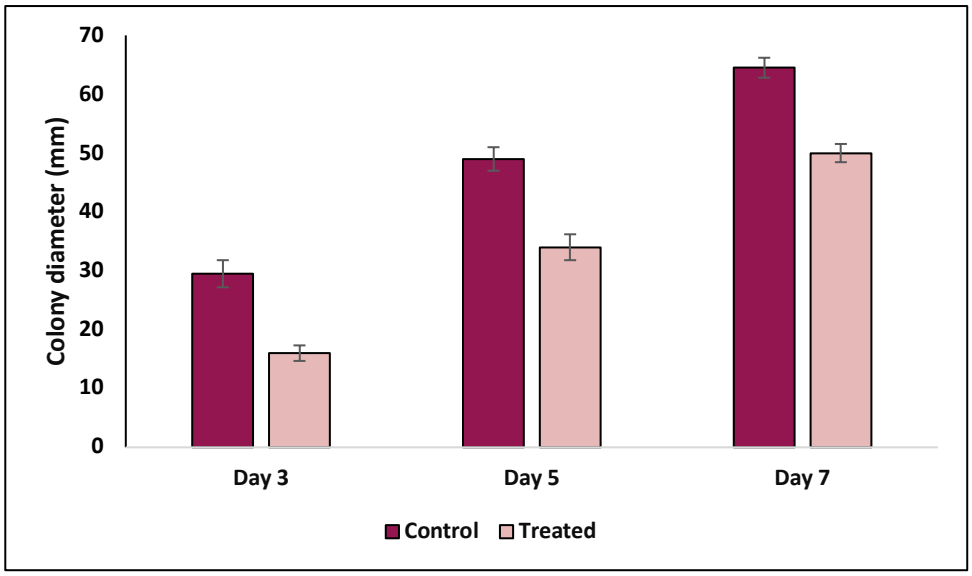


Figure 9: Effect of VOCs on the colonies sizes of point inoculated *F. graminearum FGr14.*

[The sizes of the *F. graminearum FGr14* colonies were measured after 3, 5 and 7 days of sealing]

Figure 10: Effect of yeast VOCs on the characteristics of *F. graminearum FGr14.*

[A: control fungal colony without VOCs; B: fungal colony with VOCs]

4.1.3 Effect of yeast's VOCs on *P. verrucosum TF11* growth

P. verrucosum TF11 point inoculated colonies sealed to yeast showed a great inhibition in their radial diameter making *P. verrucosum TF11* the most sensitive towards exposure to yeast's VOCs compared to *A. parasiticus AF82* and *F. graminearum FGr14*. On the 3rd, 5th and 7th day post-sealing, the diameters of the treated *P. verrucosum TF11* colonies reached to 6 mm, 9.5 mm and 10 mm accounting for an inhibition percentage of 32%, 37.91%, and 43.72%, respectively (Figure 11). The colony development rate in the colonies exposed to the yeast's VOCs was faster after the 3rd day. Moreover, the VOCs affected the pigmentation of the fungal colonies exposed to the yeast's VOCs, where the treated colonies were pale white compared to the colonies of the control which appeared pigmented in dark green (Figure 12).

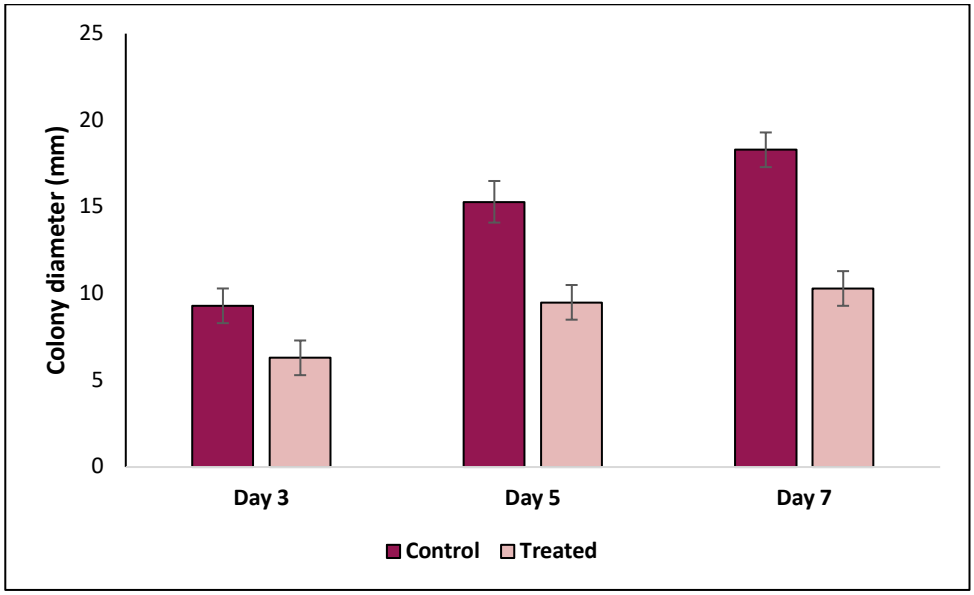


Figure 11: Effect of VOCs on the colonies sizes of point inoculated *P. verrucosum TF11.*

[The sizes of the *F. graminearum FGr14*colonies were measured after 3, 5 and 7 days of sealing]

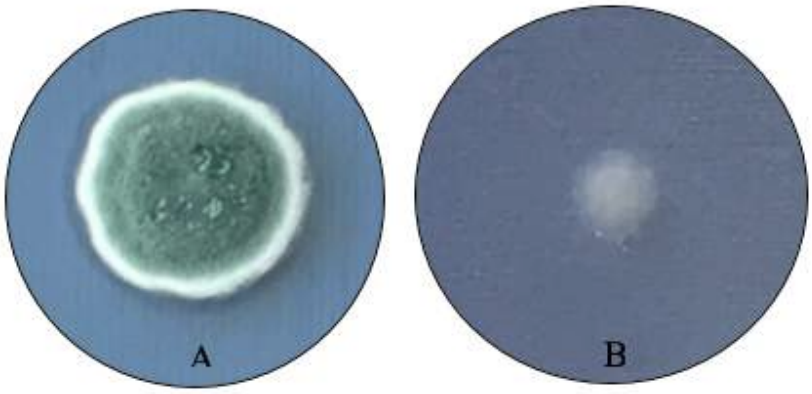


Figure 12: Effect of yeast VOCs on the characteristics of *P. verrucosum TF11.*

[A: control fungal colony without VOCs; B: fungal colony exposed to VOCs]

4.2 Effect of yeast's VOCs on individual fungal spores' germination spread on PDA.

4.2.1 Effect of yeast's VOCs on individual colonies of *A. parasiticus AF82*

Appropriate volumes of diluted spores' suspension (10^6 spore/mL) of *A. parasiticus AF82* were plated on PDA in order to obtain isolated colonies on the surface of the plate and were sealed to the yeast cells (10^6 /mL) on YPDA; the diameters of fungal colonies were recorded. The results showed a slight inhibition of fungal growth. On the third day post to sealing; the fungal colonies development rate was high and individual colony sizes continued to increase to become almost as close in size (17 mm) as those which were not exposed to the VOCs (18.8 mm). Due to the fungal cells fast developing rate, there was a slight change in the diameter growth of the treated colonies accounting for 9.7% on the 3rd day of sealing (Figure 13). However, the shape of the individual developing colonies of *A. parasiticus AF82* was different upon the exposure to yeast's VOCs compared to the colonies which weren't exposed to VOCs. The colonies of the treatment have totally lost their color to white and appeared in a cottony buffed shape compared to the untreated colonies which have appeared in a brown yellow color concentrated in the center of each colony (Figure 14).

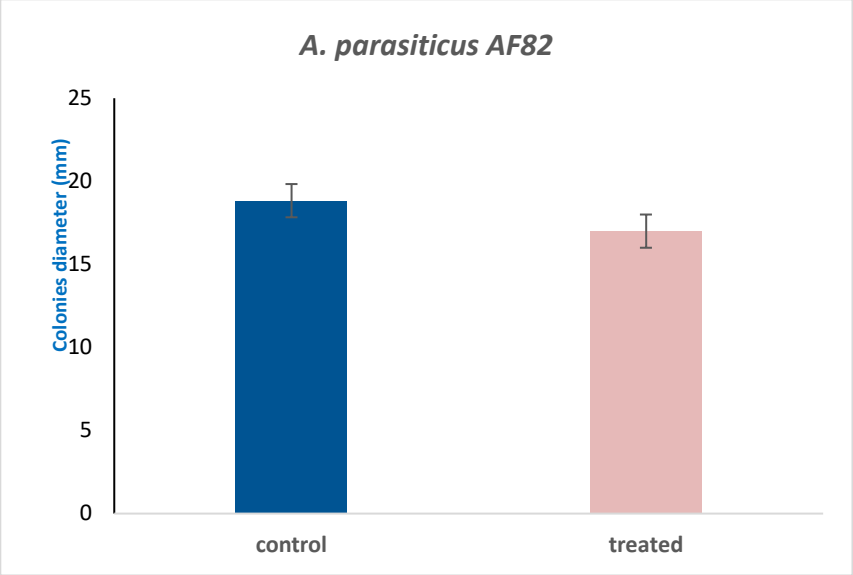


Figure 13: Effect of yeast's VOCs on the growth of individual colonies of *A. parasiticus AF82*

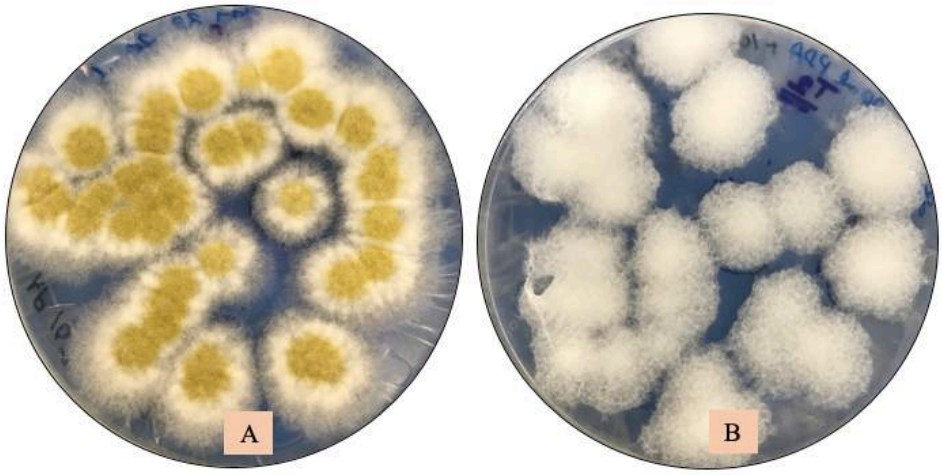


Figure 14: Effect of yeast VOCs on individual cells of *A. parasiticus AF82.*

[A: control fungal colony without VOCs; B: fungal colony exposed to VOCs]

4.2.2 Effect of yeast's VOCs on individual colonies of *F. graminearum FGr14*

The diameters of *F. graminearum FGr14* colonies exposed and non-exposed to yeast cells VOCs were measured and compared. The growth of *F. graminearum FGr14* individual cells sealed to yeast cells (10^6 cells) was 100% inhibited compared to the cells growth in the control which reached up to 26.6 mm on the third day of sealing (Figure 15). No visible germination of the spores was noticed for those exposed to the yeast VOCs, unlike the spores in the control (not exposed to VOCs), where they germinated and gave big colonies having a pink color in the center. The colonies exposed to the VOCs were returned back to the incubator to see if the spores could germinate with the time. However, even after a month of the incubation at 26ºC; the spores exposed to the VOCs didn't germinate at all. In contrast, colonies which were not exposed to the VOCs continued to grow normally.

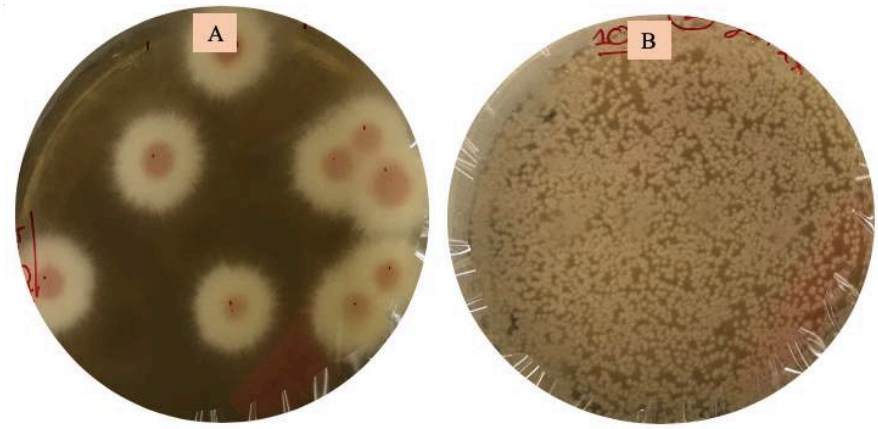


Figure 15: Effect of yeast VOCs on individual cells of *F. graminearum FGr14*
[A: control fungal colony without VOCs; B: fungal spores' inoculum exposed to VOCs]

4.2.3 Growth inhibitory effect of yeast's VOCs on individual colonies of *P. verrucosum TF11*

The diameters of *P. verrucosum TF11* colonies exposed and non-exposed to yeast cells VOCs were measured and compared. When individual spores of *P. verrucosum TF11* were sealed to yeast cells, the spores which were spread on PDA didn't germinate at all on the third day of sealing, and the *P. verrucosum TF11* was 100% inhibited. In the control (without yeast's VOCs), the spores germinated and continued to grow reaching to 7.5 mm on the third day of sealing. Hence, yeast's VOCs were able to completely suppress the spores' germination of *P. verrucosum TF11* and the plates appeared clear from any fungal growth (Figure 16). The plates which had the spores exposed to the yeast VOCs were kept incubated at 26ºC for a month, but the spores didn't germinate at all.

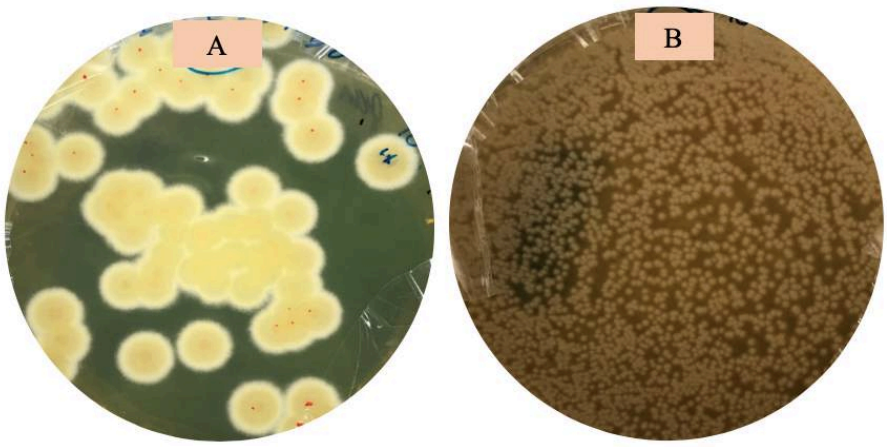


Figure 16: Effect of yeast VOCs on individual cells of *P. verrucosum TF11*

[A: control fungal colony without VOCs; B: fungal spores' inoculum exposed to VOCs]

4.3 Effect of yeast's cells concentration on the growth of *F. graminearum FGr14* individual colonies

Two yeast cells concentrations were tested to investigate their effect on the production of VOCs and hence the levels of the fungal growth. Yeast cells suspensions having a concentration of 10^6 cells/mL and 10^4 cell/mL were spread on YPDA plates which were sealed to PDA plates having spores of *F. graminearum FGr14* spread on them. *F. graminearum FGr14* was chosen to be the reference strain to be tested in this experiment and the latter ones. The sensitivity of *F. graminearum FGr14* toward yeast's VOCs falls in between that of *P. verrucosum TF11* and *A. parasiticus AF82*, whereas *P. verrucosum TF11* was too sensitive and its spores weren't germinating upon the treatment with VOCs, and *A. parasiticus AF82* was growing with minimal inhibition in its radial growth. The yeast suspension of 10^6 cell/mL and 10^4 cell/mL were supposed to account for the large and lower yeast cells numbers; hence more crowded and less crowded plates of yeast growth would be obtained, respectively.

The growth of the fungal colonies exposed to VOCs of yeast cells of both concentrations were compared to the spores which were not exposed to VOCs. The VOCs produced by yeast cells (10^3) in the lower concentration were able to inhibit the fungal growth completely and the fungus was not able to grow at all. In higher yeast CFUs (10^5), the spores could germinate and grow but the shape of colonies was different from those in the control. The colonies treated with higher yeast CFUs had lost their pigmentation and had foggy boarders (Figure 17). It was shown that very low yeast CFUs (10^4/mL) inhibited the growth and germination of *F. graminearum FGr14* completely.

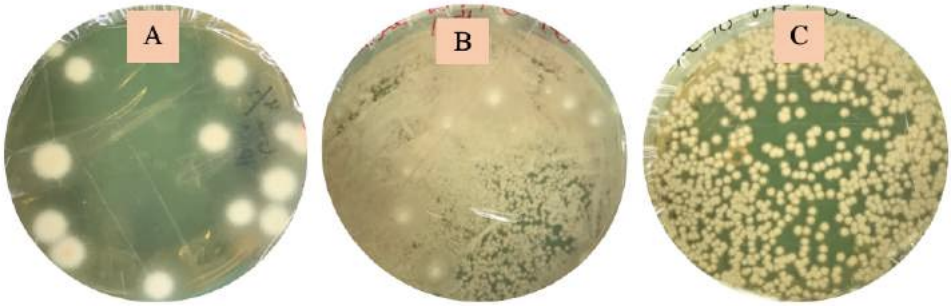


Figure 17: Effect of different yeast's CFUs on *F. graminearum FGr14* growth.
[A: negative control, no yeast; B: fungal cells exposed to 10^5 yeast CFUs; C: fungal cells exposed to 10^3 yeast]

4.4 Effect of nutrients availability on the production of yeast's VOCs for the inhibition of *F. graminearum FGr14*

In order to study the effect of nutrients availability on the antagonistic effect of yeast on *F. graminearum FGr14* growth, the yeast growth medium was diluted to 2 and 10 folds. The ability of yeast cells' VOCs to inhibit the fungal growth on the diluted media was compared to their ability to inhibit the fungal growth on normal non-diluted YPDA. Yeast CFUs counting was done for both media, and the plates having close CFUs were sealed with *F. graminearum FGr14.* The CFUs for the 2 folds diluted media were close to those of the normal YPDA, but they were less. After measuring the diameter for the fungal colonies after the sealing, it turned out that the less numbers of yeast CFUs on the 2 folds diluted YPDA were able to inhibit the fungal growth more than the higher CFUs on the normal media (data not shown). As result to that, the ½ diluted YPDA was chosen for the latter experiments.

4.5 Effect of increasing yeast's CFUs on the growth kinetics of *F. graminearum FGr14*.

The potential for yeast VOCs to inhibit the growth of *F. graminearum FGr14*

was explored in a range of yeast's CFUs spread on ½ YPDA and sealed to point inoculated fungal inoculum. Yeast cells range started from 7 CFUs to 598 CFUs. The ½ YPDA medium was chosen based on the results obtained in the previous experiment (section 4.4). The diameters of all fungal colonies were recorded for 5 consecutive days. The yeast CFUs counted in all plates allowed to work with different ranges of CFUs. Diameters of *F. graminearum FGr14* colonies exposed to VOCs from different yeast CFUs were compared to the ones in the control plates which were not exposed to yeast's VOCs. It was shown that the inhibition of the fungal colony growth of *F. graminearum FGr14* has started even from 7 yeast CFUs (Figure 18). The inhibition of the radial growth continued to decrease gradually with increasing the yeast's CFUs, and the highest inhibition in colony diameters (48.8%) was shown in the range of 145-305 CFUs. The fungal diameter (15.08 mm) decreased to almost half the diameters of colonies in the control plates (29.8 mm). The diameters of the fungal colonies were increased with yeast CFUs of 402-500. From 500 to 598 yeast CFUs, the diameters of the fungal colonies didn't increase (22.5 mm). The results of this experiment confirm that there is an optimum yeast CFUs affecting fungal growth, and that the increase of yeast's CFUs inhibit fungal cell growth.

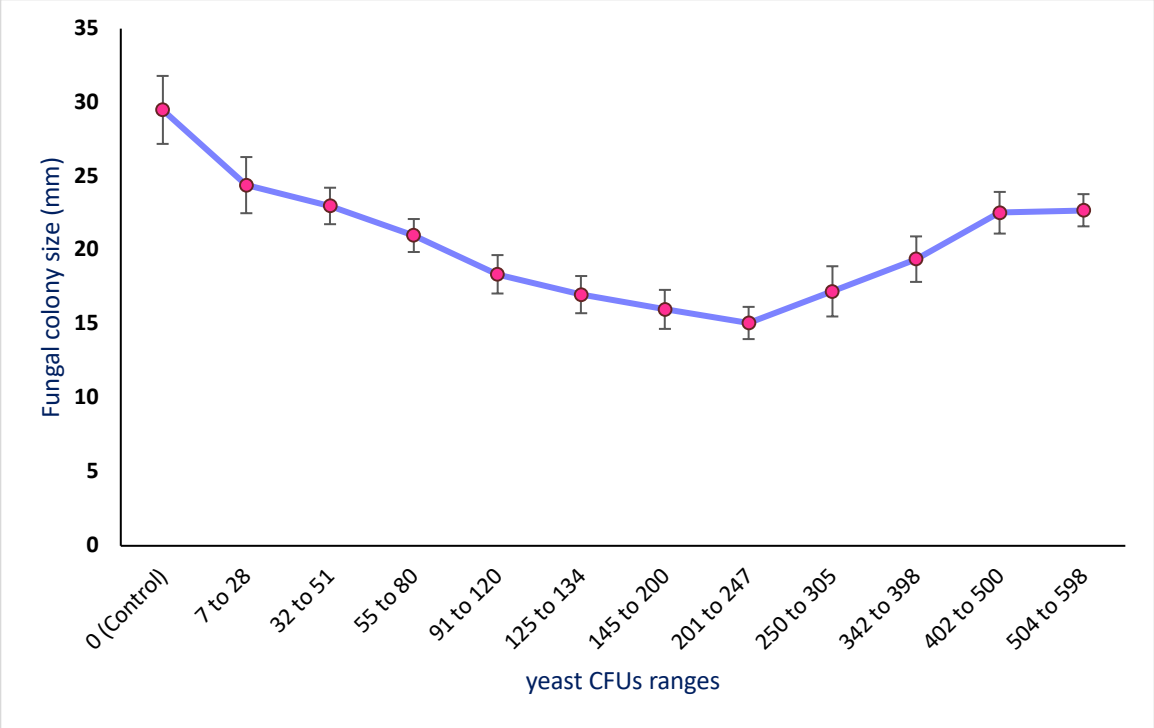


Figure 18: Effect of increasing yeast CFUs on *F. graminearum FGr14*

4.6 Effect of the yeast VOCs on the fungal synthesis of mycotoxins

4.6.1 Determination of Aflatoxin's concentrations in point inoculated and individual colonies

The synthesis of Aflatoxins was measured in point inoculated and individual colonies of *A. parasiticus AF82* which were exposed to VOCs produced by 10^5 yeast CFUs. Three plugs were extracted from the solid medium for AFB_1 extraction. AFB_1 was extracted and the concentration of AFB_1 was determined by ELISA. Although the radial growth for both individual and point inoculated colonies wasn't significantly different from the control after exposure to the yeast VOCs, AFB_1 concentration in the treated colonies was significantly decreased. In the point inoculated treated colonies (7 mm), AFB_1 was detected as 1.43 µg/kg compared to the control which had 8.01 µg/kg. This accounts for 82.1% reduction in the synthesis of AFB_1 in point inoculated colonies.

In the individual colonies of the control (19.25 mm); the concentration of AFB_1 was 7.09 µg/kg, while in the treated colonies it was 0.03 µg/kg (Figure 19). The reduction percentage of AFB_1 synthesis from individual colonies reached up to 99.5%.

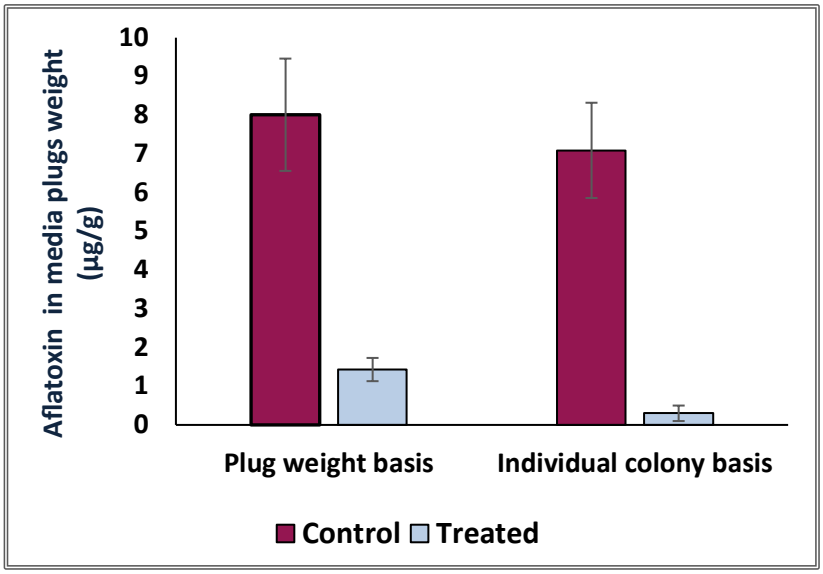


Figure 19: Effect of yeast VOCs on Aflatoxin synthesis by *A. parasiticus AF82.*
[AFB_1 was measured in samples of point inoculated and individual colonies of *A. parasiticus AF82*]

4.6.2 Detection of Deoxynivalenol concentrations in *F. graminearum FGr14* as an effect of increasing yeast's CFUs

DON concentration was measured in *F. graminearum FGr14* colonies which were exposed to VOCs from a wide range of yeast CFUs. For the determination of DON concentration; plugs were cut using a cork borer (7 mm) and were soaked with 70% methanol. The extract was dried and was resuspended with water and DON concentration was determined by mycotoxin's ELISA kit. Interestingly, the concentration of DON was gradually decreasing with increasing yeast CFUs, following

the same trend of the growth inhibition (results of section 4.5). As shown in figure 20; starting from the lowest yeast CFUs of 7 reduction of the synthesis of DON in *F. graminearum FGr14* colonies was noticed. The concentration of DON started to decrease gradually from 7 CFUs and down to 200 CFUs. Colonies of *F. graminearum FGr14* sealed to yeast CFUs from 145 to 398 cells have shown the most reduction in the synthesis of DON, where the concentration was below the detectable limit of ELISA. From 201 to 398 cells, DON's concentration was below the detectable limits of ELISA. DON's concentration started to gradually increase again from 402 to 598 CFUs.

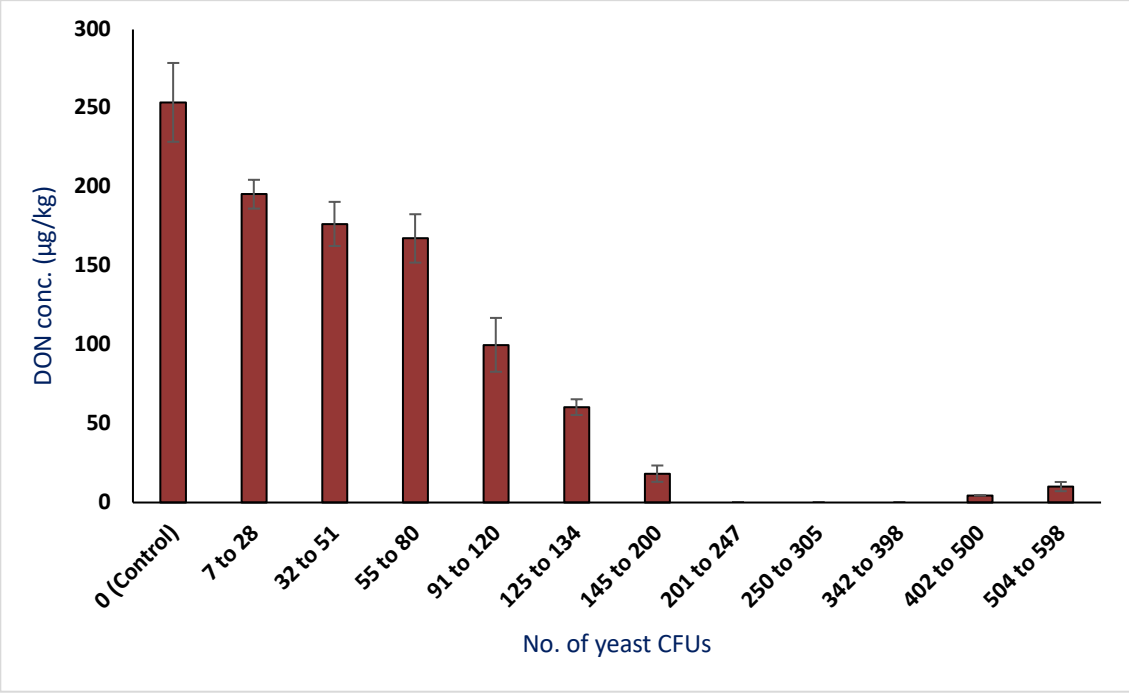


Figure 20: Effect of yeast CFUs on DON concentration measured in *F. graminearum FGr14*

Discussion:

In order to explore the yeast's VOCs potentials to inhibit the growth of mycotoxigenic fungi, yeast (*L. thermotolerans 751*) VOCs from a concentration of 10^7/mL were able to inhibit the radial growth of individual colonies of *P. verrucosum TF11, A. parasiticus AF82* and *F. graminearum FGr14*. The inhibition rate for the individual spores of *F. graminearum FGr14* and *P. verrucosum TF11* (100% both) was higher than the rate of inhibition in point inoculated colonies.

According to a study done by Virgili *et al*., (2012), when they used different yeast strains isolated from dried ham for the biocontrol of *P. nordicum*, they were able to demonstrate that yeast cells (10^8 /mL) sealed to fungal colonies of variable conidia concentration were able to inhibit the lesser concentration of fungal conidia (10^2/mL) better than other two higher concentration (10^4 and 10^6/mL). The fungal growth was almost vanished for the conidia having a concentration of 10^2/mL. Their findings confirmed the results obtained for the co-culture experiment of *F. graminearum FGr14* and *P. verrucosum TF11,* where the individual spore colonies were 100% inhibited by the VOCs. However, their findings don't agree with our results for sealing individual spores of *A. parasiticus AF82* with yeast cells. The inhibition of the individual colonies' growth (9%) wasn't bigger than the inhibition of the radial growth of the point inoculated colonies (25%) for the 10^6 spore/mL concentration. Nevertheless, the concentration of the synthesis of AFs had an opposite pattern. From the individual colonies, lower concentrations of AFs were detected compared to those measured in point inoculated colonies.

Similar to our results obtained on the morphology of the fungal colonies exposed to yeast's VOCs, Fiori *et al*., (2014) studied the antagonistic activity of four yeast strains including *L. thermotolerans.* They proved the effect of the yeast's VOCs to

inhibit the growth of point inoculated *A. carbonarius* colonies sealed to yeast cells (10^8 /mL). They also proved that upon the treatment with yeast VOCs; fungal colonies of *A. carbonarius* lost their color, sporulation and the borders of the colonies become uneven or undefined. This comes in line with our findings where all the treated fungal colonies (individual and point inoculated) have lost their color to white and became uneven in the margins and mycelia were shown scattered.

From a yeast called *K. apiculate,* Liu *et al*., (2014) managed to identify an antifungal compound (2-phenylethanol) and they described its mode of action on *Penicillium* species. From the analysis of the transcriptome of exposing *P. italicum* culture to 2-phenylethanol, they found that 1304 genes in the treated culture were differentially regulated compared to the transcriptome analyzed from the control culture. Through functional analysis, they managed to find the pathways which have been altered after the treatment of 2-phenylethanol. Some of these pathways is related to the fungal programmed cell death. In addition, some genes responsible for the DNA replication and cell cycle were found down-regulated when the transcriptome of *P. italicum* exposed to the VOCs was analyzed. These findings came supporting to the obtained results conducted in our work about *P. verrucosum TF11* being so sensitive against the VOCs of yeast hence it couldn't grow at all.

In a transcriptomic based work; Hua *et al.*, (2014) confirmed that 2-phenylethanol produced by the yeast (*P. anomala*) was responsible for a down regulation for more than 10,000 folds in the genes of *A. flavus*. 2-phenylethanol has hindered the production of AFB_1 by reducing the expression of its structural genes. They also managed to elucidate the effect of 2-phenylethanol on the expression of chromatin modifying genes, and according to their results, the down regulation in those genes is directly linked to the down regulation of the genes responsible for the biosynthesis of AFB_1 in *A. flavus*.

This confirms the reduction of AFB_1 measured in the point inoculated and individual colonies of *A. parasiticus*.

In an i*n-vitro* application of yeast to reduce the vegetative growth of two *Aspergilli* species, yeast's VOCs were successful to inhibit the radial growth of the colonies sealed to the yeast but also inhibit the sporulation and the synthesis of OTA as well (Farbo *et al*., 2018). Farbo *et al*., (2018) studied the effect of VOCs from yeast having an antifungal activity. They demonstrated that 2-phenylethanol is the major compound responsible for the inhibition of the radial growth and synthesis of OTA in *Aspergilli* species. The effect of 2-phenylethanol on OTA genes expression was experimented and it was proved that the yeast has down regulated the genes responsible for the biosynthesis of OTA, in addition to some regulatory genes. VOCs of yeast were able to cause a 99% demolition in the expression of *acpks* gene, and this gene is responsible for the synthesis of OTA in *A. ochraceous*.

Similar to what we found on the effect of yeast's VOCs the reduction of DON synthesis, Armando *et al*., (2013) experimented the inhibitory effect of two *Saccharomyces cerevisiae* strains in different conditions. A significant reduction in the radial growth of *F. graminearum* and *A. carbonarius* was obtained on a solid medium inoculated with a yeast inoculum. The concentration of DON was measured and found significantly reduced compared to the control, proving yeast as a good biocontrol agent to reduce concentrations of DON by *F. graminearum*, which comes in line with our findings.

We explored the effect of yeast VOCs on the growth kinetics and DON synthesis by *F. graminearum*, and the results showed that there was an optimum yeast CFUs range at which the highest inhibition for the fungal growth and DON synthesis were exhibited. However, above that yeast CFUs range, the inhibition of the fungal growth

started to decrease and DON's concentrations were started to increase as well. This could be explained by the fact that higher yeast CFUs account for more oxygen consumption, and due to the fact that the sealed plates create anaerobic medium, the oxygen will eventually deplete and synthesis of VOCs by the yeast cells would decrease, hence, less effect on the fungal growth at higher yeast CFUs would result, in addition to less inhibition in DON synthesis as well.

Similar to what we deduced in our results about having an optimum yeast CFU range where best inhibition can occur; Petersson *et al*., (1998) tested the impact of increasing yeast (*Saccharomyces cerevisiae*) CFU in the media on the growth of *P. verrucosum* and the synthesis of mycotoxin. They found that CFUs of yeast as low as 10^3 /mL were able to show an inhibition in the radial growth of *P. verrucosum* and that from as low as 10^2/mL yeast cells, reduction in the concentration of OTA to below detectable limits was achieved.

Conclusion:

Our findings demonstrated that *L. thermotolerans 751* has a great potential for the inhibition of fungal growth (specially *P. verrucosum TF11*) and the synthesis of mycotoxins. The results also showed that yeast's VOCs can reduce the synthesis of mycotoxins more in individual colonies of *A. parasiticus AF82* rather than in point inoculated colonies. It was also shown that the nutrients availability for the yeast cells has an impact on the synthesis of VOCs and that yeast can still synthesis VOCs on ½ diluted media as efficiently as it can on normal non-diluted media. Antagonistic activity of the yeast cells was demonstrated to start even at 7 yeast cells, there's a certain colonies range for an optimum fungal growth inhibition in *F. graminearum FGr14* to occur.

CHAPTER 5: DETERMINATION OF YEAST CELLS ADSORPTION POTENTIAL TO MYCOTOXINS (AFS, DON AND OTA)

Introduction

Mycotoxins are secondary metabolites known to contaminate food and feed, especially those from plant origin such as cereals and other agricultural feed or food consumed by the human beings or by animals. Food is not the only source of mycotoxins transfer to humans' body; where the carry-over can help transferring the mycotoxins from animal products such as milk and eggs to humans (Ji *et al*., 2016). Mycotoxins can impose many health issues due to their carcinogenic, mutagenic, teratogenic and immunosuppressive effect (Vila-Donat *et al.,* 2018). Apart from their dangerous effects; mycotoxins can also affect the economic status by leading to the loss of livestock, crops and feed (Ji *et al*., 2016). There have been different approaches utilized to reduce the harmful effects of mycotoxins, where the biological ones were the most successful (Ringot *et al*., 2005). Detoxification methods using biological control agents are thought to be more efficient when compared to organic and inorganic adsorbents and this is due to the need of applying the antagonistic microorganisms post and pre to harvest which reduces the use of synthetic chemical in the environment (Droby *et al*., 2009).

In this chapter, we explored the adsorption potentials of *L. thermotolerans 751* yeast cells and its derivatives in decontaminating mycotoxins. Heat inactivated and live yeast cells were used as binders to AFs, DON and OTA, where two different concentrations for each mycotoxin were used in the adsorption process. In addition to that, the effect of pH on the adsorption of the three mycotoxins was studied. The removal percentage of the mycotoxins from the buffer and the adsorption percentage of mycotoxins to the sediments of both types of yeast cells were obtained in the mentioned conditions.

5.1 Exploration of Aflatoxin binding onto yeast cells

5.1.1 Aflatoxin binding to living yeast cells

Living yeast cells ($2x10^6$) were transferred to two buffers of pH 5 and 7 which were contaminated with two concentrations of AFs, 0.2 and 0.4 µg/L (Figure 21). After 30 min of shaking at 37ºC, the mixture was centrifuged, and the supernatant was separated from the formed pellet and concentration of AFs was measured in supernatant and pellet. At pH 5 for the buffer contaminated with 0.2 µg/L AFs, 15% of AFs were removed from the supernatant and 10% were detected in the pellet (Figure 21, A). The percentage of AFs which was detected in the pellet accounts for the percentage of adsorption of AFs to the cell wall of the live yeast cells. Removal of AFs at pH 5 was better than at pH 7 which had a removal percentage of 7.97%. The percentage of AFs detected in the pellet at pH7 was 2.34%, which is lower compared to what was detected in pellet of pH 5. When the buffers were contaminated with higher concentration of AFs (0.4 µg/L), the percentages of removal and adsorption for AFs were found higher in pH 5 and higher than when low concentration of AFs (0.2 µg/L) were used (Figure 21, B). At pH 5 in the buffer contaminated with 0.4 µg/L, live yeast cells were able to remove 34.7% of AFs, and the percentage of AFs which was detected in the pellet was 25%. In contrast to the higher removal and adsorption of AFs at pH 5; pH 7 had the lowest percentages of removal and adsorption of AFs. The percentage of AFs' removal from the supernatant was only 5.25% and what was detected in the pellet was 3.63%. The results showed that pH 5 has increased the percentage of binding of AFs to the cell wall of the live yeasts, unlike the binding capacity of AFs at pH7, which was the least at both contamination levels.

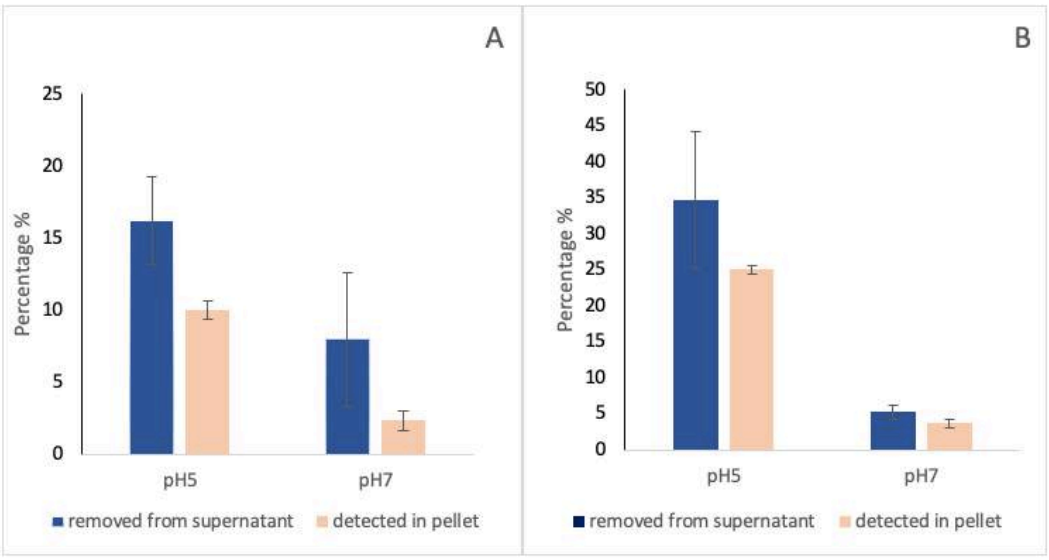


Figure 21: Adsorption potentials of living yeast cells (*L. thermotolerans 751*) to AFs. [The removal and adsorption rates at pHs 5 and 7 of AFs ((A): 0.2 µg/L; (B): 0.4 µg/L) to yeast cells were determined in the supernatant and the pellet of the suspension.]

5.1.2 Aflatoxin binding to inactive yeast cells

Dried inactive yeast cells were used to study their adsorption potentials to AFs (Figure 22). At pH 5 in the buffer having lower concentration of AFs (0.2 µg/L) (Figure 22, A), the removal of AFs from the supernatant has reached 20.45% and 18.75% of which were found in the pellet. The concentration that was detected in the pellet accounted for the binding of AFs to the cell walls of the inactive yeast cells.

At pH 7 of the same concentration of AFs, removal of AFs of 9.17%, and 8.27% was found adsorbed to the yeast cell walls in the pellet. When the buffer of pH 5 was contaminated with higher concentration of AFs, the removal percentage of AFs was 28%, and what was found bound to the yeast cells in the pellet was 24.45% (Figure 22, B). Both removal and adsorption of AFs at high concentration were higher in pH 5 than in pH 7. At pH 7, removal percentage of AFs was 9.71% and what was found bound to

the inactive yeast cells was 7.23%. Binding of the inactive yeast cells to AFs was always best at pH 5 than at pH 7.

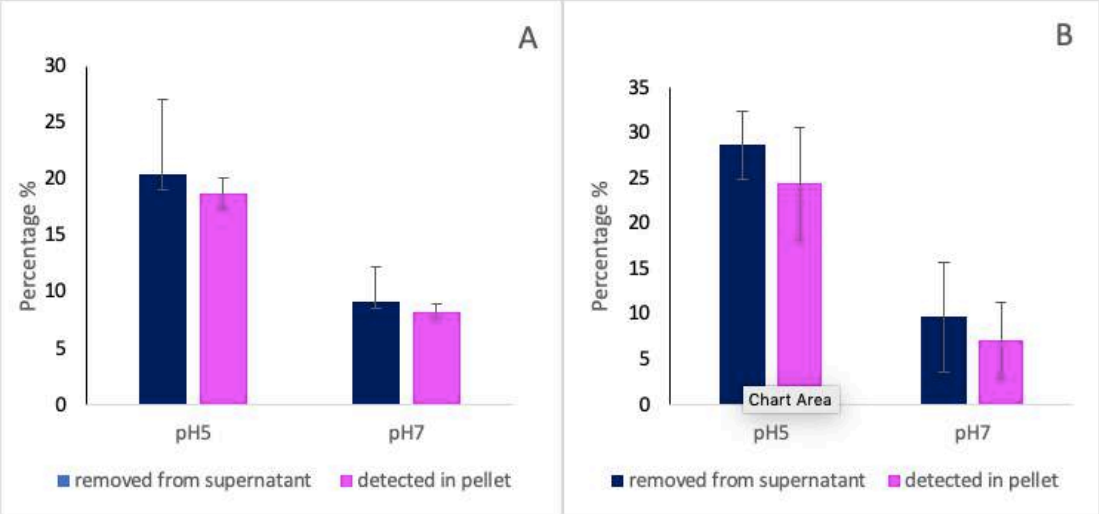


Figure 22: The adsorption potential of inactive yeast cells (*L. thermotolerans 751*) to AFs.

[The removal and adsorption rates at pHs 5 and 7 of AFs ((A): 0.2 µg/L; (B): 0.4 µg/L) to inactive yeast cells were determined in the supernatant and the pellet of the suspension.]

5.2 Exploration of the Deoxynivalenol binding to yeast cells

5.2.1 Deoxynivalenol binding to living yeast cells

Living yeast cell adsorption capacity to DON was determined in buffers of pH 5 and pH 7 which were contaminated with DON at two concentrations (40 and 80 µg/L) (Figure 23). The binding capacity of the living yeast cell wall to DON was best in the buffer of pH 7 at which better removal of DON from the supernatant has occurred in both contamination levels (Figure 23). In the buffer having a concentration of 40 µg/L

of DON, 10.1% of DON was removed at pH 5 and no detectable concentration of DON was found bound in the pellet (Figure 23, A). Higher removal percentage of DON has occurred at pH 7 where 52.11% was removed from the supernatant of the buffer contaminated with 40 µg/L of DON, and the pellet showed no detectable concentrations of DON bound to the yeast cells. In buffer of pH 5 which was contaminated with higher concentrations of DON (80 µg/L), 17.05% of DON were removed from the supernatant while in the pellet, DON's concentration was below the detectable limit, therefore the binding percentage of the living yeast cells to DON in the pellet was zero (Figure 23, B). At pH 7 there was higher removal percentage of DON (42.72%) and no detectable concentration was found bound to the yeast cells of the pellet.

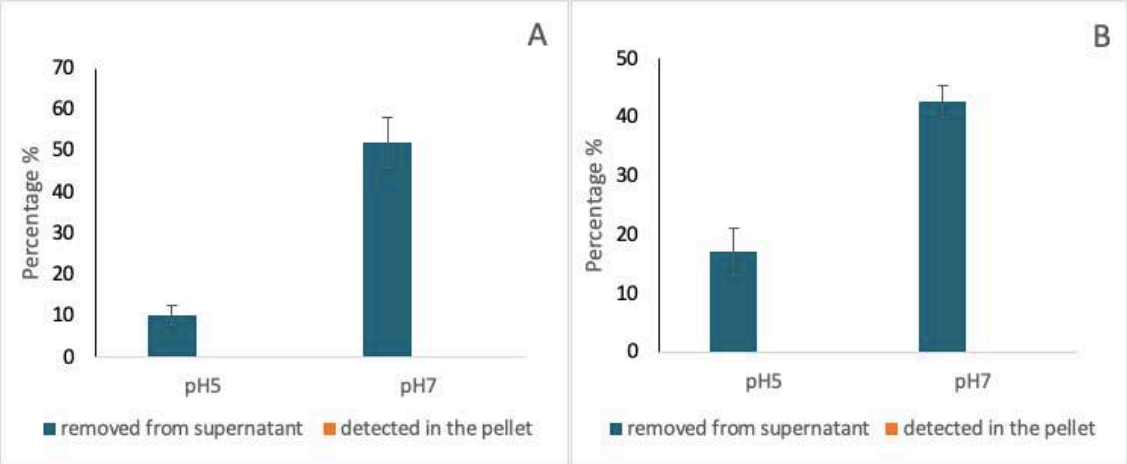


Figure 23: Adsorption potentials of living yeast cells (*L. thermotolerans 751*) to DON. [The removal and adsorption rates at pHs 5 and 7 of DON ((A): 40 µg/L; (B): 80 µg/L) to yeast cells were determined in the supernatant and the pellet of the suspension.]

5.2.2 Deoxynivalenol binding to inactive yeast cells

DON binding potential to inactive yeast cells was tested in buffers of pH 5 and

7 at lower and higher level of contamination, which were 40 and 80 µg/L, respectively (Figure 24). At a lower contamination level (40 µg/L), pH 5 has shown less removal and adsorption level of DON to yeast's cells compared to pH 7 (Figure 24, A). At pH 5, the removal percentage of DON from the supernatant was 3.17% and what was found bound to the pellet accounted for 2.5%. Removal and adsorption of DON were shown to happen at pH 7 better than pH 5. The buffer of pH 7 which was contaminated with 40 µg/L of DON had 18.17% removal from the supernatant and 14.08% were bound to inactive yeast cells in the pellet (Figure 24, A). In the buffers contaminated with higher percentage of DON (80 µg/L), pH 5 showed less removal and adsorption capacity than pH 7 (Figure 24, B). In the supernatant, 2.5% were removed of DON and 4.3% were detected in the pellet. pH7 had shown higher adsorption than in pH 5. In the supernatant of pH 7 buffer, 15.01% of DON was removed and 6.42% were detected in the pellet.

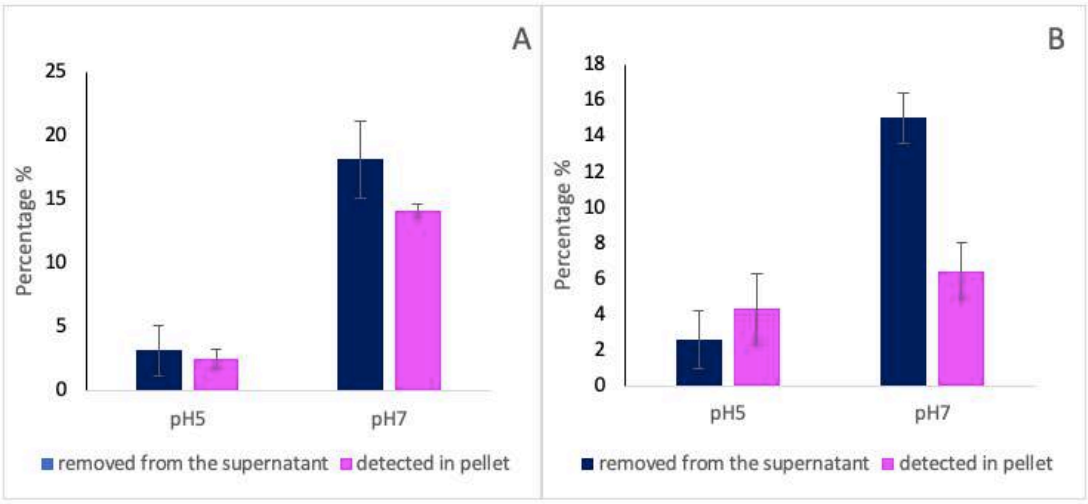


Figure 24: Adsorption potentials of inactive yeast cells (*L. thermotolerans 751*) to DON.

[The removal and adsorption rates at pHs 5 and 7 of DON ((A): 40 µg/L; (B): 80 µg/L) to inactive yeast cells were determined in the supernatant and the pellet of the suspension.]

5.3 Exploration of the Ochratoxin binding to yeast cells

5.3.1 Ochratoxin binding to living yeast cells

The removal and adsorption of OTA having the concentration of 0.9 µg/L were not greatly different at pH 5 and 7 (Figure 25, A). The removal percentage of OTA from the supernatant was 63%, and 29% was found adsorbed to yeast cells in the pellet. However, at pH7, 67% of OTA was removed from the supernatant and 30% was found adsorbed to the pellet. Higher percentage of adsorption was shown for the higher concentration of OTA (1.8 µg/L) (Figure 25, B). At pH 5, 75% of OTA was removed from the supernatant and 32% were found adsorbed to the live yeast cells in the pellet. At pH 7, higher adsorption percentage was shown for OTA where 49% were detected in the pellet and 71% were removed from the supernatant (Figure 25, B).

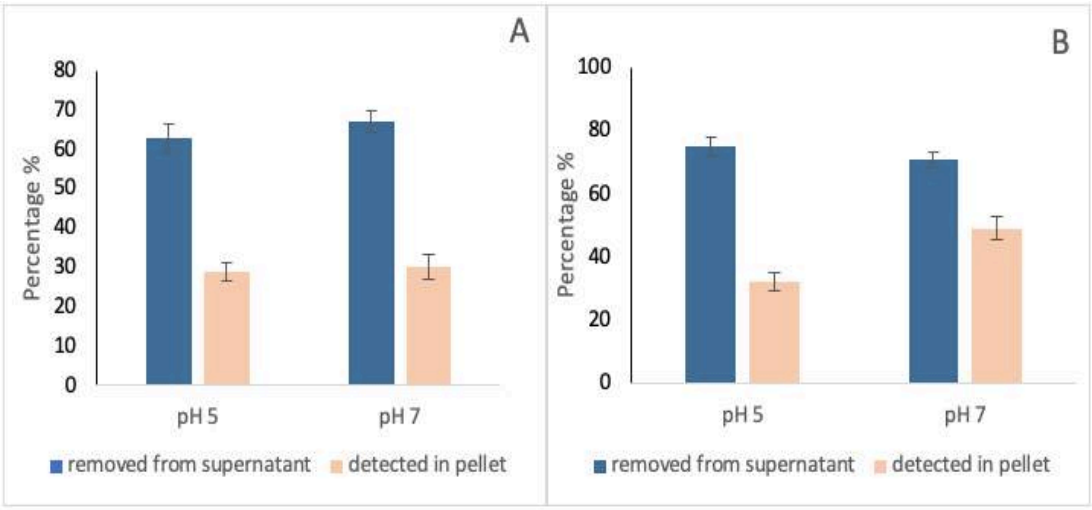


Figure 25: Adsorption potentials of living yeast cells (*L. thermotolerans 751*) to OTA. [The removal and adsorption rates at pHs 5 and 7 of OTA ((A): 0.9 µg/L; (B): 1.8 µg/L) to yeast cells were determined in the supernatant and the pellet of the suspension.]

5.3.2 Ochratoxin binding to inactive yeast cells

The results obtained (Figure 26) show that at low concentration of OTA (0.9 µg/L) and at pH 5, 71% of OTA were removed from the supernatant and 68% were detected in the pellet. pH 7 showed better removal and adsorption of OTA, where 74% was removed from the supernatant and 71% were detected in the pellet (Figure 26, A). When the buffers were contaminated with higher concentration of OTA (1.8 µg/L), 82% of it were removed from the supernatant and 80% were found adsorbed to the pellet (Figure 26, B). pH 7 showed less removal and adsorption potentials of OTA than at pH 5, and 79% of OTA were removed from the supernatant and 75% were detected in the pellet (Figure 26). It might be concluded that high adsorption potentials were evidenced for OTA to the inactive yeast cells compared to the live yeast cells.

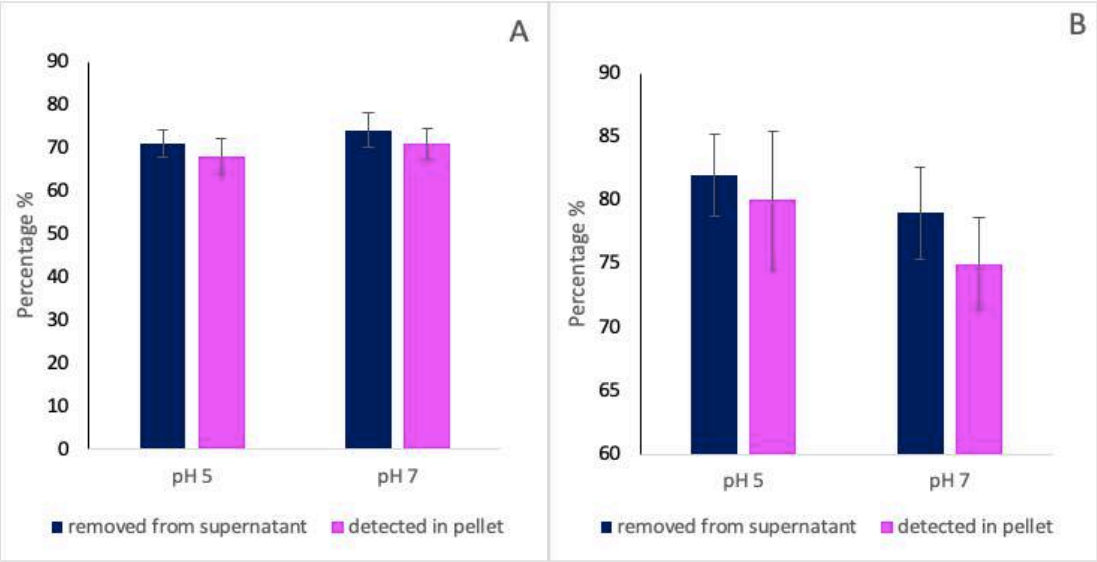


Figure 26: Adsorption potentials of inactive yeast cells (*L. thermotolerans 751*) to OTA. [The removal and adsorption rates at pHs 5 and 7 of DON ((A): 0.9 µg/L; (B): 1.8 µg/L) to inactive yeast cells, were determined in the supernatant and the pellet of the suspension.]

Discussion:

The potentials of mycotoxins AFs, DON and OTA to adsorb to *L. thermotolerans 751* were explored *in vitro*, where both living and inactive yeast cells' adsorption rates were explored. An inoculum from living yeast cell culture and heat inactivated yeast cells were used to inoculate two buffers of pH 5 and 7 which were later contaminated with mycotoxins. The tubes were then shake incubated for 30 min at 37ºC and were centrifuged, and the supernatants were separated from pellets. The adsorption of mycotoxins to the yeast cells was determined by dividing the detected concentration of mycotoxin in the pellet over the original concentration used in the buffer solution and multiplied with 100% to obtain the adsorption percentage. The removal percentage of the mycotoxin was calculated by referring the detected mycotoxin concentration to the original mycotoxin concertation added to the buffer as described in the equation below.

$$\%\ of\ mycotoxin\ removal = \frac{original\ concentration\ -detected\ concentration}{original\ ceoncentration} * 100\%$$

Based on the calculated adsorption rates for the mycotoxins in the pellet of living yeast cells; OTA has shown the greatest binding affinity to the living yeast cell walls, where OTA adsorption percentage was the highest compared to AFs and DON. Adsorption efficacy of AFs to the living yeast cells was less than OTA, but better than DON. DON was not detected in the living yeast cells although it was greatly removed from the supernatant of the living yeast cells at pH 7. For the inactive yeast cells, in pH 5 buffer and at concentration of 80µg; higher numerical value of DON adsorbed to the pellet was more than what was removed from the supernatant. When the yeast cells were heat dried; the adsorption potentials of the mycotoxins to the yeast cell wall has been generally enhanced, and more removal of mycotoxins from the supernatant was noticed. OTA showed the highest adsorption potentials to the heat inactivated yeast cells compared to AFs and DON. However, DON showed the least adsorption potentials to the yeast cell wall among the three mycotoxins.

There are many factors to which the adsorption of mycotoxins to yeast depends on. Some characteristics of the yeast cell wall such as the porosity, surface area and the charge and its distribution are considered very important features of the adsorbent (Dogi *et al.,* 2011). However, some characteristics of the mycotoxins like their shape, polarity and charge are considered important too. In addition, the pH of the buffer is also considered as an important factor which can greatly affect the adsorption process. The cell wall of the yeast is derived from important components such as the glucans. The glucans are found in the inner layer of the cell wall attached to the chitin which is another component in the cell wall of the yeast. The outer cell wall is composed of some proteins which are called mannoproteins. The adsorption process of the mycotoxins can be explained by the fact that the functional groups found on the cell wall of the yeast

cells get attached to some other functional groups found on the structure of the mycotoxins. The type of interactions which allow the binding of the mycotoxins and yeast cell wall are weak hydrophobic interaction, ionic exchange forces and complexations. The β-glucan part of the yeast cell wall is responsible for the binding which occurs between the mycotoxins and yeast. These forces can be van der Wal forces which are the ones responsible for the stacking that happens between the β-glucan fraction and functional groups found in the mycotoxins structure, just like lactones, ketones and hydroxyl groups (Faucet-Marquis *et al*., 2014; Kolosova & Stroka, 2012).

We demonstrated in our results that OTA adsorption was the highest compared to AFs and DON, which agrees with what came in the study of the binding potential of OTA, AFB_1 and ZEA to baker's yeast cell wall by Joannis-Cassan *et al.,* (2011), where they showed that AFB_1 has less binding potential to the yeast cell wall (29%) compared to ZEA and OTA which had adsorption rates of 68% and 62%, respectively. However, the adsorption of the OTA to the yeast cells increased with increasing its concentration in the buffer solution. The same results were concluded by Pereyra *et al*., (2015) who proved that yeast cell wall can be a good adsorbent to OTA, by studying two types of yeast cell wall to bind with OTA in different concentrations. They found that the adsorption percentage of OTA reached the highest (89.3%) when the concentration of the yeast was the highest.

In our study, when the adsorption of both active and heat inactive *L. thermotolerans 751* was studied against AFs, DON and OTA, the pellet of the heat inactive yeast had more mycotoxins adsorption efficacy compared to the active yeast cells. These findings come confirming to what was found by Bejaoui *et al*., (2004) who demonstrated that heat killed yeast were able to remove mycotoxin (OTA) from grape juice better than the living yeast cells. The heat killed cells were able to remove 90%

of OTA compared to the active yeast cells which removed only 35%. In our case, *L. thermotolerans 751* was able to remove OTA up to 82% and 79% at higher level of OTA contamination, even when the medium's pH different (pH 5 and 7 respectively). Enhanced adsorption in the heat-killed yeast could be attributed to the fact that with heating, more proteins are being denatured and therefore more products of the cell wall are being removed leaving more space for mycotoxins to adsorb to cell wall (Bejaoui *et al*., 2004). Therefore, the viability of the yeast cells is not a pre-requisite for better adsorption to occur, since heat treatment of yeast cells up to 120ºC is known to increase the adsorption potential of AFB_1 to the yeast's cell wall (Shetty *et al*., 2007; Gonçalves *et al.,* 2015). In this research, binding of AFs to the dried yeast at pH5 was almost the same as its binding to the viable yeast cells at concentration 0.4 µg/L, which was 24.45% and 25%, respectively.

The adsorption of DON to the viable and non-viable *L. thermotolerans 751* was shown to be lower when compared to the adsorption of AFs and OTA, which was similar to what was obtained by Dvegowda *et al*., (1998) when they studied the adsorption of DON to yeast cell wall and found that DON's adsorption (12.6%) was less than that for other mycotoxins like ZEA (66.7%), fumonisins (67%) and T-2 toxin (33%). The molecular geometry of the mycotoxin plays an important role in its adsorption to the yeast cell wall; therefore, some mycotoxins can bind better to the yeast cell wall than others. D-glucans have a 3D helical shape which allows for the mycotoxins that have similar shape to inter their helices and strongly bind to it. The structure of AFB_1 which is similar to the D-glucan structure helps the lactone, ketone and aromatic ring of AFB_1 to form polar or electron bonds with the glucose found on the glucan helices resulting in more affinity and stronger binding. In contrast to AFB_1; DON's geometrical shape has less similarity to the helical shape of the D-glucans, hence; less van der Waals forces will form between it and D-glucan on the yeast's cell

wall, which explains why it had less affinity to the yeast cell wall compared to OTA and AFs in our results. However, DON can still bind to D-glucans through the interaction of two of its hydroxyl bonds with the glucose group on the D-glucans (Jouany *et al*., 2005).

One way of the mycotoxins' detoxification is biotransformation of the mycotoxins into putative metabolites which possess less toxigenic effect than their original forms, such as 3-epi-DON and 3-epi-DOM1 which are transformed forms of DON (Nathanail *et al*., 2016; Vanhoutte *et al*., 2017). This attribution can explain the results obtained in our study on DON's adsorption to the viable yeast cells where DON was not detected at all in the sediments of the live yeast cells although it was still greatly removed from the supernatant. In this case, it's believed that the yeast has transformed DON into other putative metabolites (Nathanail *et al*., 2016).

Conclusion:

This part of the research study focused on the adsorption efficacy of three mycotoxins AFs, DON and OTA to a low-fermenting yeast *L. thermotolerans 751*. Our results obtained on this part from the *in-vitro* studies of yeast cells adsorption to mycotoxins postulated that the yeast *L. thermotolerans 751* is proved to be a great biocontrol agent in mycotoxins decontamination. Even when the contaminating concentration of the mycotoxins increased; both living and heat-treated yeast cells were still able to decontaminate the mycotoxins and efficiently remove them from the supernatant. Changing the pH of the buffer has also been shown to affect the adsorption process of both active and inactive yeast cells. Yeasts are known to have the potential to adsorb mycotoxins in *in-vitro* experiments, but more studies need to be conducted on the efficacy of yeast cells adsorption potentials to mycotoxins in *in-vivo* experiments (Oliveira, 2013). In addition, more studies need to be conducted on the mechanism of

mycotoxins binding to the yeast and biotransformation (bioconversion) of these mycotoxins in living yeast cell bioreactors, specifically for DON.

CHAPTER 6: *IN-VITRO* BIOCONTROL APPLICATION OF YEAST *L. THERMOTOLERANS 751* VOCS AGAINST *F. OXYSPORUM* CONTAMINATION IN TOMATO FRUITS

Introduction

The application of the biocontrol approach in controlling many fungi causing diseases is now achieving more attention compared to other approaches. In general, microorganisms can develop resistance to chemicals, and these chemicals, in turn, can be accumulated and therefore affect the environment. As best alternative, using biological agents to control pest population has been a favorable option recently (Wisniewski & Wilson, 1992; Parafati *et al*., 2015). Mycotoxigenic fungi which contaminate the food and feed post- and pre- harvest pose a great concern due to their ability to synthesize mycotoxins which contaminate the harvest. Therefore, many public concerns have been raised regarding mycotoxigenic fungal contamination and more demand is directed towards biological approaches (Milićević *et al*., 2010; Klich *et al*., 1991). One of the biocontrol methods that concerns using microorganisms as antagonists to mycotoxigenic fungi is employing the yeast and its VOCs in such an approach. The research on that subject matter has increased over the time to reach hundreds each year (Wisniewski *et al*., 2016; Zeidan *et al.,* 2018).

In this part of the research, the yeast VOCs synthesized by *L. thermotolerans 751* have been used *in-vitro* to study their potential effect on the growth of one of the mycotoxigenic fungi *F. oxysporum*. To study the effect of the VOCs produced by the yeast cells, a 48 h yeast streaked plate was kept with tomato fruits in a glass box which was tightly sealed and incubated at 26ºC for 33 days. The growth of mycelia on the tomato fruits surface was compared to those which were incubated with only YPDA plate having no yeast cells (control), and the contamination percentage was estimated based on the mycelial coverage.

6.1 Study of *in-vitro* effect of yeast's VOCs on the growth of *F. oxysporum* on tomato fruits surface:

The yeast's VOCs potential to affect the fungal growth and spores' germination of *F. oxysporum* contaminating tomato fruits surface was evaluated. An inoculum of 5 µL of *F. oxysporum* spores' suspension (10^4 spore/mL) was loaded on the surface of five tomato fruits which were transferred to a glass box containing a YPDA plate having 48 h streaked yeast cells, and the box was tightly sealed and closed. In another box serving as the control; five tomatoes were also contaminated with 5 µL of *F. oxysporum* and were transferred to a glass box having a plate of only YPDA medium without yeast. Both boxes were incubated at 26ºC for 33 days and the activity of yeast's VOCs was evaluated after that. When the incubation period was complete, the boxes were opened and the tomatoes in the VOCs treated box was compared to the non-treated ones (control). The tomatoes which were exposed to yeast's VOCs remained intact and rigid, and the fungal spores did not even germinate on the surface of the tomato which remained fresh. In contrast with the tomatoes which were not exposed to yeast's VOCs; the spores germinated, and the fungal mycelia was seen obvious covering the tomatoes surface. The average of the mycelia covering the tomatoes surface in the control was used to estimate the contamination rate which reached an average of 76% compared to 0% for the tomatoes which were exposed to yeast's VOCs. Figure 27 shows the difference between the VOCs exposed and non-exposed tomatoes.

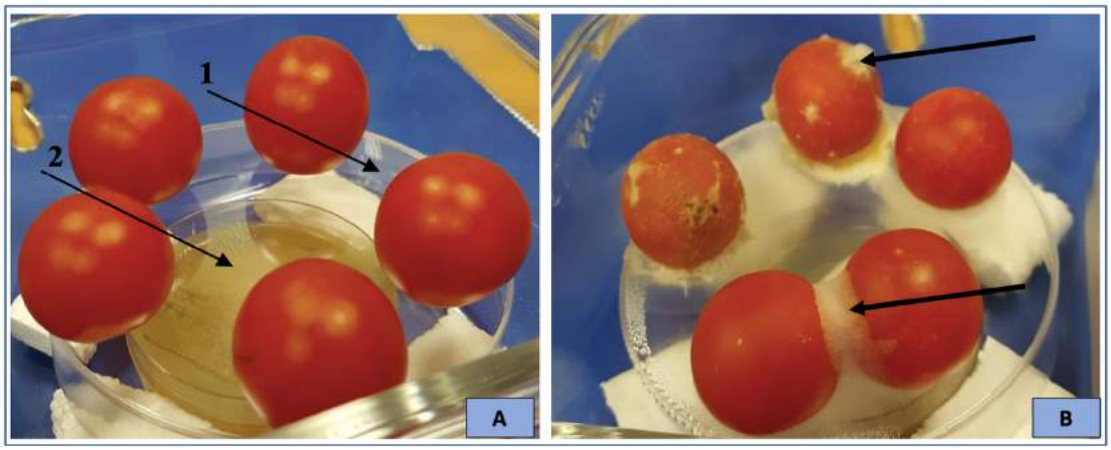


Figure 27: *In-vitro* Biocontrol of *F. oxysporum* contaminating the surface of tomato fruits by the application of *L. thermotolerans 751* VOCs.

[A*:* tomatoes contaminated with *F. oxysporum* and exposed to yeast's VOCs. Arrow #1 shows the Petri dish cover used as a stand for the tomato, arrow #2 shows the plate of YPDA streaked with yeast*;* B: tomatoes contaminated with *F. oxysporum* which were not exposed to yeast's VOCs. The arrows indicate the fungal growth contamination on the surface of the tomatoes.]

Discussion:

The potential of the VOCs produced by *L. thermotolerans 751* was *in-vitro* explored against the fungal contamination of *F. oxysporum* on tomato fruits surface. After the tomatoes were infected with the fungus, they were incubated in two boxes where in one of them the tomatoes were exposed to yeast's VOCs, and in the second the tomatoes were not exposed to the VOCs at all. After 33 days of incubation at 26ºC, the boxes were opened and the VOCs effect on the fungal growth was evaluated. Yeast VOCs managed to completely inhibit the fungal growth on the surface of the tomatoes and the spores' germination was completely ceased. The tomatoes which were treated

with the VOCs remained solid and intact as if they were preserved or bought freshly, while the contaminated tomatoes were very fragile and were watery from inside and their walls were breaking quickly.

There are many techniques by which the yeast can control and antagonize the contaminating mycotoxigenic fungi, and one of them is the production of VOCs which can affect the fungal growth (Pfliegler *et al*., 2015; Farbo *et al*., 2018). Yeast is known to produce volatiles such as 2-phenylthanol which inhibits the mycotoxigenic fungal growth (Farbo *et al*., 2018). The antifungal compound 2-phenylthanol is known to affect the fungus growth through differentially regulating the genes of the fungus, and hence inhibiting its growth (Liu *et al*., 2014).

Our results on the inhibition of *F. oxysporum* on the surface of tomatoes agrees with the findings of Fiori *et al*., (2014), who studied the effect of VOCs produced by the yeast to inhibit the growth of *A. carbonarius* infecting grape bunches in an *in-vitro* study. The yeast managed to significantly inhibit the fungal contamination on the grape bunches compared to the control where the bunches were not exposed to yeast VOCs. In a recent study which was conducted by Chen *et al*., (2018) to investigate the inhibitory effect of yeast's VOCs on *B. cinerea,* strawberry fruits surface was injured and infected with *B. cinerea*, and the fruits were then placed in a container having yeast plates. The yeast managed to control the rot in the strawberry contaminated fruits, and it was significantly lesser than the rot covering the strawberry fruits which were not exposed to the yeast's VOCs.

Conclusion:

In this part, we demonstrated the effect of yeast VOCs on the growth of *F. oxysporum* in an *in-vitro* study. Our results showed that yeast's VOCs were able to significantly inhibit the growth of *F. oxysporum* spores inoculated on the tomatoes when they were sealed incubated with yeast streaked plate. In this type of *in-vitro*

experiment, we proved the yeast VOCs as an excellent antagonist to mycotoxigenic fungi where *L. thermotolerans 751* VOCs were able to 100% inhibit the fungal growth on the tomatoes for 33 days, while also keeping their quality intact and rigid as if they were freshly harvested. Therefore, we consider *L. thermotolerans 751* as an excellent biocontrol agent against the mycotoxigenic fungi, that it might be safely used for many food and fruit preservation application as well as in fruit and vegetable product's safe exportation worldwide. We consider those fungicide very applicable in short term preservations.

CHAPTER 7: INVESTIGATION OF THE ANTIFUNGAL ACTIVITIES OF A LOCAL STRAIN *B. CEPACIA* (QBC03) AGAINST MYCOTOXIGENIC FUNGI

Introduction

The biological control of the mycotoxigenic fungi has received good attention in order to reduce the use of the chemical treatments for better preservation to the environment and to reduce plant diseases. Bacteria can act against mycotoxigenic fungi by possessing some mechanisms like having antagonistic potential, predation, antibiosis or hyper-parasitism and many other mechanisms more (Kilani-Feki *et al.*, 2010). Many bacterial genera such *Burkholderia* and *Pseudomonas* are known for producing antifungal compounds which is directly correlated to the biocontrol process. These compounds can be developed later to help in the control of the agricultural chemicals (Ligon *et al.*, 2000). *Burkholderia* spp. are common to inhibit the soil and they are especially present in the rhizosphere. They can also be endophytic to grapevines and to some legumes. *Burkholderia* spp. have been reported to have a good biocontrol effect against soil borne diseases. However, it also has been regarded as plant growth promoting bacteria (PGPB), where it promotes the plants growth by producing many substances that can support the plants growth directly or indirectly.

In this part of the study, a bacterial strain *QBC03* isolated from maize grains by our team (Jaoua *et al.* unpublished) belonging to the *Burkholderia* genus was used in the biocontrol of the mycotoxigenic fungi and their mycotoxins. *Burkholderia cepacia* antifungal activities were screened against mycotoxigenic fungi from different genera, and the effect of its antifungal compounds on the fungal biomass formation and spores' germination was evaluated too. Moreover, the stability of the antifungal compounds produced by this bacterium was also evaluated through treating the antifungal compounds with a range of different temperatures.

7.1 Optimization of *QBC03* growth conditions and anti-fungal compound sterilization methods

7.1.1 Evaluation of *QBC03's* anti-fungal compound synthesis in nutrient broth and NBY broth

In order to evaluate the activity of *QBC03* anti-fungal compound existing in the culture extract, the choice of the broth of the bacteria was defined. The bacteria were cultured in two types of broth, NB and NBY to define which broth is more suitable for the bacterial growth and production of antifungal compounds. To evaluate the bacterial antifungal production in both broths, well-diffusion method was used, where the supernatant was loaded into wells drilled on PDA media that was spread with 10^5 spores of *P. verrucosum TF11*. For accurate results and in order to avoid bacterial biofilm formation around the wells, the supernatant was sterilized using different sterilization methods.

When the supernatant of the cultures was UV sterilized, the inhibition zone appeared with bacterial growth around the wells, and the extract wasn't 100% sterile and it gave an inhibition zone of 33 mm. However, the inhibition zones resulted from the extract which was filtered in the 0.2 µm and 0.45 µm syringe filters, and UV sterilizations were compared in figure 28. It was found that 0.2 µm filter prevented the antifungal compounds from passing through it, hence, there was no zone appearing around the wells. Filters of 0.45 µm prevented part of the extract from passing through and the zones of. Inhibition appeared smaller. However, the 0.45 µm didn't prevent the bacteria from growing around the wells. To evaluate the antifungal compounds produced by *QBC03* in NB and NBY, the zones of inhibition of both extracts were compared together (Figure 29), and it was obvious that the supernatant of NBY had bigger zones of inhibition compared to the small zone for the extract of NB.

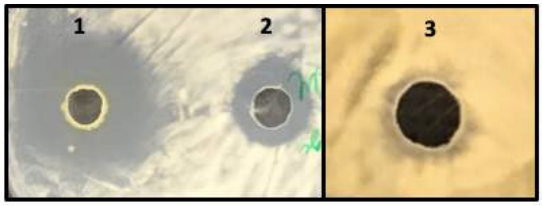


Figure 28: Evaluation of different sterilization methods of *QBC03's* antifungal extract in well-diffusion method.

[1: zone of the well loaded with the extract sterilized by UV; 2: zone of the well loaded with the extract sterilized with 0.45 µm syringe filter; 3: zone of the well loaded with the extract sterilized with 0.2 µm syringe filter]

Figure 29: Evaluation of the antifungal activity of *QBC03*'s extract harvested from NBY and NB culture in well-diffusion method.

[A: the inhibition zone of *P. verrucosum TF11* by *QBC03's* culture extract in NBY; B: the inhibition of *P. verrucosum TF11* by *QBC03's* culture extract in NB]

7.2 Screening for the antifungal activity of *QBC03* strain

7.2.1 Determination of the spectrum of the anti-fungal activity of *QBC03* strain against *Aspergillus* species

The activity of *QBC03* antifungal extract was evaluated against 7 *Aspergillus* species which were *A. carbonarius AC82, A. westerdijkiae AW82, A. parasiticus AF82*, *A. niger AN8*, *A. flavus CECT 2687*, *A. ochraceous CECT 2948* and *A. fumigatus Af14.*

QBC03 was streaked on NA plate for 24 h, and from an individual colony the bacteria were tooth-picked on the center of new NA plates. The plates were incubated at 30ºC for 48 h and they were later overlaid with 3 mL (10^4 spores) of fungal spores' suspension. The overlaid plates were incubated at 26ºC for 3 days and the average of the diameters for the inhibition zones was recorded. Figure 30 represents the zone of inhibition recorded for each of the *Aspergillus* species after three days of incubation.

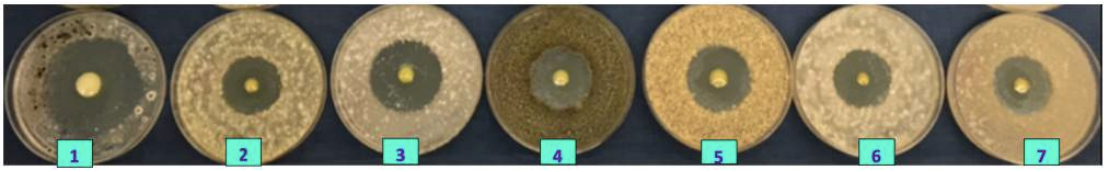


Figure 30: Evaluation of *QBC03's* antifungal compounds agaisnt 7 *Aspergillus* species in overlaying assay method.

[1: *A. carbonarius*; 2: *A. flavus*; 3: *A. fumigatus*; 4: *A. niger*; 5: *A. ochraceus*; 6: *A. parasoticus* 7: *A. westerdijikae*]

To compare the inhibition zones for each species, Duncan test was done (Figure 31), and it was found that the highest inhibition zone which was also significantly different from the rest of the species belongs to *A. carbonarius AC82* was greatly sensitive towards *QBC03's* diffusible compounds, and its zone of inhibition reached up to 59.1 mm. *A. fumigatus* had the second largest inhibition zone which was 41.9 mm, and that zone was significantly different from the rest. *A. ochraceous* was the third most affected species and its zone of inhibition reached 37.7 mm which was not significant from *A. westerdijikae* (36.7 mm). The diameters of the inhibition zones for *A. parasiticus*, *A. niger AN8* and *A. flavus CECT 2687* were relatively close and

corresponded to 35 and 34.1 mm respectively.

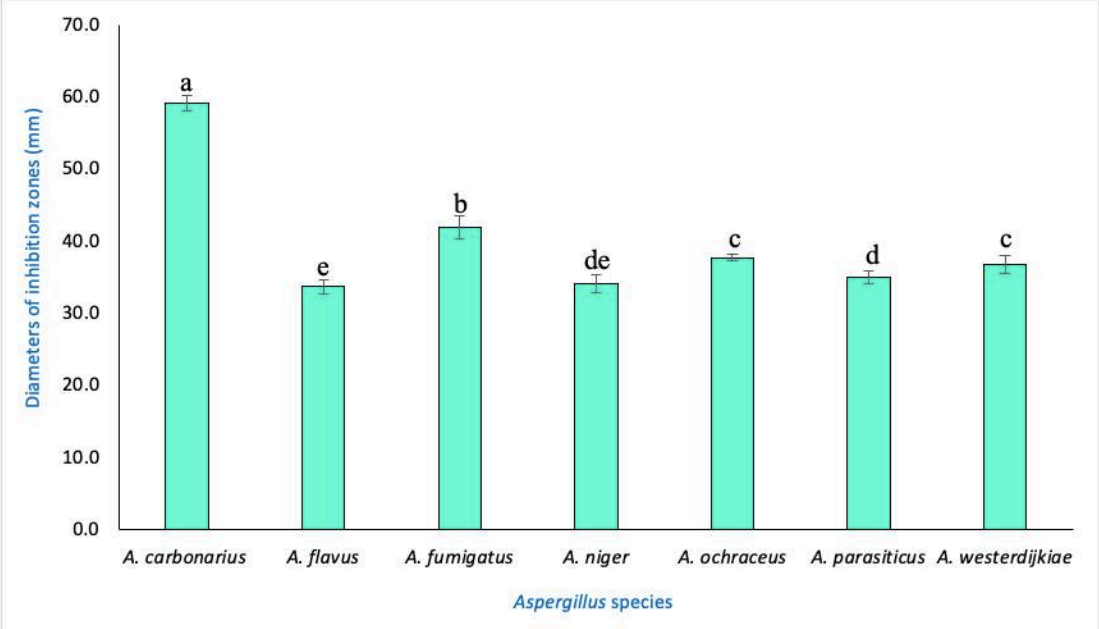


Figure 31: Determination of the spectrum of anti-fungal activity of *QBC03* strain against *Aspergillus* species.

[The diameters of the zones of inhibtion of *Aspergillus* species exposed to *QBC03's* antifungal compounds were measured. Duncan test was done to comapre the average of the inhibition zones, and those sharing the same letter are not significantly different from each other.]

7.2.2 Determination of the spectrum of anti-fungal activity of *QBC03* strain against *Fusarium* species

QBC03 antifungal activity has been studied against 9 *Fusarium* species (*F. anthophilum, F. chlamodosporum, F. culmorum, F. graminearum, F. oxysporum, F. solani, F. subglutinus, F. prolifiratum* and *F. verticilliod*) in the overlaying assay method. The diameters of the inhibition zones were measured around *QBC03* colony.

To compare the diameters of the inhibition zones of the *Fusarium* species together, Duncan test was done (Figure 32). *F. chlamodosporum* was one of the most sensitive species to *QBC03* diffusible compounds and it had the largest inhibition zone (36.8 mm) which was significantly higher than the inhibition zones for the rest of the species. *F. culmorum* (34.8 mm) and *F. graminearum* (35 mm) showed very close diameters in their zone of inhibition and were not significantly different from each other. The antifungal activity of *QBC03* corresponded to less inhibition zones in the rest of the species, but the least sensitive two species were *F. prolifiratum* and *F. verticilliod* and their inhibition zones were significantly lower than that for the rest of the species and their inhibition zones were 6.7 and 2.5 mm, respectively.

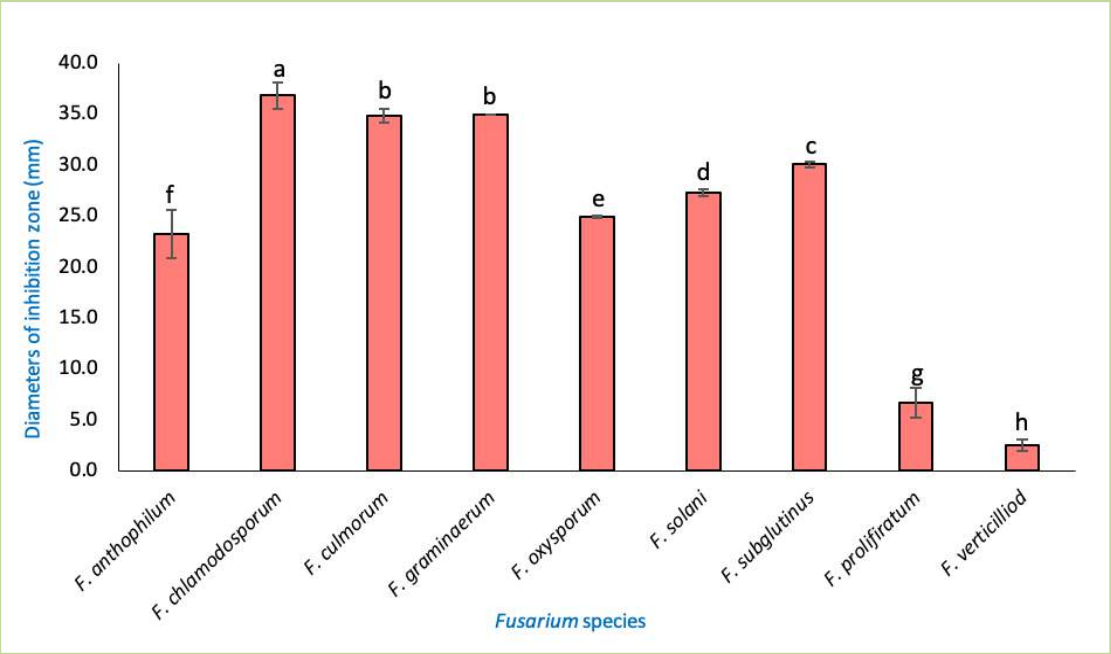


Figure 32: Determination of the spectrum of anti-fungal activity of *QBC03* strain against *Fusarium* species.

[The diameters of the zones of inhibtion of *Fusarium* species exposed to *QBC03's* antifungal compounds were measured. Duncan test was done to comapre the average of the inhibition zones, and those sharing the same letter are not significantly different from each other.]

7.2.3 Determination of the spectrum of anti-fungal activity of *QBC03* strain against *Penicillium* species.

Through co-culture overlaying assay, the antifungal potential of *QBC03* was explored against five species of *Penicillium* (*P. cambeberti, P. digetatum, P. expansum, P. italicum* and *P. verrucosum*). *QBC03* was inoculated in the center of the NA plate and was incubated for 48 h, after that the spores of the *Penicillium* (10^4) were assayed around the bacteria, and the plates were incubated at 26ºC for three days. The zone of inhibition was measured around the wells for all the species as they appear in figure 33.

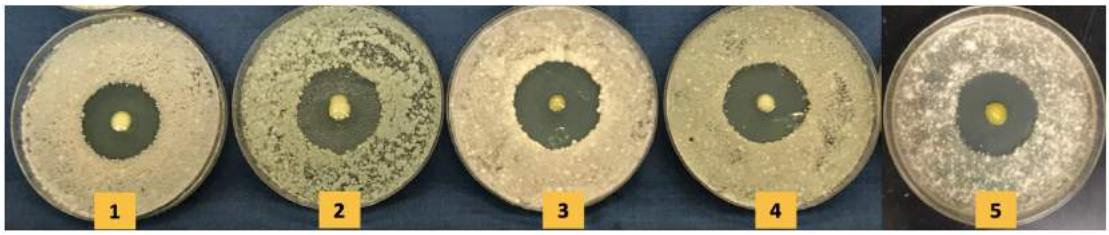


Figure 33: Determination of the spectrum of anti-fungal activity of *QBC03* strain against *Penicillium* species.

[The diameters of the zones of inhibition of *Penicillium* species exposed to *QBC03's* antifungal compounds were measured. 1*: P. camemberti,* 2*: P. digitatum,* 3*: P. expansum,* 4*: P. italicum,* 5*: P. verrucosum*]

To compare the inhibition zones between the different species, Duncan test was conducted, and significant and non-significant species are labeled on figure 34. Among the five studied species of *Penicillium*, *P. cambeberti* turned to be the most sensitive species and was significantly higher than the rest and it had the largest inhibition zone that reached to 37.3 mm. The species *P digitatum, P. italicum* and *P. verrucousm* had inhibition zones which were not significantly different from each other, and their inhibition zones were 35.8, 35.3 and 35.1 mm, respectively. The least sensitive *Penicillium* specie was *P. expansum* which had an inhibition zone's diameter of 34.1 mm.

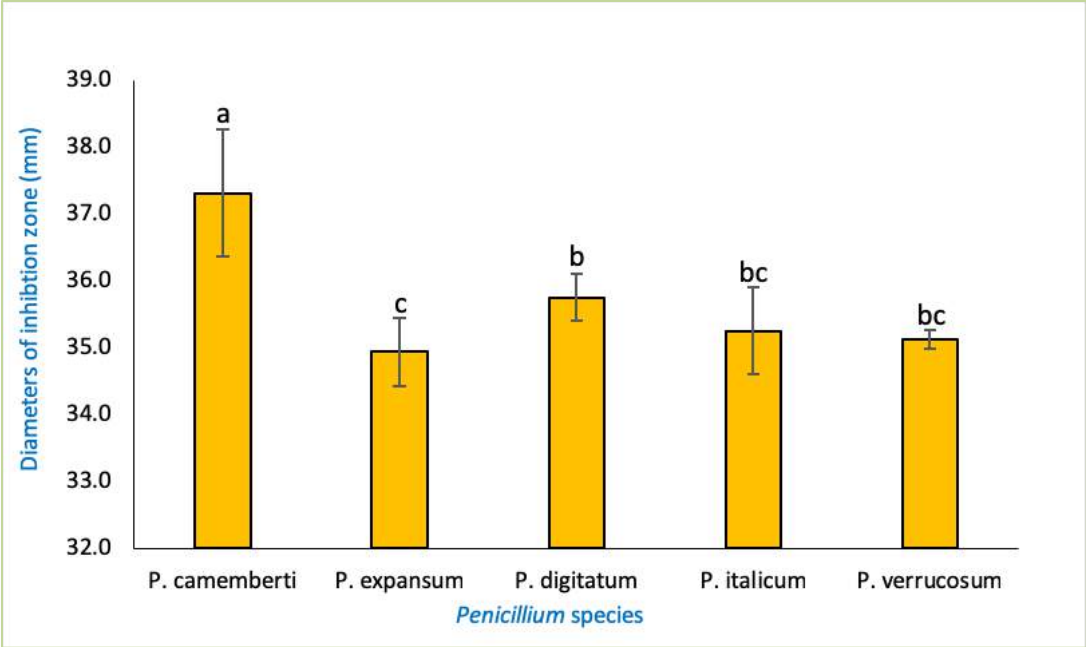


Figure 34: Determination of the spectrum of anti-fungal activity of *QBC03* strain against *Penicillium* species.

[The diameters of the zones of inhibtion of *Penicillium* species exposed to *QBC03's* antifungal compounds were measured. Duncan test was done to comapre the average of the inhibition zones, and those sharing the same letter are not significantly different from each other.]

7.3 Evaluation of the antifungal activity of the supernatant of *QBC03* strain in PDA

7.3.1 Evaluation of *QBC03's* antifungal compounds in well-diffusion method at different incubation periods

The antifungal compounds produced by *QBC03* were evaluated against three species; *A. carbonarius, F. culmorum* and *P. verrucosum* in well-diffusion method. *QBC03* extract was prepared from 24, 48 and 72 h culture of *QBC03* in 10 mL NBY that was incubated at 30ºC/140 rpm in 50 mL conical tubes. The tubes were centrifuged

at 5500g for 20 min and the supernatant was harvested. PDA amended with 100 µg/L chloramphenicol was poured in Petri dishes and 200 µL from the fungal spores' suspension (10^6/mL) were spread on the media. Thereafter, 7 mm wells were drilled in the media and different concentration of *QBC03's* extract (1%, 20%, 60%, 80% and 100%) were loaded in those wells. The plates were incubated at 26ºC for 3 days and diameters of the inhibition zones were recorded.

7.3.1.1 Evaluation of QBC03's antifungal compounds against A. carbonarius in well-diffusion method

The extracts of 24, 48 and 72 h of *QBC03* were evaluated against *A. carbonarius* in well-diffusion method. The extract was diluted to 1%, 20%, 60%, 80% and 100%, and 100 µL were loaded in all wells. After 3 days of incubation at 26ºC, the diameters around the inhibition zones were measured and recorded around the wells. As summarized in the graph of figure 35. The 1% and 20% extract of the 24 h *QBC03* culture didn't show any inhibition zone around the wells, thus both percentages of the extract corresponded to zero inhibition. The extract's activity of the 24 h culture started to appear at 60% were an average of 15.2 mm inhibition zone was recorded. The extract's activity was increasing with both percentages 80% and 100%, and the inhibition zones recorded for both treatments were 17.1 mm and 19.4 mm, respectively. The antifungal activity of the 48 h culture was the best compared to the 24 h and 72 h extracts. The extract's antifungal activity started at 20% where an inhibition zone of 13.5 mm was measured around the wells. The antifungal activity of the extract started increasing gradually while increasing the percentage of the extract added to the wells, to reach 17.4 mm and 20.4 mm for 60% and 80% of the extract, respectively. The largest inhibition diameter recorded for the 48 h culture was when 100% of the extract was used and the diameter was 22.4 mm. The 72 h culture extract had an antifungal activity that was less than the activity of the 48 h cultural extract more than 24 h culture extract.

However, the antifungal activity of the 72 h culture extract had started at 20% at an inhibition zone of 12.2 mm, and the activity had continued to increase while increasing the percentages of the extract. At 60%, 80% and 100% extract, the inhibition zones were 16, 18.6 and 20.3 mm, respectively.

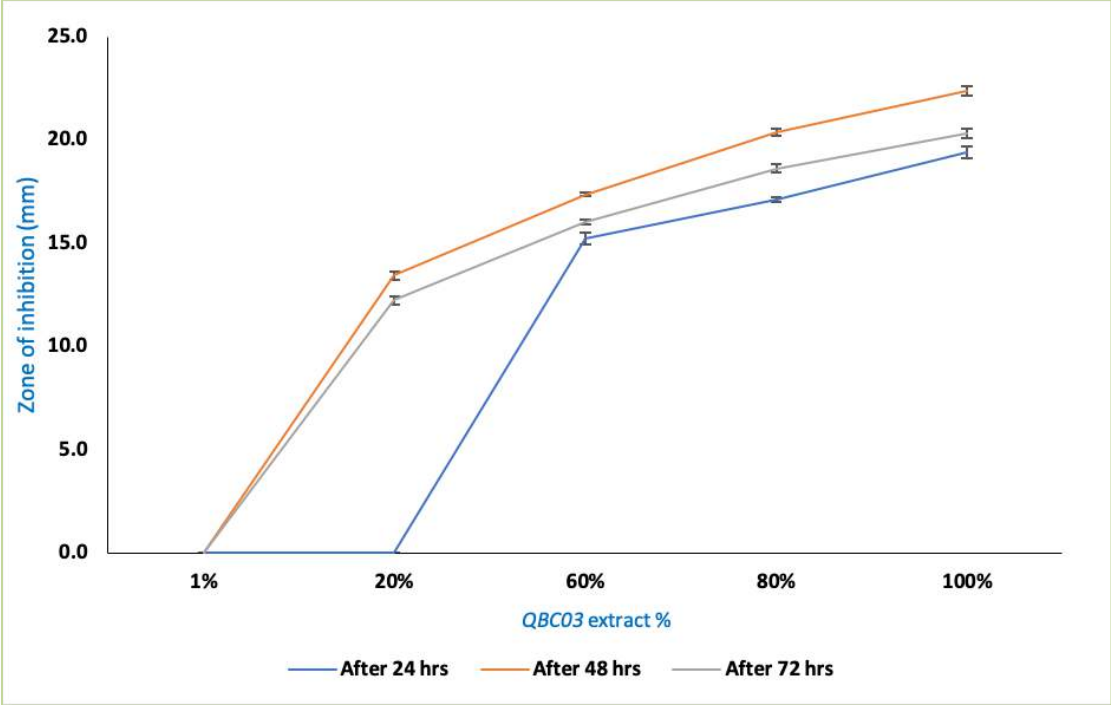


Figure 35: Effect of the antifungal compounds concentration on the growth of *A. carbonarius AC82* .

[The diameters of the zones of inhibition for *A. carbonarius AC82* by the antifungal compounds of 24, 48 and 72 h cultures of *QBC03* in well-diffusion method.]

7.3.1.2 Evaluation of QBC03's antifungal compounds against F. culmorum

The well-diffusion method was used to evaluate the extract antifungal activity against *F. culmorum. QBC03* was cultured in NBY and the supernatants of 24, 48 and

72 h cultures were harvested and loaded in wells of PDA spread with 200 µL spores of *F. culmorum* spores' suspension (10^6/mL). For each culture extract, five concentrations were used (1%, 20%, 60%, 80% and 100%) and loaded in the wells. The diameters of the inhibition zones were measured and recorded after 3 days for incubation at 26ºC. In figure 36, the antifungal activity of the three cultures started at 20% of the extract, where for all, 1% of the extract showed no inhibition zones at all. Nevertheless, the *F. culmorum* growth rate was fast and the mycelia were growing quickly and therefore they were able to cover zones around the wells of 20% treatment of the extract. At 20% of the extract, the highest inhibition zone was measured for the 48 h culture (14.2 mm), and the zones for the 24 and 72 h culture extracts were almost the same (12.4 and 12.6 mm, respectively). The antifungal activity started increasing for the three cultures when the percentages of the extract were increasing. At 60%, the 48h culture had the highest inhibition zone that corresponded to 18.2 mm, and it was followed by the diameters of 24 h culture then the 72 h culture which corresponded to 16.4 and 14.6 mm, respectively. When 80% of the extract was used, the cultures of 24 h and 72 h had almost the same antifungal activity and diameters for the zones of inhibition (20.2 and 20.1, respectively) followed by the activity of the 72 h culture that had smaller inhibition zone (18.3 mm). Using the 100% extract for the three cultures resulted in higher inhibition zones in the extract of the 48 h culture (25 mm), followed by the activity of the 24 h culture (23.1 mm). The lowest activity was shown for the 72h culture, where it corresponded to an inhibition zone of 18.8 mm.

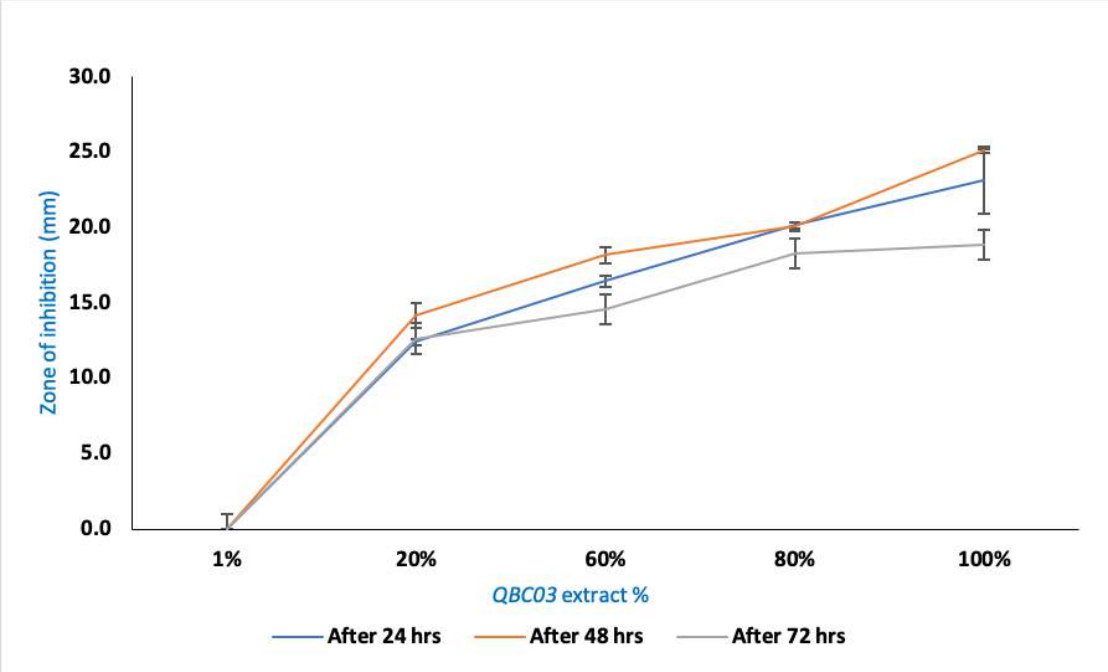


Figure 36: Effect of the antifungal compounds concentration on the growth of *F. culmorum*.

[The diameters of the zones of inhibition for *F. culmorum* by the antifungal compounds of 24, 48 and 72 h cultures of *QBC03* in well-diffusion method.]

7.3.1.3 Evaluation of QBC03's antifungal compounds against P. verrucosum

The extracts of three cultures of *QBC03* which were incubated from 24, 48 and 72 h were collected to study their antifungal activity against *P. verrucosum* in well-diffusion method. The extract of the three cultures were loaded into wells in different percentages (1%, 20%, 60%, 80% an 100%), and the zones of inhibition around the wells were recorded after three days of incubation at 26ºC. As shown in figure 37, at 1% of the extract, there was no antifungal activity for the extract collected from the three cultures and therefore zero inhibition zone was recorded. The 24 h culture extract had the best antifungal activity, where the inhibition zones were recorded starting from using 20% of the antifungal extract, unlike the activity of the 48 and 72 h culture extract

that started after 20%. However, the antifungal activity of the extract was increasing gradually with the increasing percentages, and the zones of 16.4, 11.4 and 8.2 mm were recorded for the 48, 72 and 24 h culture, respectively. Higher inhibition zone was recorded for the 48 h culture using 80% of the extract (18.1 mm) compared to those recorded for 72 and 24 h culture extract (13 and 13.1 mm, respectively). Using 100% of the extract taken from the three cultures resulted in higher activity for the 48 h culture extract giving 19.5 mm inhibition zone compared to the activity of the other two cultures of the extract which had the same antifungal that gave 17.2 mm zone of inhibition.

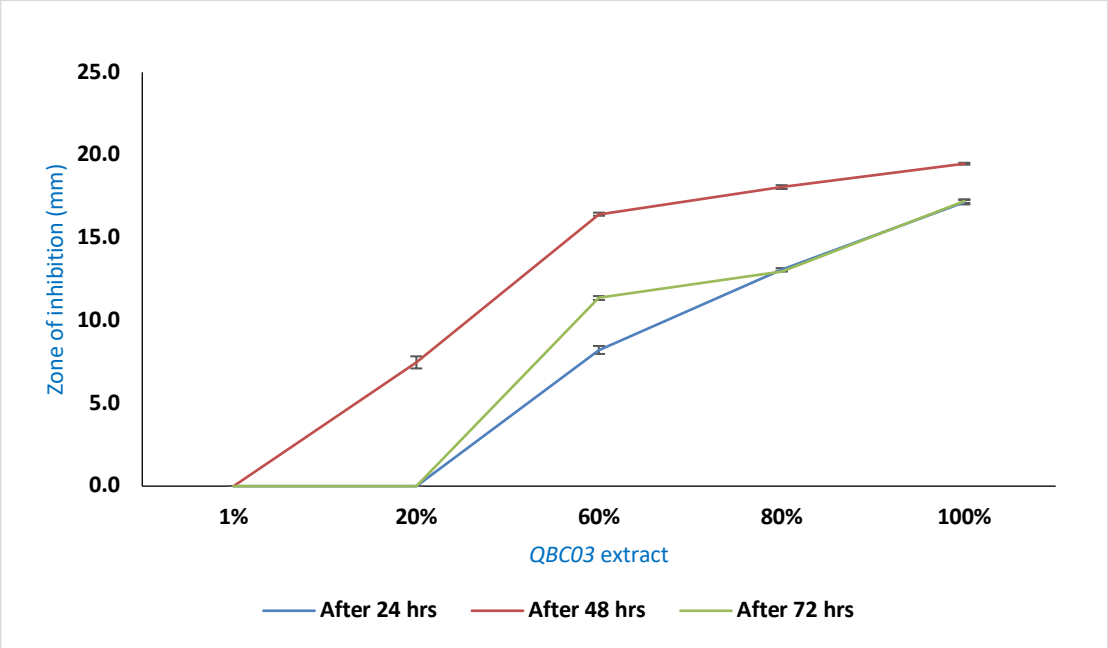


Figure 37: Effect of the antifungal compounds concentration on the growth of *P. verrucosum TF11*.

[The diameters of the zones of inhibition for *P. verrucosum TF11* by the antifungal compounds of 24, 48 and 72 h cultures of *QBC03* in well-diffusion method.]

7.3.2 Evaluation of *QBC03*'s antifungal compounds through the incorporation of the supernatant with PDA

The antifungal activity of 48 h *QBC03* culture extract was evaluated by incorporating different volumes of the extract in molten PDA. The supernatant of *QBC03* was collected by centrifuging the cultures at 5500g/20 min. Hereafter, the supernatant was added in different percentages to molten PDA amended with 100 µg/L of chloramphenicol. The media was poured in equal volumes in the Petri dishes (20 mL) and 3 µL of the fungal spores' suspension (10^6/mL) was loaded in the center of the PDA plate. The plates were incubated at 26ºC for five days and the diameters of the fungal growth on the media were measured at day 2nd and 5th day of incubation. The inhibition ratio for the radial growth diameters was calculated by referring to the control that didn't have bacterial extract (NBY broth instead).

7.3.2.1 Effect of increasing the volume of QBC03's supernatant on the inhibition of A. carbonarius growth

The antifungal activity of *QBC03's* culture extract was evaluated by inoculated spores of *A. carbonarius* on the PDA incorporated with the bacterial extract. PDA plates containing different concentrations (2.5%, 3.5%, 4.5%, 5.5%, 6.5%, 7.5%, 8.5%, 9.5% and 10.5%) of *QBC03's* culture extract were used to study their effect of the growth of *A. carbonarius*. The diameters were recorded on 2nd and 5th day post the incubation. On the second day of incubation, the fungal growth was gradually decreasing on the PDA having the following percentages: 2.5%, 3.5%, 4.5% and 6.5%. The 100% inhibition of the fungal spores' germination was observed from the extract percentages of 7.5% to 10.5%.

On the fifth day of incubation, the diameters of the fungal colonies for the concentrations 2.5%, 3.5%, 4.5% and 6.5%, were increasing in a slower rate compared to that on the control. However, starting from 7.5% of the bacterial extract, the fungal

spores didn't germinate at all, hence the 100% inhibition was achieved at 7.5%, 8.5%, 9.5% and 10.5%. In figure 38, the fungal colony growing on the 2.5% treatment was significantly different from the control, and the colonies margins appear white having the spores concentrated in the center. The colonies grow on the plates amended with 6.5% extract, they had few spores germinating in the center, but their radial diameter was slightly increasing with the times.

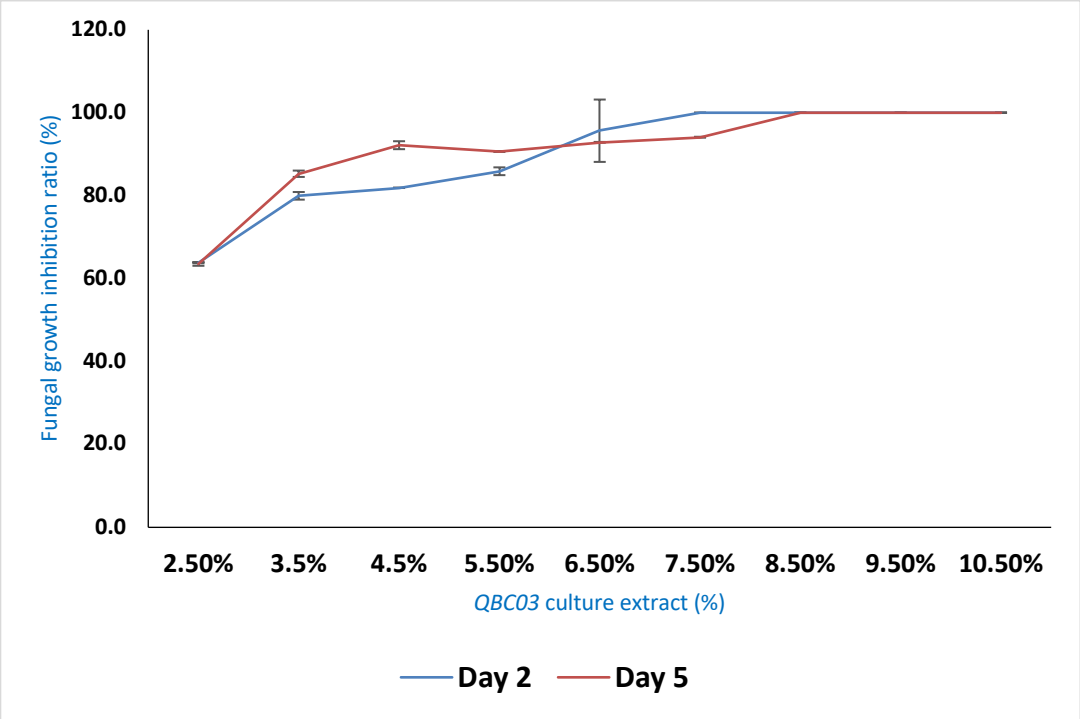


Figure 38: The inhibition of the radial growth of *A. carbonarius AW82* on PDA by *QBC03*'s antifungal compounds.

On the 1st day post incubation, the fungal spores of *A. carbonarius* inoculated on PDA of the treatment 2.5% and 7.5% were observed under the light microscope at 40X and they were compared to spores of the control (Figure 39). The spores in the inoculum of the control have germinated finely and the long, thin ramified mycelia can

be seen protruding from the margin of the colony. In the PDA having 2.5% extract, the fungal spores were seen swollen and the germination tube was seen some of the spores, but not in all of the spores. The spores inoculated on the 7.5% PDA with extract, the spores have remained the way are, and the germination was completely hindered in all the spores, and it remained in the same way even after weeks from the incubation.

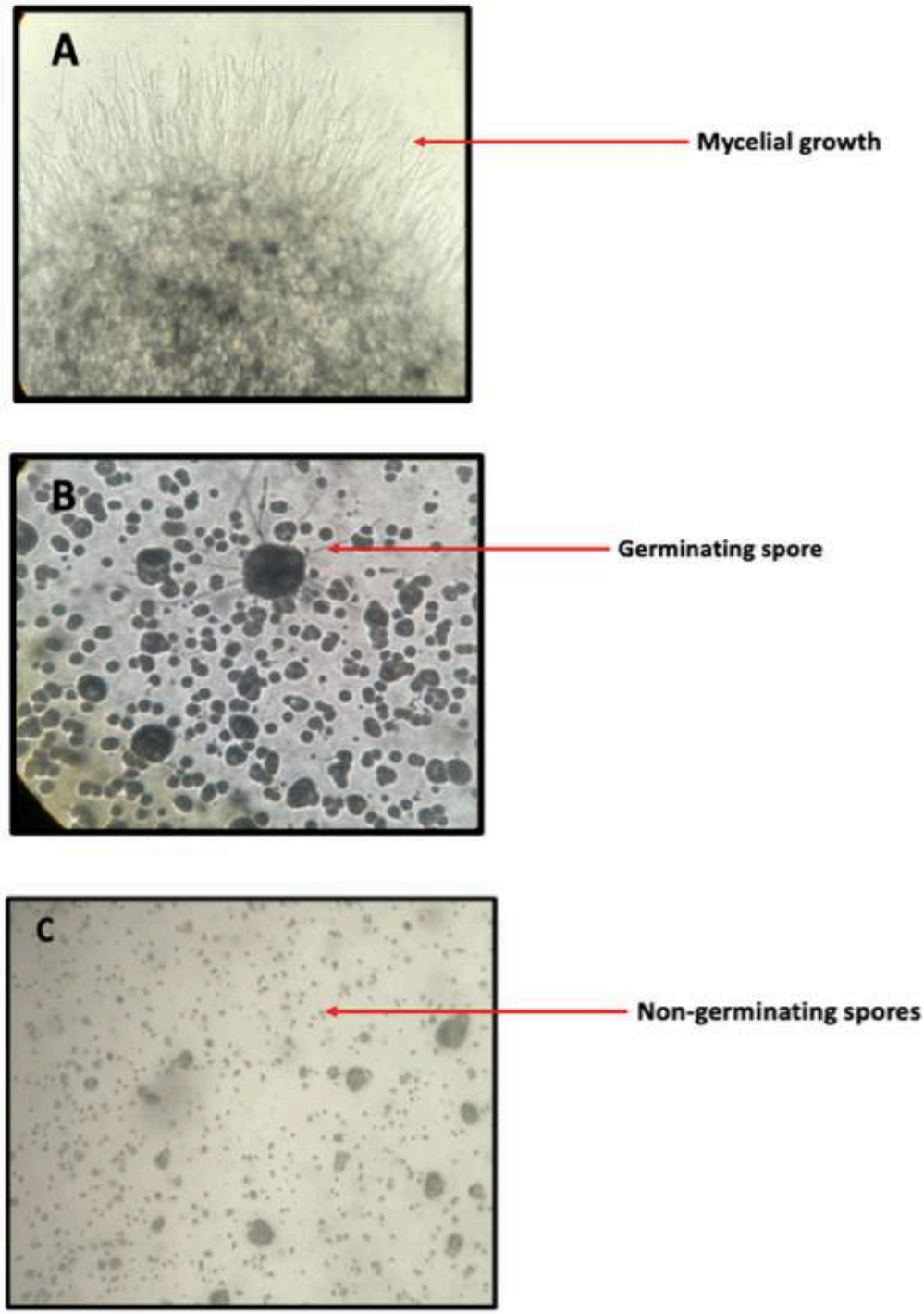


Figure 39: Effect of the antifungal compounds of *QBC03* on the spores germination of *A. carbonarius AW82* inoculated on PDA.

[A: control (only PDA); B: PDA having 2.5% of *QBC03's* extract; C: PDA having 7.5% of *QBC03's* extract]

7.3.2.2 Effect of increasing the volume of QBC03's supernatant on the inhibition of F. culmorum growth

The extract of *QBC03* was also used to study its antifungal effect on *F. culmorum*. The spores were inoculated on PDA medium that was amended with the extract in different concentrations ranging from 2.5% to 15.5%. The plates were incubated at 26ºC and diameters were taken on the 2nd and 5th day post to incubation. On the 2nd day post to incubation, the decrease of the diameters was significantly noticed decreasing even in the colonies growing on the PDA having the lowest extract concentration (2.5%). In figure 40, the fungal growth on the plates having extract percentages of 2.5% to 10.5% was generally decreasing with increasing extract concentration. The fungal colony diameter has decreased to more than half its size in the control (56%) starting at 2.5% of the extract. It was observed that on the PDA that had extract percentage of 11.5%, 12.5%, 13.5%, the fungus spores were almost about to germinate but the process was hindered, therefore some white prints appeared exactly on the same place where the spores were loaded, but they never continued to germinate and there was no mycelia growing out of the colony's circumference, therefore, the percentages of 11.5%, 12.5%, 13.5%, 14.5% and 15.5% corresponded to 100% inhibition.

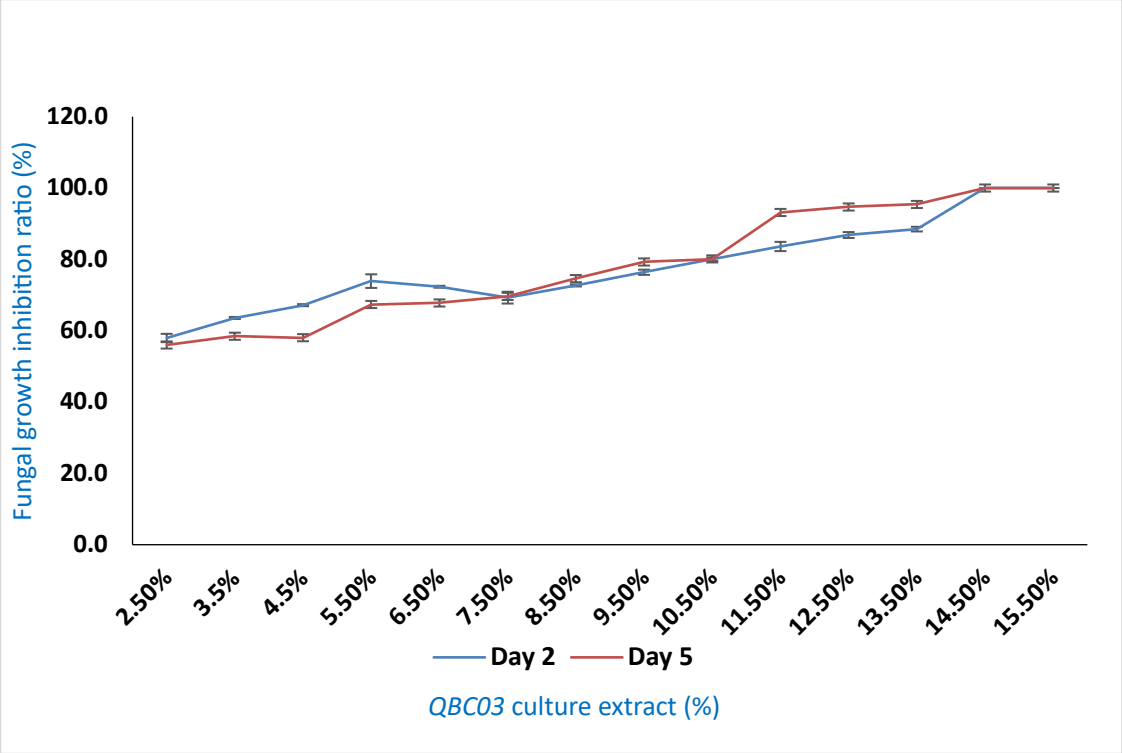


Figure 40: Inhibition of the radial growth of *F. culmorum* on PDA by *QBC03*'s antifungal compounds

On the 5th day post to the incubation, the fungal growth was gradually decreasing with increasing the concentration of the extract, and 58% inhibition ratio was obtained at the lowest extract concentration (2.5%). The fungus inoculated on the PDA having extract from 2.5% to 10.5% was growing in a slow rate, and the fungal colonies never grew further on the PDA having 11.5%, 12.5%, 13.5%, 14.5% and 15.5% of extract, hence the 100% inhibition of *F. culmorum* was achieved at 11.5% of the extract. Even after keeping the plates incubated for a month, the fungal colonies never grew on the previously mentioned percentages. Figure 41 shows the inhibition zones obtained on the 5th day of incubation.

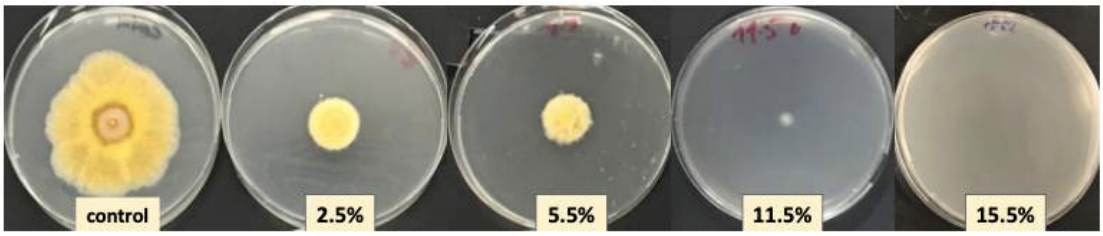


Figure 41: Reduction of *F. culmorum* growth on PDA containing different concentrations of *QBC03*'s antifungal compoundsranging from 0 % to 15.5%

7.3.2.3 Effect of increasing the volume of QBC03's supernatant on the inhibition of P. verrucosum growth

The effect of *QBC03's* extract was studied against *P. verrucosum*. The extract was added to PDA medium in different concentrations (2.5%, 3.5%, 4.5%, 5.5%, 6.5%, 7.5%, 8.5%, 9.5% and 10.5%), and the 3 µL inoculum was loaded on the surface of these PDA plates. The inhibition ratios were calculated by referring to the control and were plotted in figure 42. On the second day post-incubation, the fungal growth was observed only on the medium plates having 2.5% of the bacterial extract, and there was 28.5% inhibition in the growth of the fungus for the 2.5% extract. The fungal spores were not germinating at the 2nd day on the plates having extract concentration from 3.5% to 10.5%. On the 5th day post incubation, the decreased fungal growth was seen only on the PDA having the percentages of 2.5% to 5.5%. The 100% inhibition was witnessed on the PDA having 6.5%, 7.5%, 8.5%, 9.5% and 10.5% extract, where the fungal spores didn't germinate at all (figure 43).

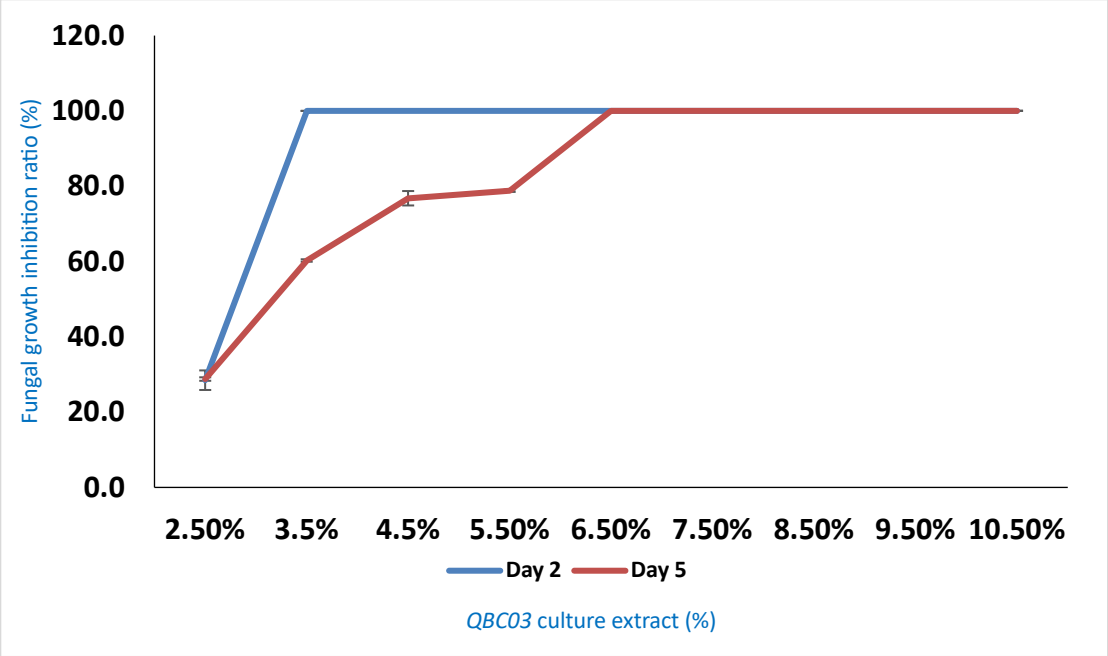


Figure 42: Inhibition of the radial growth of *P. verrucosum TF11* on PDA by *QBC03*'s antifungal compounds

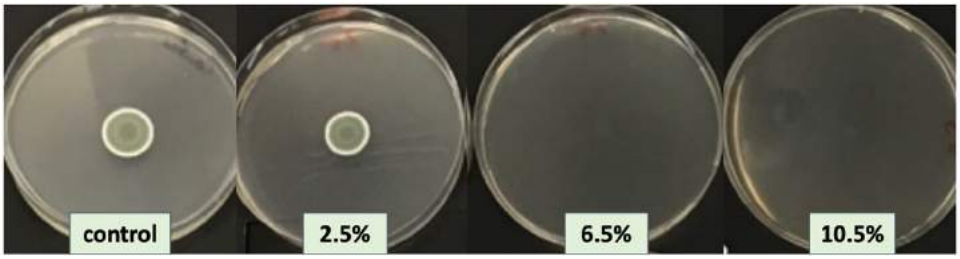


Figure 43: Reduction of *P. verrucosum TF11* growth on PDA containing different concentrations of *QBC03*'s antifungal compounds ranging from 0 % to 10.5%

After a microscopic observation of the fungal inoculum on the PDA medium containing the extract, the spores' germination was compared in the control and in the PDA amended with the extract. Figure 44 shows the difference in the spores' shape of the control and PDA having 2.5% and 6.5% bacterial extract.

The spores in the control have started to germinate at day one post to incubation, and the mycelia ware already growing appearing tall and thin. The spores which were inoculated on the PDA that had 2.5% bacterial extract were starting to germinate, but the mycelia that were growing were shorter than those in the control. However, these mycelia showed more fragmentation hence appeared shorter than those grown in the control. There was no obvious germination for the spores for the treatment with 6.5% and above when observed under the microscope, and no mycelia were shown in any of these treatments.

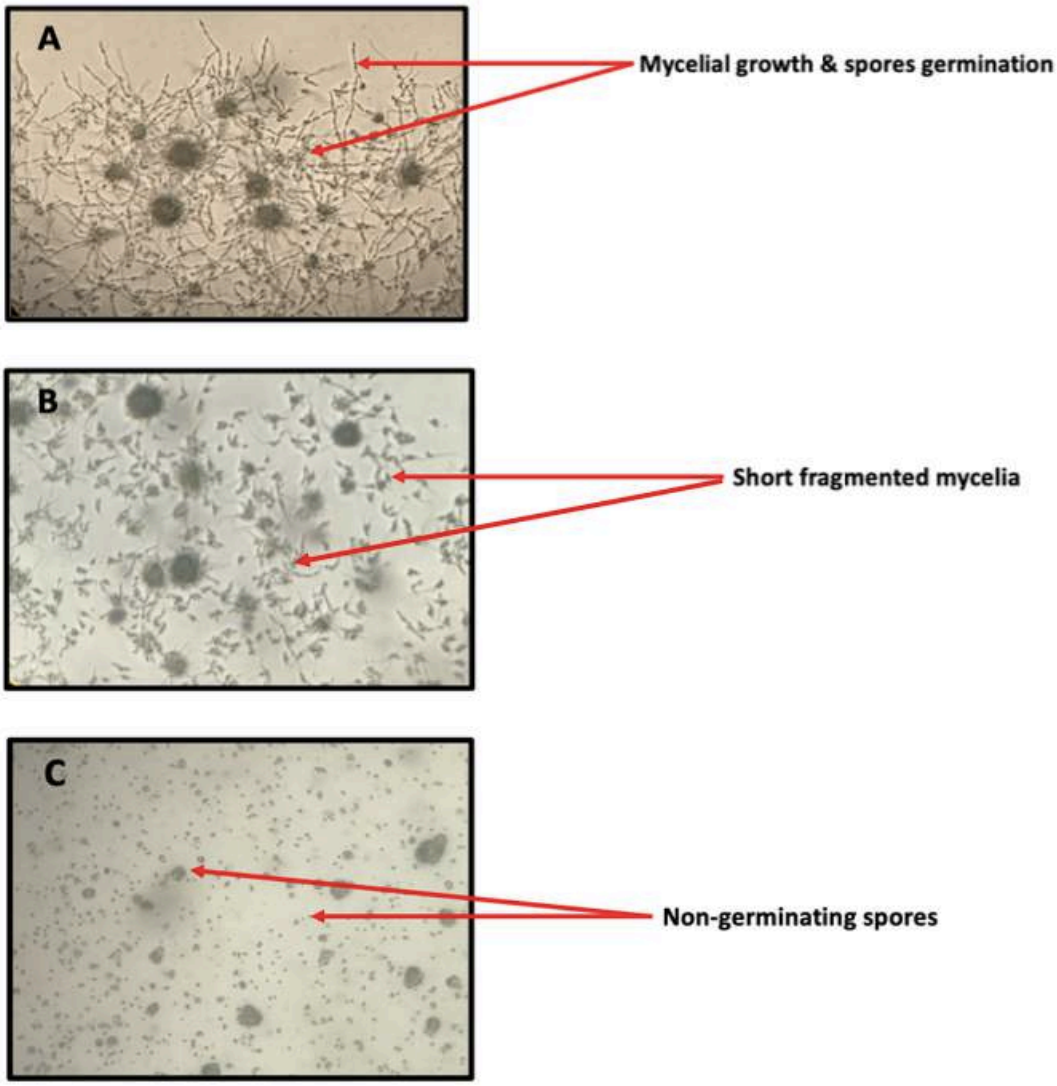


Figure 44: The effect of the antifungal compounds of *QBC03* on the spores germination of *P. verrucosum TF11* on PDA.

[A: control (only PDA); B: PDA having 2.5% of *QBC03's* extract; C: PDA having 6.5% of *QBC03's* extract]

7.4 Evaluation of *QBC03*'s antifungal compounds effect on the mycelial biomass of mycotoxigenic fungal strains in PDB

In this part of the study, the effect of *QBC03's* antifungal compounds on the fungal growth was investigated by the measurement of the biomass of the fungal cells exposed

to different concentrations of *QBC03* extract. The activity of *QBC03's* antifungal extract was tested against two fungal species, *A. carbonarius* and *P. verrucosum*. The fungal biomass that grew in the flasks was measured and was compared to the fungal biomass of the control which was not treated with anything (only PDB).

7.4.1 Evaluation of *QBC03's* antifungal compounds effect on the mycelial biomass and OTA synthesis of *A. carbonarius*

The antifungal activity of 48 h culture extract of *QBC03* was evaluated for its antifungal activity against *A. carbonarius* in 20 mL PDB. 10 µL from a 10^6 spore/mL *A. carbonarius* spores' suspension were seeded in flasks having different concentrations of *QBC03* extract (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% and 100%). After 72 h, the morphology of the treated mycelia was observed by light microscopy and their morphology was compared to those in the control. Then, the fungal biomass was estimated by measuring the dry weight of the filtered mycelia. In addition to the biomass determination, the synthesis of OTA by the treated and non-treated fungal biomass of *A. carbonarius* was determined. The concentration of OTA in the extract treatments was compared to its concentration in the control.

After 72 h of incubation of the spores with the filtrate, there was an obvious decrease in the biomass of the fungus among the treatments while increasing the percentage of the extract in PDB. In figure 45, the fungal biomass dramatically decreased into half when only 1% of the extract was used, where the biomass measured in the control was 60.45 g and 30.43 g measured in 1% treatment. The fungal biomass continued to decrease where it reached 14.17 g when the extract percentage was increased to 2%. Even lower biomass was measured for the fungus in the presence of 3% of the extract in PDB, and the fungal biomass reached to 1.83 g. The fungal growth had started to become unseen starting from 3% of the extract and above, therefore, the point of 3% extract can be considered as the minimum inhibitory concentration for *A. carbonarius*.

The spores weren't able to germinate and grow in the 100% extract at all.

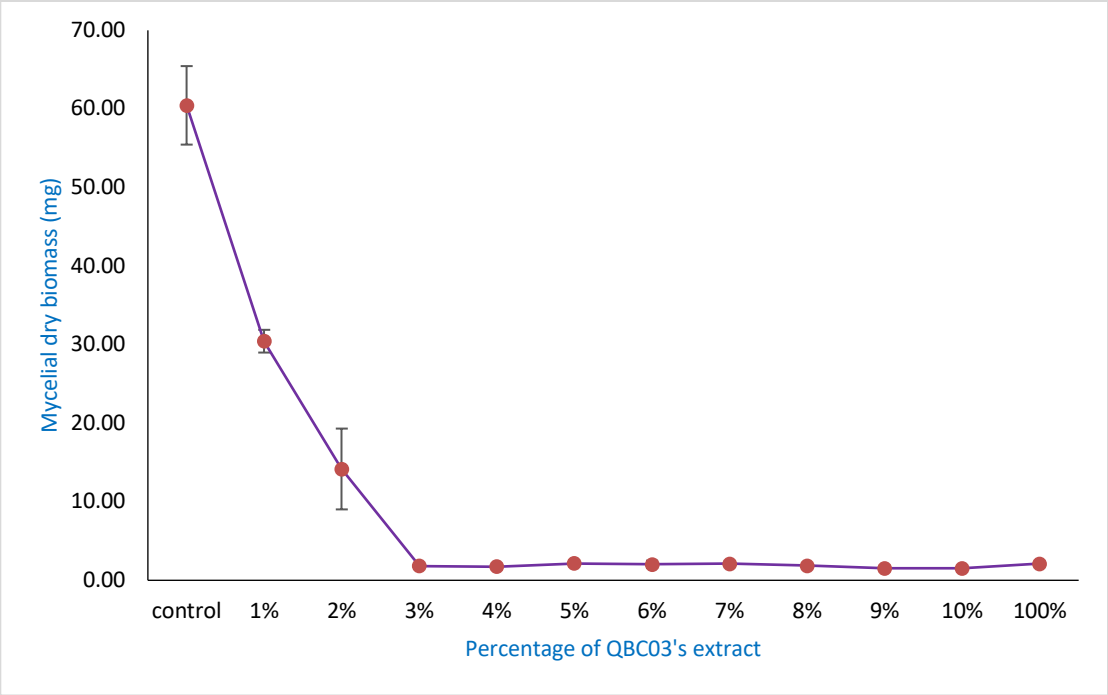


Figure 45: Effect of *QBC03*'s antifungal compounds on the biomass of *A. carbonarius AW82.*

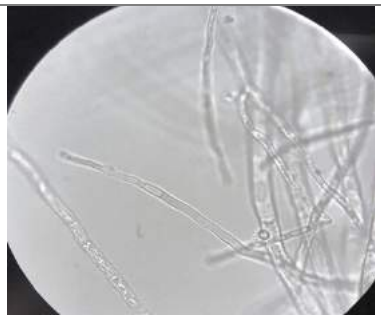
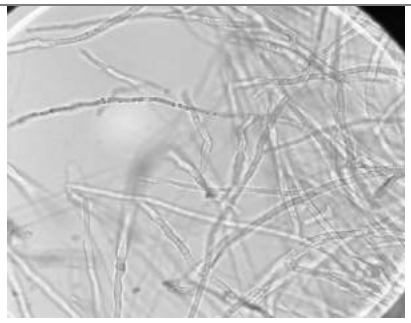
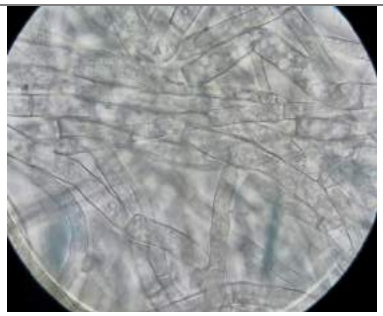
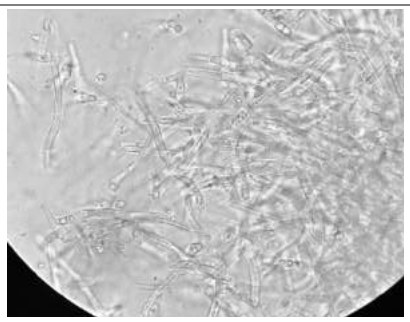
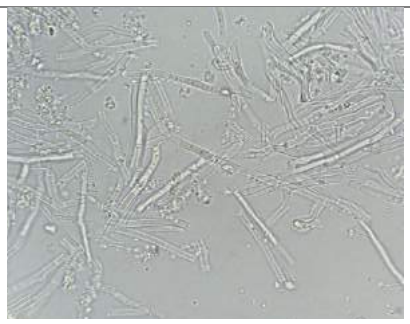
[The dry weight of the mycelium of *A. carbonarius AW82* was measured for different concentrations of *QBC03*'s antifungal compounds in PDB.]

From the treatment of 1% and 2%, the effect of the extract on the morphology of the mycelia was visualized under the microscope. Table 6 demonstrates the effect of the extract on the mycelia that were treated with 1% and 2% compared to the mycelia of the control (0% extract). In the control where the mycelia were not treated with the extract, the mycelia appeared thin and long, compared to the treated one. With increasing the percentage of the extract, the thickness of the mycelia was increasing, and the formation of bulbous structures protruding from the mycelia was also evident.

Moreover, the mycelia treated with the extract was shown to have branched, while that was not seen in the mycelia of the control that appeared longer and unbranched.

Table 6: Effect of *QBC03*'s antifungal compounds on the morphology of the mycelium of *A. carbonarius AW82* and *P. verrucosum.*

[The treatment was done with 1% and 2% of *QBC03*'s antifungal compounds and compared to the non-treated mycelia.]

	A. carbonarius	***P. verrucosum***
Control		
1% extract		
2% extract		

The concentration of OTA produced by *A. carbonarius* was determined by ELISA, and the concentration of OTA was estimated in the control and all treatments. Figure 46 represent the concentrations of OTA synthesized by the biomass of *A. carbonarius* in the control and those treated with different concentrations of the extract. In the control, the concentration of OTA was higher than the detectable limit concentration of ELISA (> 36 ng/20 mL), and when 1% of the extract was added, the concentration of OTA has decreased a little bit to become 34.51 ng/20 ml. At 2% of the extract, OTA concentration was above the detectable limit, however, in the treatment of only 3% of the extract, there was an apparent sharp decline in the concentration of OTA detected. The trend was similar for the rest of the percentages (4% to 10%), where OTA synthesis was greatly reduced compared to the control, and OTA was not detected at all in the 100% extract treatment.

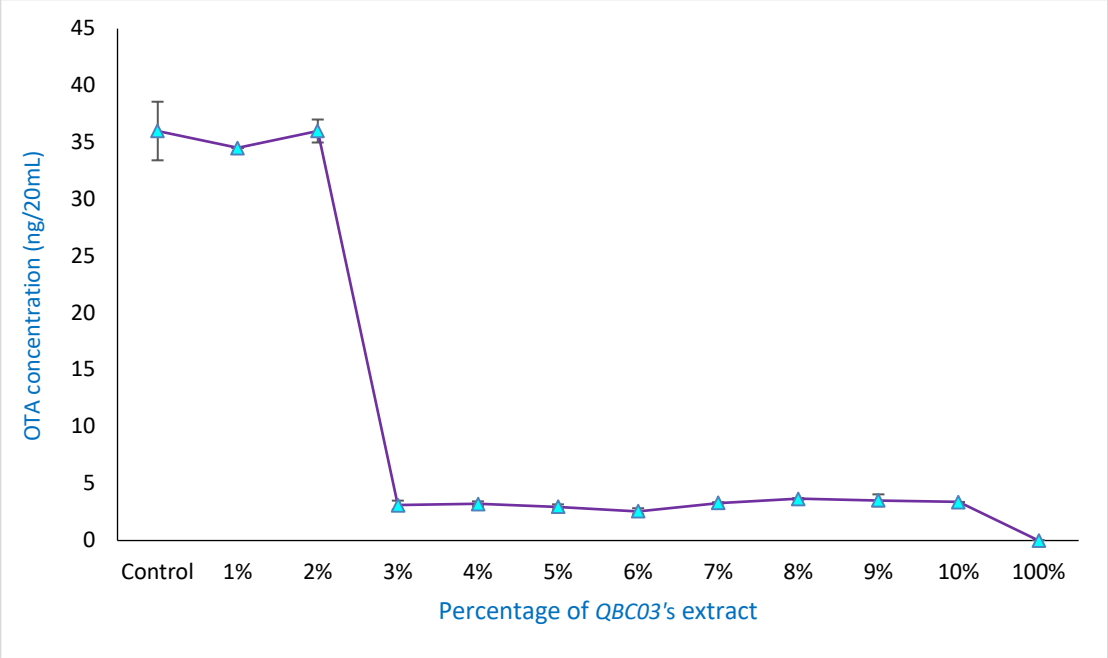


Figure 46: Effect of *QBC03*'s antifungal compounds on OTA synthesis by *A. carbonarius AW82* upon treatment with different concentrations of *QBC03*'s antifungal compounds in PDB.

7.4.2 Evaluation of *QBC03's* antifungal compounds effect on the mycelial biomass of *P. verrucosum.*

The effect of the antifungal compounds produced by *QBC03* was studied on the growth of *P. verrucosum*. The extract of a 48 h *QBC03* culture was collected. When different concentrations of the extract were used, the fungal biomass growth was different than the control, and there was a general decreasing trend in the biomass of *P. verrucosum*. As shown in figure 47, the incorporation of 1% and 2% of the bacterial extract in the 20 mL PDB didn't result in a big difference in the inhibition of the fungal biomass, and the measured dry weight of the fungal biomass was almost similar to the dry weight of the control. Hereafter, increasing the volume of the extract to 3% resulted in a great decrease of the fungal biomass to 1.4 mg, compared to the control biomass

weight which was 65.37 mg. However, when the concentration of the antifungal compounds was increased to only 3%, the dry mycelial biomass was reduced to half of the dry weight of the mycelia measured in the control, and the weight was 35.9 mg compared to the control which had 65.3 mg. Increasing the concentration of the extract further to 4% resulted in even more decrease in the dry biomass weight where it reached 1.4 mg. The fungal cells were not seen in the further percentages after 4%, hence, 4% of the extract can be considered as the minimal inhibitory concentration. When the weight of the dry fungal mycelia was measured in the rest of the concentrations, the weight was reduced to more than half of the mycelial weight in the control.

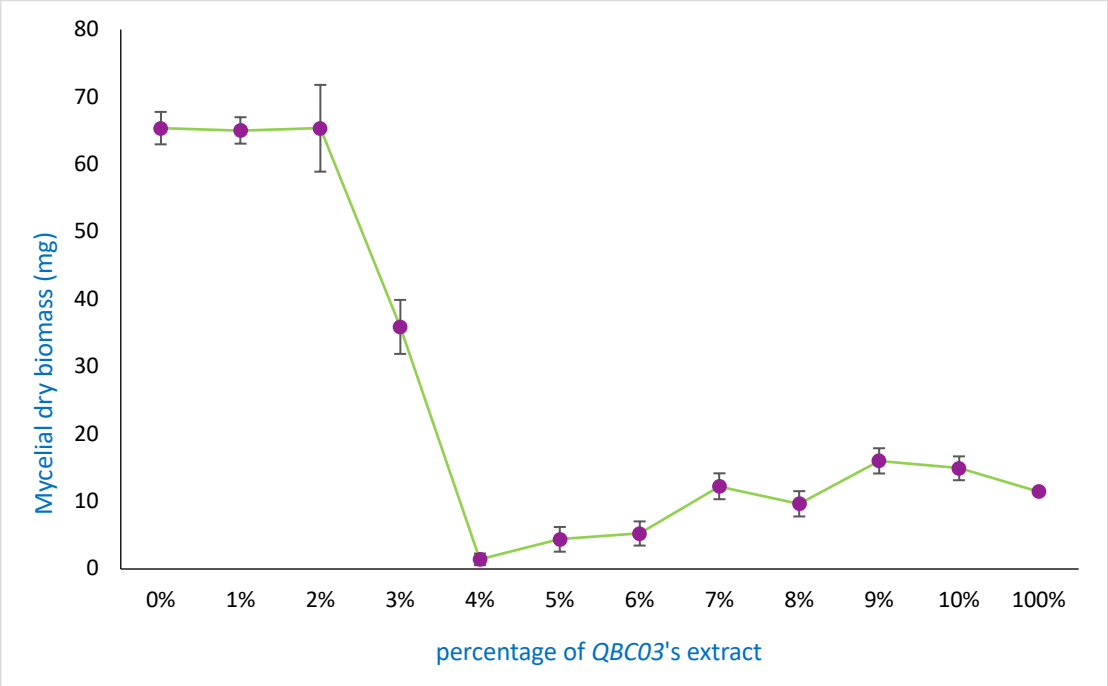


Figure 47: Effect of *QBC03*'s antifungal compounds on the biomass of *P. verrucosum.* **[**The dry weight of the mycelium of *P. verrucosum* was measured for different concentrations of *QBC03*'s antifungal compounds in PDB.]

7.5 Effect of the temperature on the stability of *QBC03*'s antifungal compounds

In this study, the effect of different temperature treatments on the antifungal compounds of *QBC03* was explored in well-diffusion method. The extract of *QBC03* was incubated at different temperatures for 30 min. The extract was then poured into wells drilled in PDA media amended with 100 µg/L of chloramphenicol. The plates were incubated at 26ºC and the diameters of the inhibition zones around the wells were measured after 3 days post to incubation.

7.5.1 Effect of the temperature on *QBC03*'s antifungal compounds activity against *A. carbonarius* growth

The effect of different heat treatments on the activity of the antifungal compounds was studied in this part. The antifungal compounds with different heat treatment were tested against *A. carbonarius*. The extracts were incubated at -80º, -20º, 4º, 26º, 30º, 40º, 60º, 80º and 100ºC for 30 min. 200 µL of *A. carbonarius* spores' suspension (10^6/mL) were spread on PDA having 100 µg/L chloramphenicol. Wells were drilled in PDA and 100 µL of treated extract were loaded in wells in PDA. The plates were later incubated at 26ºC for 72 h and the inhibition zones for the treatments were measured and compared to the inhibition zone around the well which was loaded with *QBC03* supernatant harvested from the culture that was not exposed to any heat treatment. Figure 48 shows different inhibition zones in PDA around the wells as a result of different heat treatment of *QBC03's* antifungal compounds.

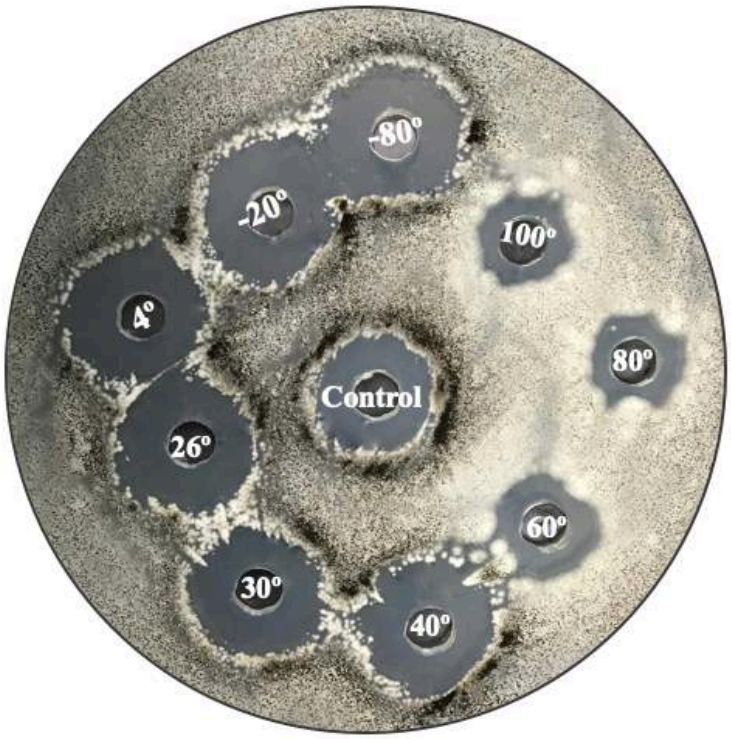


Figure 48: Effect of the temperature on *QBC03*'s antifungal compounds activity against *A. carbonarius AW82.*

[Inhibition zones of *A. carbonarius AW82* by the antifungal compounds of *QBC03* treated with different temperatures (-80º, -20º, -4º, 26º, 30º, 40º, 60º, 80º and 100ºC).]

Duncan test was used to compare the effect of applying different temperatures on the extract and it was found that (Figure 49), when the extract was treated with low temperatures such as -80º, -20º and 4ºC, its activity remained close to that of the control (no heat treatment was applied), and the zones of inhibition were 22.5, 22.1 and 21.8 mm, respectively, also, they weren't significantly different from each other. Increasing the temperature of the extract to 26ºC has shown the best inhibition activity that corresponded to the largest zone of inhibition (24.8 mm), which was significantly different from all other treatments and the control.

However, increasing the temperatures more than 26ºC resulted in a gradual

decrease in the antifungal compounds activity and the zones of inhibition were getting smaller. The extract's antifungal activity has significantly decreased when it was treated with 100ºC, thus, it had the smallest inhibition zone appeared around its wells (15.1 mm) which was significantly lower than any other diameter of the other treatments.

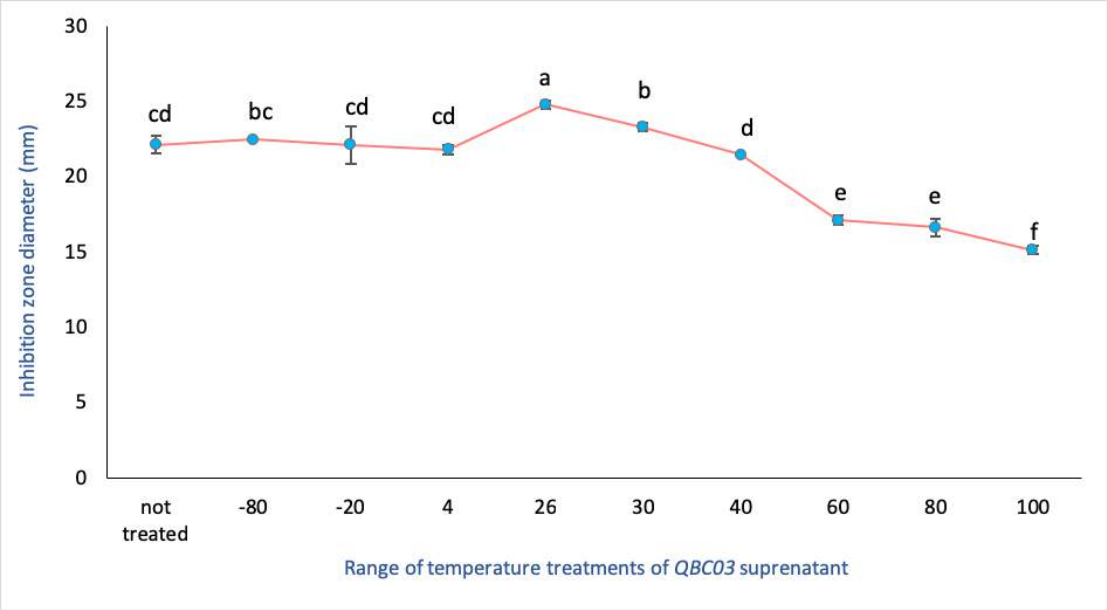


Figure 49: Effect of the temperature on *QBC03*'s antifungal compounds activities against *A. carbonarius AW82*

[The inhibition zone diameters of *A. carbonarius AW82* by heat treated *QBC03's* antifungal compounds were measured. Duncan test was done to compare the average of the inhibtion zones, and those sharing the same letter are not significantly different from each other.]

7.5.2 Effect of the temperature on *QBC03's* antifungal compounds activity against *F. culmorum* growth

The antifungal compounds with different heat treatments were tested against *F.*

culmorum. The extracts were incubated at -80º, -20º, 4º, 26º, 30º, 40º, 60º, 80º and 100ºC for 30 min. 200 µL of *F. culmorum* spores' suspension (10^6/mL) were spread on PDA having 100 µg/L chloramphenicol. Spores' suspension of *F. culmorum* was prepared (10^6/mL) and 200 µL were spread on PDA, and wells were then created in the plates using a cork borer. 100 µL of the antifungal extract incubated at different temperatures were loaded into the wells, and the inhibition zones around these wells were measured and compared to the zone around the well having the antifungal compounds extract that harvested from the centrifuged culture (control). After 3 days incubation of plates at 26ºC, the inhibition zones were very clear around the wells as shown in figure 50.

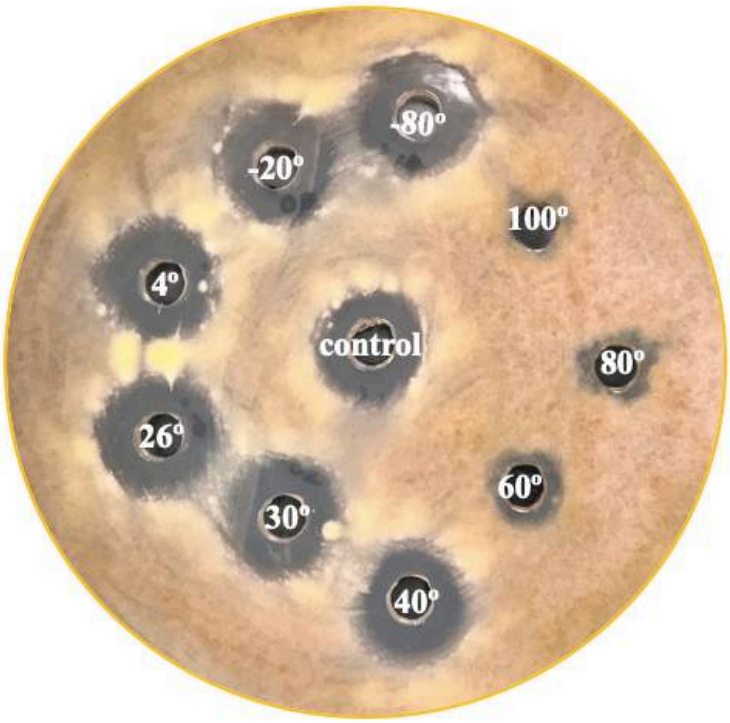


Figure 50: Effect of the temperature on *QBC03*'s antifungal compounds activities against *F. culmorum.*

[The inhibition zones of *F. culmorum* by the heat treated antifungal compounds of *QBC03* were measured . Temperatures were -80º, -20º, -4º, 26º, 30º, 40º, 60º, 80º and 100ºC.]

As a result of different heat treatment of *QBC03's* antifungal compounds, the activity of this extract was influenced, where some heat treatments led to enhancement of the activity, and some other treatment decreased the efficiency of the extract (Figure 51). Duncan test was done to compare the diameters of the inhibition zones resulted from the different temperature treatments. When the extract was treated with low temperatures, its activity was affected in that it was gradually decreasing. Therefore, treatments at -80º, -20º and 4ºC of the extract showed diameters which were significantly different from each other, and diameters recorded for them were 17, 18

and 20 mm, respectively. Increasing the temperatures from 26ºC to 30ºC resulted in the increase of the extract activity where it was at its highest at 30ºC, and the diameters for both treatments were 21 and 21.6 mm. Increasing the temperatures further, starting from 40ºC to 100ºC has caused a gradual decline in the activity of the extract, and the lowest activity was at 100ºC making an inhibition zone of 9.8 mm.

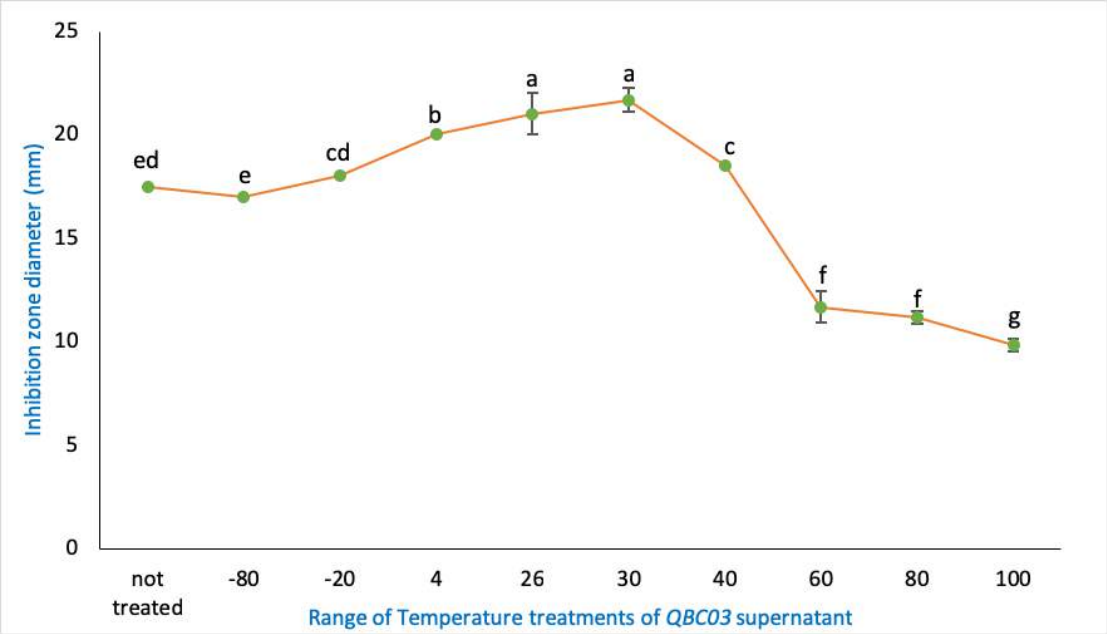


Figure 51: Effect of the temperature on *QBC03*'s antifungal compounds activities against *F. culmorum*

[The inhibition zone diameters of *F. culmorum* by heat treated *QBC03's* antifungal compounds were measured. Duncan test was done to compare the average of the inhibtion zones, and those sharing the same letter are not significantly different from each other.]

7.5.3 Effect of the temperature on *QBC03*'s antifungal compounds activity against *P. verrucosum TF11*growth

The antifungal compounds with different heat treatments were tested against *P. verrucosum TF11*. The extracts were incubated at -80º, -20º, 4º, 26º, 30º, 40º, 60º, 80º and 100ºC for 30 min. 200 µL of *P. verrucosum TF11* spores' suspension (10^6/mL) were spread on PDA having 100 µg/L chloramphenicol. Wells were created in PDA using a cork borer, and they were filled with 100 µL of the heat-treated extract. The inhibition zones around the wells were measured and compared to the zone of the extract which wasn't heat-treated (control). Figure 52 shows the different inhibition zones around the wells which were recorded and compared.

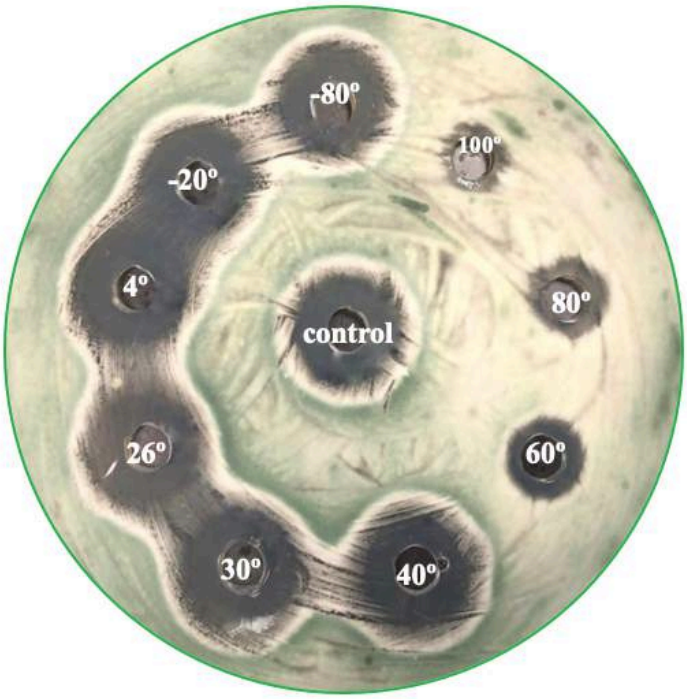


Figure 52: Effect of the temperature on *QBC03*'s antifungal compounds activities against *P. verrucosum.*

[The inhibition zones of *P. verrucosum* by the heat treated antifungal compounds of *QBC03* were measured. Temperatures were -80º, -20º, -4º, 26º, 30º, 40º, 60º, 80º and 100ºC.]

Duncan test was done to compare the diameters for the zones of inhibition resulting from the different temperature treatments of the extract (Figure 53). The antifungal compounds of *QBC03* exposed to low temperatures such as -80º, -20º and 4ºC stayed as efficient as control in inhibiting the growth of *P. verrucosum* around the wells, and the diameters recorded for them were 26.3, 26.7 and 26 mm, respectively, and the control had a zone of 25.6 mm. However, increasing the temperatures furthermore resulted in increasing the activity of the antifungal compounds were the highest activity was achieved at 30ºC and it has created an inhibition zone of 28.1 mm, which was significantly higher those of the rest of the treatments. However, with

increasing the temperatures starting from 40ºC and above, the extract's antifungal activity started to gradually decrease reaching its lowest at 100ºC, at which the lowest inhibition zone was 14.5mm. Furthermore, the diameters of the inhibition zones of 60º, 80º and 100ºC were not significantly different from each other.

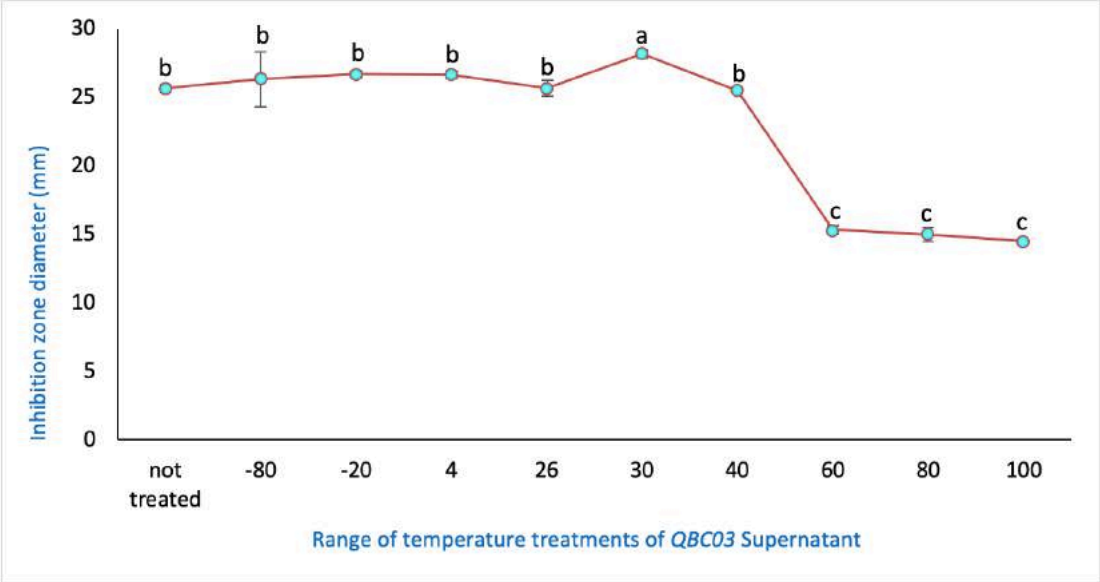


Figure 53: Effect of the temperature on *QBC03*'s antifungal compounds activities against *P. verrucosum.*

[The inhibition zones of *P. verrucosum* by the heat treated antifungal compounds of *QBC03* were measured. Temperatures were -80º, -20º, -4º, 26º, 30º, 40º, 60º, 80º and 100ºC. Duncan test was done to compare the average of the inhibtion zones, and those sharing the same letter are not significantly different from each other.]

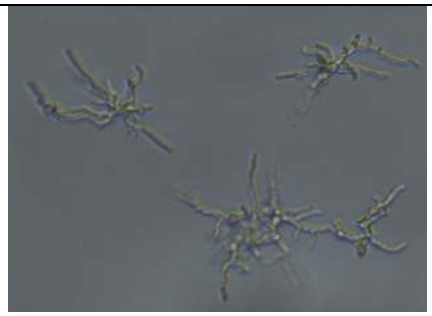
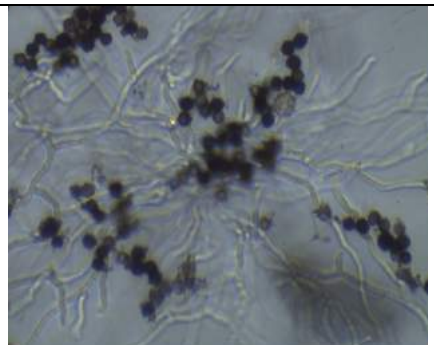
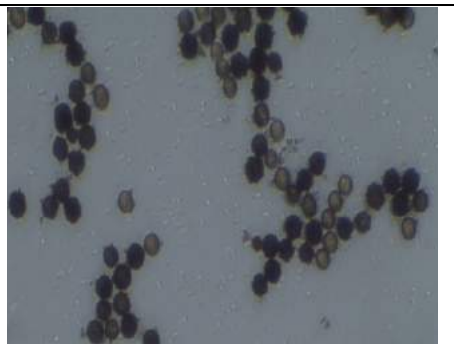
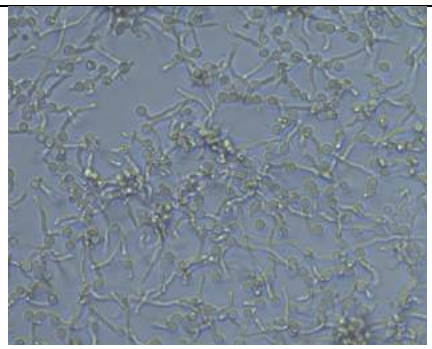
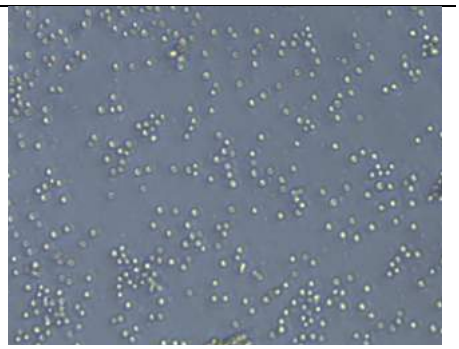
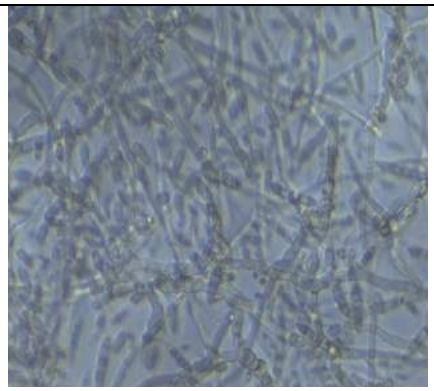
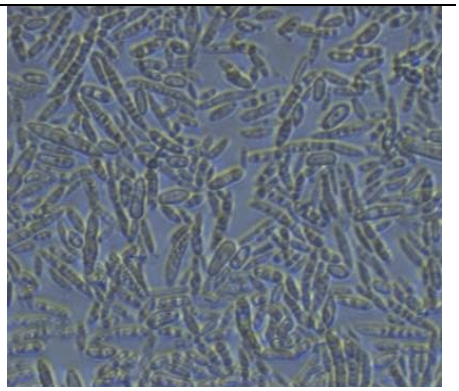
7.6 Effect of *QBC03*'s culture extract on the germination of fungal spores

In this part of the work, we studied the effect of the antifungal compounds of *QBC03's* on the germination of fungal spores of four fungal species, *P. verrucosum A.*

carbonarius AW82, A. westerdijikae and *F. oxysporum* by culturing the spores in the presence of *QBC03's* extract. The microscopic images of the treatment vs. the control for each fungal species were summed in table 7. There were significant differences between the spores that were treated with the extract and the spores of the control. The effect of the antifungal compounds produced by *QBC03's* in the 48 h culture had significantly affected the spores' germination and growth of the four studied fungal specie. There was a 100% inhibition in the spores' germination of *P. verrucosum A. carbonarius, A. westerdijikae* and *F. oxysporum* after the treatment with the 48 h bacterial extract, where the spores couldn't germinate in any of the treated wells of the three replicates. Unlike the treated fungal spores, in the control where the spores were not treated with the extract, all the spores finally germinated, and the germination was seen protruding from their conidia.

In the treated spores for *P. verrucosum A. carbonarius* and *A. westerdijikae,* the spores couldn't germinate, and they were characterized by a spherical shape. In contrast to the untreated conidia of *P. verrucosum A. carbonarius* and *A. westerdijikae*, the germination tube was clearly seen emerging from the conidia for the four species and was forming small extensions. For *F. oxysporum*, the treated conidia appeared swollen upon the treatment of the bacterial extract and looked short and stumpy compared to the morphology of the non-treated conidia that appeared thin and long. Nevertheless, the spores were kept in the incubator for longer time (3 weeks) to check if the spores can germinate with the time, but the result was that no conidial germination was noticed in the treated spore.

Table 7: Effect of *QBC03*'s antifungal compounds on spore germination of *P. verrucosum, A. carbonarius, A. westerdijikae* and *F. oxysporum*

	Control	**Treated**
P. verrucosum		
A. carbonarius		
A. westerdijikae		
F. oxysporum		

Discussion:

In this part, the antifungal activity of our *Qatari Burkholderia cepacia* (*QBC03*) strain was investigated. The effect of *QBC03's* antifungal compounds was screened on several species of three fungal genera (*Aspergillus*, *Fusarium* and *Penicillium*), and their stability was evaluated. The bacterial antifungal spectrum of *QBC03* was studied through overlaying assay of the fungal spores against 20 fungal species (*A. carbonarius, A. westerdijkiae, A. parasiticus, A. niger, A. flavus, A. ochraceous, A. fumigatus, F. anthophilum, F. chlamodosporum, F. culmorum, F. graminearum, F. oxysporum, F. solani, F. subglutinus, F. prolifiratum and F. verticilliod, P. cambeberti, P. digetatum, P. expansum, P. italicum and P. verrucosum*). All *Aspergillus* species were sensitive to *QBC03* compounds. However, *A. carbonarius AW82* was the most sensitive (59.1 mm) and *A. flavus* the less sensitive (33.7 mm). These results demonstrate that the antifungal activity efficiency is species specific.

For *Fusarium*, the results showed that *QBC03's* antifungal compounds inhibited some of the studied *Fusarium* species, where *F. verticilliod* and *F. prolifiratum* showed the smallest inhibition zones (2.6 and 6.7 mm, respectively) compared to *F. chlamodosporum* which had the largest inhibition zone (36.8 mm). The studied *Penicillium* species also showed sensitivity towards the diffusible antifungal compounds of *QBC03*, and all the five species had shown a zone of inhibition around *QBC03*. The most sensitive specie among the *Penicillium* group was *P. camemberti* having a zone of inhibition of 37.3 mm, and the least inhibited species was *P. expansum* having a zone of 34.9 mm. Several bacteria from different genera have the potential to produce cyclic lipopeptides. Lipopeptides have binding potential to the bilayer lipid membrane of the pathogen. These cyclic lipopeptides can be classified into three categories that are, surfactants, iturin or fengycin. However, fengycin and iturin possess antifungal activities against some of the pathogenic fungi, but they don't have any

antibacterial activity. Some of these lipopeptides are produced by *Burkholderia cepacia* such as cepaciamides A & B, cepacidines, siderophores, altericidin, pyrrolntrin, glidobactins and volatile compounds (Haidar *et al.,* 2016). *B. cepacia* diffusible compounds containing ammonia have been reported to inhibit the fungal growth on PDA. In addition to that, it is also known to produce volatiles on PDA and that is why it's capable of controlling the growth of soil pathogens (Rahman *et al*., 2007).

QBC03's production of antifungal compounds in the broth was optimized in NBY broth that was modified in the work of Kilani-Feki & Jaoua (2011), thus, *QBC03's* extract was always prepared from the broth of NBY. The antifungal compounds in the culture of *QBC03* in NBY were evaluated in solid media by applying two methods that were well-diffusion method and incorporation of the extract in molten media (PDA). In both methods, the results showed that increasing the concentrations of the extracts resulted in more inhibition of the three fungi *A. carbonarius*, *F. culmorum and P. verrucosum*.

When the supernatant of *QBC03* was incorporated in PDA, even very low concentrations of the extract accounted for a very significant reduction in the radial growth of the three studied fungal species (*A. carbonarius AW82, F. culmorum* or *P. verrucosum TF11*). The complete inhibition for the fungal growth was at 8.5%, 11.5% and 6.5% for *A. carbonarius AW82, F. culmorum* and *P. verrucosum TF11*, respectively, where beyond these concentrations, the extract has 100% inhibited the germination of the fungal spores on PDA and they never germinated again after that. However, after the second day of incubation, the mycelia of *P. verrucosum TF11* appeared fragmented and shorter than those growing on the control. For *A. carbonarius AW82*, on the second day, the spores had few germination tubes protruding from them and very few mycelia were seen. The antifungal compounds of the bacteria can have different modes of actions in order to disrupt the growth of the fungal cells upon the

treatment with these antifungal compounds. The antifungal compounds can either act on the cell wall of the fungi or inhibit the synthesis of some nucleic acids. Antifungal compounds can be enzymes having the ability to interfere with the DNA, RNA leading to disrupted protein synthesis (Ghannoum & Rice, 1999). However, another mechanism of action of different antifungal peptides is by acting on the cell wall integrity and permeability (Yeaman & Yount, 2003). Rahman *et al*., (2007) studied the effect of the bacterial extract of *B. cepacia* and *P. aeruginosa* strain on the spores' germination of *C. gloeosporioides* in PDA incorporated with the sterile bacterial antifungal extract or cell suspension. The cells suspension and extract of *B. cepacia* managed to completely inhibit the germination of the spores of *C. gloeosporioides* after 24 hr of incubation, while *P. aeruginosa* inhibited 3.7% and 1.31% of the spores' germination in the PDA having the cell suspension or extract, respectively.

San-Lang *et al.,* (2002) studied the effect of incorporating different volumes of the antifungal compounds of *B. subtilis W113* strain in molten PDA on the radial inhibition of *F. oxysporum*. Their results postulated that increasing the concentration of the bacterial fungicides resulted in better inhibition of *F. oxysporum*. Increasing the concentrations of the bacterial extract up to 25% resulted in 50% inhibition in the fungal growth. Compared to our results, incorporating the lowest concentration of *QBC03's* antifungal compounds resulted in more than 50% inhibition (56%) of *F. culmorum* growth on PDA.

The effect of the antifungal compounds on the biomass of the fungal mycelia was explored in this study as well. The 48 h culture extract of *QBC03* was added in different concentrations (1%-10%) to PDB. The fungal spores (10^4) of *A. carbonarius AW82* and *P. verrucosum TF11* were inoculated in the broth and the dry weight of the mycelial biomass was measured. The weight of *A. carbonarius AW82* mycelial biomass was shown to decrease dramatically at only 1% of the extract, where the biomass

decreased to 50%. The biomass continued to decrease further with increasing the concentrations of the extract in the PDB. However, no visible fungal biomass was seen after 3%. The study of the effect of *QBC03* antifungal extract on the synthesis of OTA concentration showed that 3% extract decreased the concentration of OTA drastically, and it became 3.1 ng/20 mL. However, the concentration of OTA remained close to that except for the 100%, where OTA concentration was zero. The effect of the *QBC03* antifungal extract was studied on the biomass of *P. verrucosum TF11*, and it was shown that there was a gradual decrease in the biomass of the fungal mycelia. The antifungal bacterial compounds managed to decrease the fungal biomass of *P. verrucosum TF11* to half when only 3% of the extract was used.

In another experiment which was done at the same conditions (data not shown), where fungal spores were treated with different concentrations of *QBC03's* extract (0.75-10%) in 10 mL PDB in order to study the viability of the fungal cells, 100 µL from each treated fungal cells in 10 mL PDB were transferred and spread on PDA media, and the plates were incubated for two days at 26ºC. The fungal growth was detected in the PDA of the treatments from 0.75% and up to 2.75%. The PDA which was spread with 100 µL of 3% and above, the fungal cells didn't grow on them at all. The same results were obtained when 100 µL were transferred from the 100% *QBC03* extract treatment to PDA, were the fungal cells didn't grow at all. These results can explain the weight which was measured for the treatments beyond 3%, since the fungal cells were not visible but there was still mass that was detected. This weight measured in the extract can be attributed to dead mycelia cells which were trying to grow but their growth was ceased in the presence of the extract. Hence, since an inoculum from each percentage was transferred to PDA and nothing grew, this explains the weight measured was non-viable fungal cells.

The bacterial antifungal compounds are known to cause several damages to

fungal mycelia when they are exposed to them (San-Lang *et al*., 2002). Kilani-Feki *et al*., (2011) studied the potential of *Burkholderia cepacia* strain *Cs5* to inhibit the fungal growth of *F. oxysporum*, *F. culmorum*, *F. graminearum*, in solid media and *R. solani* and *A. niger* in liquid culture. The extract of *Cs5* was increased in the PDB starting from 0.25% to 3 %. The antifungal compounds of *Cs5* were able to cause 50% inhibition in *A. niger* growth by incorporating 0.45% of the extract. The extract of *Cs5* managed to delay sporulation in *A. niger*, and the morphology of the hyphae of the fungus was altered. Some aggregations of bulbous structures were shown on the sides on the hyphae and didn't appear after treatment with 0.6% of the extract. Mycelia fragmentation was also shown upon the treatment of the antifungal compounds of *Cs5* in the liquid culture. Cell wall deterioration was observed in the treated mycelia of the *F. solani* proving that one way of the mechanism of action of the antifungal compounds is changing the permeability of the fungus cell wall. In PDA medium, 0.25% of the extract accounted for 50% inhibition in the growth of *F. graminearum* and *F. solani* on PDA.

Kilani-Feki & Jaoua, (2011) studied the effect of *B. cepacia* strain *Cs5* on the inhibition of *B. cinerea* by adding the bacterial extract in different concentrations (0-2%) in PDB, where a fungal inoculum was added too. They were able to demonstrate the effect of these antifungal compounds on *B. cinerea* by measuring the biomass of the treated mycelia. The incorporation of 0.25% of the extract accounted for more than 60% inhibition, and a 100% inhibition was reached when 1.25% of the extract was used. These results prove that *B. cepacia* has a great antifungal potential corroborating with the results that were obtained in this work. However, the effect of the extract on the fungal mycelial was taken into account for this study, where we observed several changes caused by the extract on the morphology of the mycelia. The extract has obviously caused swellings in the mycelia of *P. verrucosum* and the mycelia appeared

short and fragmented in both treatments, compared to the mycelia of the control which was thin, long and not fragmented. The mycelia of *A. carbonarius AW82* appeared very swelled and the tips of the hyphae appeared swelled too with enlarged ends. The hyphae were short, and many aggregations were seen in 2% extract treated mycelia. These aggregations were larger in width than that of the mycelia and they were characterized with thick walls. These structures are formed by the fungal conidia or hyphae and they play a major role in the survival strategy of the host and they are named as chlamydospores (Oliveira *et al*., 2012). Figure 54 shows the aggregations and morphology of the chlamydospores that appeared as a result of 2% bacterial extract treatment in *A. carbonarius AW82* hyphae. Chlamydospores were also noticed to grow terminally in the tips of *A. carbonarius* hyphae. These structures can survive in the environment, specifically in the soil better than conidia do. However, the presence of some bacteria in the soil can induce the formation of such structures. Li et *al*., (2005) have demonstrated in their studies that the treatment of very low concentration of a bacterial antifungal compound referred to as 10M has induced the formation of the chlamydospores in the conidia of *T. harzianum* in addition to their appearance in the hyphae tips. The findings of Li *et al.,* (2005) match the results obtained in this part in using the antifungal compounds of *QBC03* to inhibit the fungal growth of *A. carbonarius AW82*. The chlamydospores appeared in *A. carbonarius AW82* and were characterized with thick walls. The chlamydospores couldn't appear at treatments above 2% of *QBC03* extract.

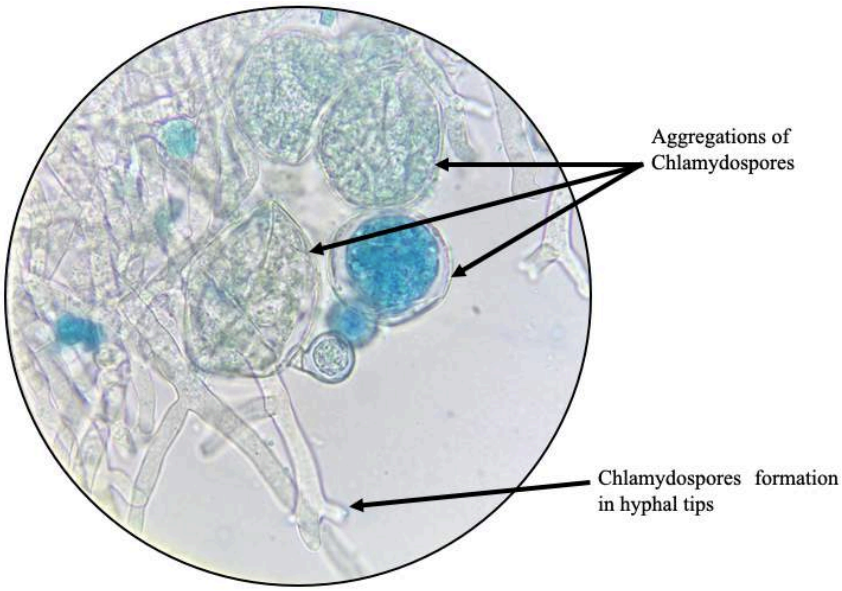


Figure 54: Effect of *QBC03*'s antifungal compounds on the mycelia of *A. carbonarius AW82* treated with 2% of the extract showing formation of chlamydospores.
[Mycelia of *A. carbonarius AW82* treated with 2% were stained with methylene blue and their morphological changes were obsereved under the oil lense (100X)]

Similar to our findings, when San-Lang *et al*., (2002) studied the effect of incorporating different volumes of *B. subtilis* extract in PDB inoculated with *F. oxysporum* spores' suspension, they showed that the bacterial extract causes several morphological changes in the hyphae of the fungus, such as swellings, lysis and degradation of the fungal cells. In another study regarding the effect of the bacterial extract on the fungi, Rahman *et al*., (2007) evaluated the effect of *B. cepacia* antifungal compounds on the mycelial morphology of *C. gloeosporioides* and they found that the morphology of the treated mycelia had changed in that the hyphae became thicker with vacuolation appearing in the tips, unlike the hyphae in the control which appeared normal and smooth.

In another part of this work, we studied the effect of heat treatment on *QBC03's*

antifungal compounds stability. When *QBC03's* extract was tested against *A. carbonarius*, *F. culmorum* and *P. verrucosum*, the effect of decreasing the temperatures of the extract on *A. carbonarius* from 4ºC down to -80ºC didn't affect the extract efficiency. Increasing the temperatures caused a decrease of the antifungal activity of the extract, specially at 100ºC. For *F. culmorum*, decreasing the temperatures of the extract from 4ºC to -80ºC has affected the antifungal activity of the extract. The optimum temperature at which the extract had shown the best activity was at 30ºC. However, increasing the temperature after 30ºC was causing a decline in the activity and smallest inhibition zone appeared at 100ºC (9.8 mm). In *P. verrucosum*, the extract's antifungal activity remained constant when it was treated with lower temperatures from 4ºC to -80ºC. The activity remained the same from -80º until 40ºC, except at 30ºC where the highest activity was noticed.

Li *et al*., (2005) studied the thermostability of a purified antifungal compound termed 10M by applying different heat treatments. 10M was tested against *T. harzianum* and *G. roseum*, and they found that keeping the extract at room temperature for a month didn't reduce its antifungal activity. On the other hand, treating the extract with a 100ºC for 30 min has reduced the activity of the extract to 40%.

Kadir *et al*., (2008) also studied the effect of different temperatures treatments on the crude extract of *B. cepacia* against *C. gloeosporioides*. The antifungal compounds were treated with several different temperatures ranging from 28ºC to 121ºC, and their results showed that activity of *B. cepacia* supernatant didn't decrease and it retained its activity after being treated with high temperatures.

For our results, treating the extract with 100ºC had significantly decreased its activity, especially against *F. culmorum* where the inhibition zone of the 100ºC treated supernatant was 9.8 mm, compared to *A. carbonarius* and *P. verrucosum* that had inhibition zones of 15.1 mm and 14.5 mm, respectively.

Another study that confirms our findings was conducted by San-Lang *et al.,* (2002) who studied the effect of pH and temperatures on the stability of the antifungal compounds produced by two strains of *B. subtilis* against *F. oxysporum* and found that even after heating the antifungal compounds at 100%, they still showed an antifungal activity. However, increasing the temperatures of the antifungal compounds has caused a drastic decrease in their activity. These findings come along with our results, regarding to what was found in the thermostability of the antifungal compounds of *QBC03* against the fungal growth, especially against *F. culmorum*.

Conclusion:

In this part of the study, our local *Qatari Burkholderia cepacia* strains (*QBC03*) was investigated for its antifungal spectrum and potential of inhibiting the mycotoxigenic fungi and the synthesis of mycotoxins. *QBC03*'s antifungal activities were screened against 21 mycotoxigenic fungal species belonging to the three genera *Aspergillus*, *Fusarium* and *Penicillium*. It was found that *QBC03* has a strong antifungal activity against those mycotoxigenic fungi, except for two of the *Fusarium* species (*F. verticilliod* and *F. prolifiratum*). The antifungal compounds were able to completely inhibit the growth of three mycotoxigenic fungi *A. carbonarius*, *F. culmorum* and *P. verrucosum* at low concentrations. The use of 8.5%, 6.5% and 11.5% has inhibited the fungal growth of *A. carbonarius, F. culmorum* and *P. verrucosum* on solid media, respectively. In PDB, very low concentrations of the extract inhibited the fungal growth, were the fungal mycelia were not visualized by naked eye. The incorporation of only 3% and 4% of the antifungal compounds of *QBC03* in the liquid media caused a drastic decrease in the fungal biomass of *A. carbonarius* and *P. verrucosum*, and beyond these treatments, the fungal growth was not being visual to the eye. Moreover, very low concentrations of the extract (1% and 2%) were able to cause the fragmentation and swelling of the mycelia of both *A. carbonarius* and *P. verrucosum*.

In addition to that, the extract of *QBC03* was able to cause a 100% inhibition of the germination of the fungal spores, where even after being kept for longer time incubated, still the spores never managed to germinate. The antifungal compounds of *QBC03* are very stable at very high and low temperatures. This thermostability of *QBC03's* antifungal compounds make them promising potential candidates for the biocontrol of mycotoxigenic fungi. All these very interesting characteristics of *QBC03's* antifungal compounds make them very promising biocontrol agents useful for the control of mycotoxigenic fungi in Qatar particularly due to their high activities at wide ranges of temperatures and environmental conditions.

CHAPTER 8: CONCLUSION AND FUTURE PERSPECTIVES

In this M. Sc. Thesis, we explored the biocontrol potentialities of two microbial strains, a low fermenting yeast *L. thermotolerans 751* and a local strain of *Burkholderia cepacia* (*QBC03*). The antifungal potentials of both *Lachancea* and *Burkholderia* to control the growth of mycotoxigenic fungal species of *Aspergillus*, *Fusarium* and *penicillium* and the synthesis of their mycotoxins was investigated.

The low fermenting yeast *L. thermotolerans 751* had shown a great antifungal potential through the synthesis of VOCs which are able to act on the mycotoxigenic fungi by either inhibiting their growth, sporulation, spores' germination or most importantly, the synthesis of their mycotoxins. *L. thermotolerans 751* also has shown a great adsorption potential to mycotoxins in *in-vitro* experiments. Production of VOCs by *L. thermotolerans 751* was assessed in an *in-vitro* experiments to inhibit the fungal growth and spores' germination of the fungi inoculated on the tomato fruit. This has resulted in very positive results where the yeast was able to inhibit the growth of the fungus completely on the tomato fruits.

In addition, we demonstrated for the first time that depending on the yeast's CFUs, the inhibition of the mycotoxigenic fungal growth and the synthesis of mycotoxins varies a lot. What's more significant in this study in using the yeast as a biocontrol agent was the part dealing with the preservation of the tomato fruits from the fungal infection by the VOCs, where the fruits were preserved at 26ºC for more than a month without any damage or spoilage. Such experiments can be developed in the future to preserve more food commodities for short periods of time, or to preserve vegetables and fruits from the post-harvest infection by the mycotoxigenic fungi during their transportation from a place to another.

We believe that more comprehensive studies should be conducted in the future to study the mechanism of action of yeast VOCs against the mycotoxigenic fungi. The

mechanism of yeast cells adsorption to the mycotoxins and the conversion of these mycotoxins to putative metabolites by the living yeast cells deserve more future attention.

Our Qatari bacterial strain *Burkholderia cepacia* (*QBC03*) has shown a broad antifungal spectrum against a wide range of mycotoxigenic fungi belonging to different genera. Through the production of very promising antifungal compounds, *QBC03* was able to inhibit the growth, spores' germination and synthesis of mycotoxins of these mycotoxigenic fungi. In addition, the antifungal compounds produced by *QBC03* have shown a distinctive thermostability at a wide range of temperatures. Those antifungal compounds can be exploited and used as very stable and highly active biofungicides in all environments particularly locally in the region, substituting the chemical fungicides that are harmful for the environment.

We believe that more researches should be conducted to purify and study the antifungal compounds produced by *QBC03* and identify the genes that are responsible for the synthesis of the antifungal compounds. In addition to that, since *QBC03* is also known to produce VOCs that have antifungal activity, studies can be conducted more on this aspect to study the mechanisms of mycotoxigenic fungal growth inhibition by *QBC03*.

REFERENCES

Abbas, H. K., Wilkinson, J. R., Zablotowicz, R. M., Accinelli, C., Abel, C. A., Bruns, H. A., & Weaver, M. A. (2009). Ecology of *Aspergillus flavus*, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. *Toxin Reviews*, *28*(2-3), 142-153.

Agriopoulou, S., Koliadima, A., Karaiskakis, G., & Kapolos, J. (2016). Kinetic study of aflatoxins' degradation in the presence of ozone. *Food Control*, *61*, 221-226.

Ameer Sumbal, G., Hussain Shar, Z., Hussain Sherazi, S. T., Nizamani, S. M., & Mahesar, S. A. (2016). Decontamination of poultry feed from ochratoxin A by UV and sunlight radiations. *Journal of the Science of Food and Agriculture*, *96*(8), 2668-2673.

Anfossi, L., Giovannoli, C., & Baggiani, C. (2016). Mycotoxin detection. *Current opinion in biotechnology*, *37*, 120-126.

Armando, M. R., Dogi, C. A., Poloni, V., Rosa, C. A. R., Dalcero, A. M., & Cavaglieri, L. R. (2013). *In vitro* study on the effect of *Saccharomyces cerevisiae* strains on growth and mycotoxin production by *Aspergillus* carbonarius and *Fusarium graminearum*. *International journal of food microbiology*, *161*(3), 182-188.

Ayyadurai, N., Ravindra Naik, P., Sreehari Rao, M., Sunish Kumar, R., Samrat, S. K., Manohar, M., & Sakthivel, N. (2006). Isolation and characterization of a novel banana rhizosphere bacterium as fungal antagonist and microbial adjuvant in micropropagation of banana. *Journal of Applied Microbiology*, *100*(5), 926-937.

Bejaoui, H., Mathieu, F., Taillandier, P., & Lebrihi, A. (2004). Ochratoxin A removal in synthetic and natural grape juices by selected oenological *Saccharomyces* strains. *Journal of applied microbiology*, *97*(5), 1038-1044.

Bhat, R., Rai, R. V., & Karim, A. A. (2010). Mycotoxins in food and feed: present status and future concerns. *Comprehensive reviews in food science and food*

safety, *9*(1), 57-81.

Bhattacharyya, P. N., & Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, *28*(4), 1327-1350.

Burkholder, W. H. (1950). Sour skin, a bacterial rot. *Phytopathology*, *40*, 115-17.

Bzducha-Wróbel, A., Błażejak, S., Kawarska, A., Stasiak-Różańska, L., Gientka, I., & Majewska, E. (2014). Evaluation of the efficiency of different disruption methods on yeast cell wall preparation for β-glucan isolation. *Molecules*, *19*(12), 20941-20961.

Calvente, V., De Orellano, M. E., Sansone, G., Benuzzi, D., & De Tosetti, M. S. (2001). Effect of nitrogen source and pH on siderophore production by *Rhodotorula* strains and their application to biocontrol of phytopathogenic moulds. *Journal of Industrial Microbiology and Biotechnology*, *26*(4), 226-229.

CAST (Council for Agricultural Science and Technology). (2003). Mycotoxins: Risks in plant, animal, and human systems. *Task Force Report No. 139*, 13-85.

Chen, P. H., Chen, R. Y., & Chou, J. Y. (2018). Screening and Evaluation of Yeast Antagonists for Biological Control of *Botrytis cinerea* on Strawberry Fruits. *Mycobiology*, *46*(1), 33-46.

Cole, M. D. (1994). Key antifungal, antibacterial and anti-insect assays—a critical review. *Biochemical Systematics and Ecology*, *22*(8), 837-856.

Compant, S., Duffy, B., Nowak, J., Clément, C., & Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.*, *71*(9), 4951-4959.

Compant, S., Nowak, J., Coenye, T., Clément, C., & Ait Barka, E. (2008). Diversity and occurrence of *Burkholderia spp*. in the natural environment. *FEMS*

microbiology reviews, *32*(4), 607-626.

Dogi, C. A., Armando, R., Ludueña, R., De Moreno de LeBlanc, A., Rosa, C. A. R., Dalcero, A., & Cavaglieri, L. (2011). *Saccharomyces cerevisiae* strains retain their viability and aflatoxin B1 binding ability under gastrointestinal conditions and improve ruminal fermentation. *Food Additives & Contaminants: Part A*, *28*(12), 1705-1711.

Droby, S., Wisniewski, M., Macarisin, D., & Wilson, C. (2009). Twenty years of postharvest biocontrol research: is it time for a new paradigm?. *Postharvest biology and technology*, *52*(2), 137-145.

Dvegowda, G., Raju, M. V. L. N., & Swamy, H. V. L. N. (1998). Mycotoxins: novel solutions for their counteraction. *Feedstuffs (USA)*.

Eloff, J. N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta medica*, *64*(08), 711-713.

Fandohan, P., Zoumenou, D., Hounhouigan, D. J., Marasas, W. F. O., Wingfield, M. J., & Hell, K. (2005). Fate of aflatoxins and fumonisins during the processing of maize into food products in Benin. *International Journal of Food Microbiology*, *98*(3), 249-259.

Farbo, M. G., Urgeghe, P. P., Fiori, S., Marcello, A., Oggiano, S., Balmas, V., Ul Hassan, Z., Jaoua, S., & Migheli, Q. (2018). Effect of yeast volatile organic compounds on ochratoxin A-producing *Aspergillus carbonarius* and *A. ochraceus*. *International journal of food microbiology*, *284*, 1-10.

Faucet-Marquis, V., Joannis-Cassan, C., Hadjeba-Medjdoub, K., Ballet, N., & Pfohl-Leszkowicz, A. (2014). Development of an *in vitro* method for the prediction of mycotoxin binding on yeast-based products: case of aflatoxin B 1, zearalenone and ochratoxin A. *Applied microbiology and*

biotechnology, *98*(17), 7583-7596.

Fiori, S., Urgeghe, P. P., Hammami, W., Razzu, S., Jaoua, S., & Migheli, Q. (2014). Biocontrol activity of four non-and low-fermenting yeast strains against *Aspergillus carbonarius* and their ability to remove ochratoxin A from grape juice. *International journal of food microbiology*, *189*, 45-50.

Freire, L., & Sant'Ana, A. S. (2018). Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. *Food and Chemical Toxicology*, *111*, 189-205.

Garcia, D., Ramos, A. J., Sanchis, V., & Marín, S. (2009). Predicting mycotoxins in foods: a review. *Food microbiology*, *26*(8), 757-769.

Ghannoum, M. A., & Rice, L. B. (1999). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clinical microbiology reviews*, *12*(4), 501-517.

Gil-Serna, J., Patiño, B., Cortés, L., González-Jaén, M. T., & Vázquez, C. (2011). Mechanisms involved in reduction of ochratoxin A produced by *Aspergillus westerdijkiae* using *Debaryomyces hansenii* CYC 1244. *International journal of food microbiology*, *151*(1), 113-118.

Gnonlonfin, G. J. B., Hell, K., Adjovi, Y., Fandohan, P., Koudande, D. O., Mensah, G. A., & Brimer, L. (2013). A review on aflatoxin contamination and its implications in the developing world: a sub-Saharan African perspective. *Critical reviews in food science and nutrition*, *53*(4), 349-365.

Gonçalves, B. L., Rosim, R. E., de Oliveira, C. A. F., & Corassin, C. H. (2015). The *in vitro* ability of different *Saccharomyces cerevisiae*–based products to bind aflatoxin B1. *Food control*, *47*, 298-300.

Haidar, R., Fermaud, M., Calvo-Garrido, C., Roudet, J., & Deschamps, A. (2016). Modes of action for biological control of *Botrytis cinerea* by antagonistic

bacteria. *Phytopathologia Mediterranea*, *55*(3), 301-322.

Hameed, M. R., Khan, M. Z., Khan, A., & Javed, I. (2013). Ochratoxin induced pathological alterations in broiler chicks: effect of dose and duration. *Pak Vet J*, *33*, 145-9.

Hershkovitz, V., Sela, N., Taha-Salaime, L., Liu, J., Rafael, G., Kessler, C., & Droby, S. (2013). De-novo assembly and characterization of the transcriptome of *Metschnikowia fructicola* reveals differences in gene expression following interaction with *Penicillium digitatum* and grapefruit peel. *BMC genomics*, *14*(1), 168.

Hua, S. S. T., Beck, J. J., Sarreal, S. B. L., & Gee, W. (2014). The major volatile compound 2-phenylethanol from the biocontrol yeast, *Pichia anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*. *Mycotoxin research*, *30*(2), 71-78.

Hussein, H. S., & Brasel, J. M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology*, *167*(2), 101-134.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, International Agency for Research on Cancer, & World Health Organization. (1993). *Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins* (Vol. 56). World Health Organization.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, World Health Organization, & International Agency for Research on Cancer. (2002). *Some traditional herbal medicines, some mycotoxins, naphthalene and styrene* (No. 82). World Health Organization.

JECFA (Joint, F. A. O., WHO Expert Committee on Food Additives, & World Health Organization). (2007). Evaluation of certain food additives and contaminants:

sixty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives.

Ji, C., Fan, Y., & Zhao, L. (2016). Review on biological degradation of mycotoxins. *Animal Nutrition*, *2*(3), 127-133.

Jin, L., Wang, W., Degroote, J., Van Noten, N., Yan, H., Majdeddin, M., & Mombaerts, R. (2017). Mycotoxin binder improves growth rate in piglets associated with reduction of toll-like receptor-4 and increase of tight junction protein gene expression in gut mucosa. *Journal of animal science and biotechnology*, *8*(1), 80.

Joannis-Cassan, C., Tozlovanu, M., Hadjeba-Medjdoub, K., Ballet, N., & Pfohl-Leszkowicz, A. (2011). Binding of zearalenone, aflatoxin B1, and ochratoxin A by yeast-based products: A method for quantification of adsorption performance. *Journal of food protection*, *74*(7), 1175-1185.

Joo, H. J., Kim, H. Y., Kim, L. H., Lee, S., Ryu, J. G., & Lee, T. (2015). A *Brevibacillus sp*. antagonistic to mycotoxigenic *Fusarium spp*. *Biological Control*, *87*, 64-70.

Jouany, J. P. (2007). Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. *Animal Feed Science and Technology*, *137*(3-4), 342-362.

Jouany, J. P., Yiannikouris, A., & Bertin, G. (2005). The chemical bonds between mycotoxins and cell wall components of *Saccharomyces cerevisiae* have been identified. *Archiva Zootechnica*, *8*, 26-50.

Kabak, B. (2009). The fate of mycotoxins during thermal food processing. *Journal of the Science of Food and Agriculture*, *89*(4), 549-554.

Kadir, J., Rahman, M. A., Mahmud, T. M. M., Abdul Rahman, R., & Begum, M. M. (2008). Extraction of antifungal substances from *Burkholderia cepacia* with antibiotic activity against *Colletotrichum gloeosporioides* on papaya (Carica

papaya). *Int. J. Agric. Biol*, *10*, 15-20.

Kilani-Feki, O., & Jaoua, S. (2011). Biological control of *Botrytis cinerea* using the antagonistic and endophytic *Burkholderia cepacia Cs5* for vine plantlet protection. *Canadian journal of microbiology*, *57*(11), 896-901.

Kilani-Feki, O., Culioli, G., Ortalo-Magné, A., Zouari, N., Blache, Y., & Jaoua, S. (2011). Environmental *Burkholderia cepacia* strain Cs5 acting by two analogous alkyl-quinolones and a didecyl-phthalate against a broad spectrum of phytopathogens fungi. *Current microbiology*, *62*(5), 1490-1495.

Kilani-Feki, O., Khiari, O., Culioli, G., Ortalo-Magné, A., Zouari, N., Blache, Y., & Jaoua, S. (2010). Antifungal activities of an endophytic *Pseudomonas fluorescens* strain Pf1TZ harbouring genes from pyoluteorin and phenazine clusters. *Biotechnology letters*, *32*(9), 1279-1285.

Kilani-Feki, O., Zouari, I., Culioli, G., Ortalo-Magné, A., Zouari, N., Blache, Y., & Jaoua, S. (2012). Correlation between synthesis variation of 2-alkylquinolones and the antifungal activity of a *Burkholderia cepacia* strain collection. *World Journal of Microbiology and Biotechnology*, *28*(1), 275-281.

Klich, M. A., Lax, A. R., & Bland, J. M. (1991). Inhibition of some mycotoxigenic fungi by iturin A, a peptidolipid produced by *Bacillus subtilis*. *Mycopathologia*, *116*(2), 77-80.

Kolosova, A., & Stroka, J. (2012). Evaluation of the effect of mycotoxin binders in animal feed on the analytical performance of standardized methods for the determination of mycotoxins in feed. *Food Additives & Contaminants: Part A*, *29*(12), 1959-1971.

Li, L., Qu, Q., Tian, B., & Zhang, K. Q. (2005). Induction of chlamydospores in *Trichoderma harzianum* and *Gliocladium roseum* by antifungal compounds produced by *Bacillus subtilis C2*. *Journal of phytopathology*, *153*(11-12), 686-

693.

Ligon, J. M., Hill, D. S., Hammer, P. E., Torkewitz, N. R., Hofmann, D., Kempf, H. J., & Pée, K. H. V. (2000). Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Management Science: formerly Pesticide Science*, *56*(8), 688-695.

Liu, P., Cheng, Y., Yang, M., Liu, Y., Chen, K., Long, C. A., & Deng, X. (2014). Mechanisms of action for 2-phenylethanol isolated from *Kloeckera apiculata* in control of *Penicillium* molds of citrus fruits. *BMC microbiology*, *14*(1), 242.

Liu, P., Luo, L., & Long, C. A. (2013). Characterization of competition for nutrients in the biocontrol of *Penicillium italicum* by *Kloeckera apiculata*. *Biological Control*, *67*(2), 157-162.

Luo, M., Purdy, H., & Avis, T. J. (2019). Compost bacteria provide antifungal activity against grey mold and *Alternaria* rot on bell pepper fruit. *Botany*, (ja).

Mari, M., Martini, C., Spadoni, A., Rouissi, W., & Bertolini, P. (2012). Biocontrol of apple postharvest decay by *Aureobasidium pullulans*. *Postharvest Biology and Technology*, *73*, 56-62.

Milani, J. M. (2013). Ecological conditions affecting mycotoxin production in cereals: a review. *Veterinarni Medicina*, *58*(8).

Milićević, D. R., Škrinjar, M., & Baltić, T. (2010). Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. *Toxins*, *2*(4), 572-592.

Morath, S. U., Hung, R., & Bennett, J. W. (2012). Fungal volatile organic compounds: a review with emphasis on their biotechnological potential. *Fungal Biology Reviews*, *26*(2-3), 73-83.

Nathanail, A. V., Gibson, B., Han, L., Peltonen, K., Ollilainen, V., Jestoi, M., & Laitila, A. (2016). The lager yeast *Saccharomyces pastorianus* removes and

transforms *Fusarium trichothecene* mycotoxins during fermentation of brewer's wort. *Food chemistry*, *203*, 448-455.

Oliveira, C. A. F., Bovo, F., Corassin, C. H., Jager, A. V., & Reddy, K. R. (2013). Recent trends in microbiological decontamination of aflatoxins in foodstuffs. In *Aflatoxins-recent advances and future prospects*. InTech.

Oliveira, R. R., Aguiar, B. D. M., Tessmann, D. J., Pujade-Renaud, V., & Vida, J. B. (2012). Chlamydospore formation by *Corynespora cassiicola*. *Tropical Plant Pathology*, *37*(6), 415-418.

Pandey, P., Kang, S. C., & Maheshwari, D. K. (2005). Isolation of endophytic plant growth promoting *Burkholderia sp*. MSSP from root nodules of *Mimosa pudica*. *Current Science*, 177-180.

Parafati, L., Vitale, A., Restuccia, C., & Cirvilleri, G. (2015). Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. *Food microbiology*, *47*, 85-92.

Peraica, M., Domijan, A. M., Jurjević, Ž., & Cvjetković, B. (2002). Prevention of exposure to mycotoxins from food and feed. *Arhiv za higijenu rada i toksikologiju*, *53*(3), 229-237.

Pereyra, C. M., Cavaglieri, L. R., Keller, K. M., Chiacchera, S. M., Rosa, C. A. D. R., & Dalcero, A. M. (2015). *In vitro* ochratoxin A adsorption by commercial yeast cell walls.

Petersson, S., Hansen, M. W., Axberg, K., Hult, K., & SchnÜRer, J. (1998). Ochratoxin A accumulation in cultures of *Penicillium* verrucosum with the antagonistic yeast *Pichia anomala* and *Saccharomyces cerevisiae*. *Mycological Research*, *102*(8), 1003-1008.

Pfliegler, W. P., Pusztahelyi, T., & Pócsi, I. (2015). Mycotoxins–prevention and decontamination by yeasts. *Journal of basic microbiology*, *55*(7), 805-818.

Quintela, S., Villarán, M. C., De Armentia, I. L., & Elejalde, E. (2012). Ochratoxin A removal from red wine by several oenological fining agents: bentonite, egg albumin, allergen-free adsorbents, chitin and chitosan. *Food Additives & Contaminants: Part A*, *29*(7), 1168-1174.

Rahman, M. A., Kadir, J., Mahmud, T. M. M., Rahman, R. A., & Begum, M. M. (2007). Screening of antagonistic bacteria for biocontrol activities on *Colletotrichum gloeosporioides* in papaya. *Asian J. Plant Sci*, *6*(1), 12-20.

Recep, K., Fikrettin, S., Erkol, D., & Cafer, E. (2009). Biological control of the potato dry rot caused by *Fusarium* species using PGPR strains. *Biological Control*, *50*(2), 194-198.

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicosis—An overview. *International journal of food microbiology*, *119*(1-2), 3-10.

Ringot, D., Lerzy, B., Bonhoure, J. P., Auclair, E., Oriol, E., & Larondelle, Y. (2005). Effect of temperature on *in vitro* ochratoxin A biosorption onto yeast cell wall derivatives. *Process biochemistry*, *40*(9), 3008-3016.

San-Lang, W., Shih, L., Wang, C. H., Tseng, K. C., Chang, W. T., Twu, Y. K., & Wang, C. L. (2002). Production of antifungal compounds from chitin by *Bacillus subtilis*. *Enzyme and Microbial Technology*, *31*(3), 321-328.

Santos, A. V., Dillon, R. J., Dillon, V. M., Reynolds, S. E., & Samuels, R. I. (2004). Occurrence of the antibiotic producing bacterium *Burkholderia sp*. in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*. *FEMS Microbiology Letters*, *239*(2), 319-323.

Schmidt, S., Blom, J. F., Pernthaler, J., Berg, G., Baldwin, A., Mahenthiralingam, E., & Eberl, L. (2009). Production of the antifungal compound pyrrolnitrin is quorum sensing-regulated in members of the *Burkholderia cepacia complex*. *Environmental microbiology*, *11*(6), 1422-1437.

Shetty, P. H., Hald, B., & Jespersen, L. (2007). Surface binding of aflatoxin B1 by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. *International journal of food microbiology*, *113*(1), 41-46.

Smedsgaard, J. (1997). Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *Journal of Chromatography A*, *760*(2), 264-270.

Turner, N. W., Subrahmanyam, S., & Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: a review. *Analytica chimica acta*, *632*(2), 168-180.

Urquhart, E. J., & Punja, Z. K. (2002). Hydrolytic enzymes and antifungal compounds produced by *Tilletiopsis* species, *phyllosphere* yeasts that are antagonists of powdery mildew fungi. *Canadian journal of microbiology*, *48*(3), 219-229.

Vanhoutte, I., De Mets, L., De Boevre, M., Uka, V., Di Mavungu, J. D., De Saeger, S., & Audenaert, K. (2017). Microbial Detoxification of Deoxynivalenol (DON), Assessed via a Lemna minor L. Bioassay, through Biotransformation to 3-epi-DON and 3-epi-DOM-1. *Toxins*, *9*(2), 63.

Vial, L., Groleau, M. C., Dekimpe, V., & Deziel, E. (2007). *Burkholderia* diversity and versatility: an inventory of the extracellular products. *Journal of microbiology and biotechnology*, *17*(9), 1407-1429.

Vila-Donat, P., Marin, S., Sanchis, V., & Ramos, A. J. (2018). A review of the mycotoxin adsorbing agents, with an emphasis on their multi-binding capacity, for animal feed decontamination. *Food and chemical toxicology*, *114*, 246-259.

Virgili, R., Simoncini, N., Toscani, T., Camardo Leggieri, M., Formenti, S., & Battilani, P. (2012). Biocontrol of *Penicillium nordicum* growth and ochratoxin A production by native yeasts of dry cured ham. *Toxins*, *4*(2), 68-82.

WHO (1998). Aflatoxins. In: *Safety Evaluation of Certain Food Additives and Contaminants: Prepared by the Forty-ninth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)*. Geneva: World Health Organization (WHO Food Additives Series, No. 40).

WHO (2001a). Deoxynivalenol. In: *Safety Evaluation of Certain Mycotoxins in Food: Prepared by the Fifty-sixth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)*. Geneva: World Health Organization (WHO Food Additives Series, No. 47). Available at http://www.inchem.org/ documents/jecfa/jecmono/v47je05.htm.

Wielogórska, E., MacDonald, S., & Elliott, C. T. (2016). A review of the efficacy of mycotoxin detoxifying agents used in feed in light of changing global environment and legislation. *World Mycotoxin Journal*, *9*(3), 419-433.

Wisniewski, M. E., & Wilson, C. L. (1992). Biological control of postharvest diseases of fruits and vegetables: recent advances. *HortScience*, *27*(2), 94-98.

Wisniewski, M., Droby, S., Norelli, J., Liu, J., & Schena, L. (2016). Alternative management technologies for postharvest disease control: the journey from simplicity to complexity. *Postharvest Biology and Technology*, *122*, 3-10.

World Health Organization. (2001). *Evaluation of Certain Mycotoxins in Food: Fifty-sixth* report of the joint FAO/WHO expert committee on food additives. *Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)*. Geneva: World Health Organization (WHO Food Additives Series, No. 47). Available at http://www.inchem.org/ documents/jecfa/jecmono/v47je05.htm.

World Health Organization. (2000). Evaluation of certain food additives and contaminants: fifty-third report of the joint FAO/WHO expert committee on food additives. In *Evaluation of certain food additives and contaminants: fifty-third report of the joint FAO/WHO expert committee on food additives*.

Yeaman, M. R., & Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacological reviews*, *55*(1), 27-55.

Yogendrarajah, P., Jacxsens, L., De Saeger, S., & De Meulenaer, B. (2014). Co-occurrence of multiple mycotoxins in dry chilli (Capsicum annum L.) samples from the markets of Sri Lanka and Belgium. *Food Control*, *46*, 26-34.

Zeidan, R., Ul-Hassan, Z., Al-Thani, R., Balmas, V., & Jaoua, S. (2018). Application of Low-Fermenting Yeast *Lachancea thermotolerans* for the Control of Toxigenic Fungi *Aspergillus parasiticus*, *Penicillium verrucosum* and *Fusarium graminearum* and Their Mycotoxins. *Toxins*, *10*(6).

Zhang, D., Spadaro, D., Valente, S., Garibaldi, A., & Gullino, M. L. (2012). Cloning, characterization, expression and antifungal activity of an alkaline serine protease of *Aureobasidium pullulans PL5* involved in the biological control of postharvest pathogens. *International journal of food microbiology*, *153*(3), 453-464.

Zhu, R., Zhao, Z., Wang, J., Bai, B., Wu, A., Yan, L., & Song, S. (2015). A simple sample pretreatment method for multi-mycotoxin determination in eggs by liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*, *1417*, 1-7.

APPENDIX

Appendix (A): Published Article

Type of article: original article

Title: Application of Low-Fermenting Yeast *Lachancea thermotolerans* for the Control of Toxigenic Fungi *Aspergillus parasiticus, Penicillium verrucosum* and *Fusarium graminearum* and Their Mycotoxins

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toxins

MDPI

Article

Application of Low-Fermenting Yeast *Lachancea thermotolerans* for the Control of Toxigenic Fungi *Aspergillus parasiticus, Penicillium verrucosum* and *Fusarium graminearum* and Their Mycotoxins

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Abstract: Mycotoxins are important contaminants of food and feed. In this study, low fermenting yeast (*Lachancea thermotolerans*) and its derivatives were applied against toxigenic fungi and their mycotoxins. *A. parasiticus*, *P. verrucosum* and *F. graminearum* and their mycotoxins were exposed to yeast volatile organic compounds (VOCs) and cells, respectively. VOCs reduced significantly the fungal growth (up to 48%) and the sporulation and mycotoxin synthesis (up to 96%). Very interestingly, it was shown that even 7 yeast colonies reduced *Fusarium's* growth and the synthesis of its mycotoxin, deoxynivalenol (DON). Moreover, decreasing yeast nutrient concentrations did not affect the inhibition of fungal growth, but reduced DON synthesis. In addition, inactivated yeast cells were able to remove up to 82% of the ochratoxin A (OTA). As an application of these findings, the potentialities of the VOCs to protect tomatoes inoculated with *F. oxysporum* was explored and showed that while in the presence of VOCs, no growth was observed of *F. oxysporum* on the inoculated surface areas of tomatoes, in the absence of VOCs, *F. oxysporum* infection reached up to 76% of the tomatoes' surface areas. These results demonstrate that the application of yeasts and their derivatives in the agriculture and food industry might be considered as a very promising and safe biocontrol approach for food contamination.

Keywords: food safety; mycotoxins; biological control; yeast; decontamination; tomatoes

Key Contribution: Yeast VOCs have been identified to affect the growth (on artificial media and on tomatoes surface areas) and mycotoxins synthesized by toxigenic *Aspergillus*, *Penicillium* and *Fusarium*. The inactivated yeast presented a very significant potential to remove the mycotoxins from contaminated matrices.

1. Introduction

Mycotoxins, the secondary fungal metabolites of toxigenic species are mainly produced by the genera *Aspergillus*, *Penicillium* and *Fusarium*. These toxigenic species predominantly contaminate cereals during pre- and post-harvest storage and many other stages [1]. The production of mycotoxins in grains depends on several factors, including humidity, temperature, water activity, mechanical damages and fungal toxigenic potentials [2]. Based on their toxicological profiles, aflatoxin B1 (AFB1), ochratoxin A (OTA), zearalenone (ZEN), fumonisins, T-2/HT-2 and deoxynivalenol (DON) have been recognized on the top of the list of more than 400 known mycotoxins [3].

The health issues in relation to mycotoxins led the food and feed control authorities to set regulatory limits to protect animal and human subjects from the toxic effects of these compounds. For each mycotoxin, based on its available toxicological data, region/country, type of commodity and intended use, the maximum limits are different. The European Union (EU) has set maximum limits of aflatoxins for cereal-based food as 2 µg/kg (AFB1) and 4 µg/kg (AF total), while in feed these limits are 20 µg/kg. Similarly, for OTA, these levels are of 3 µg/kg for food and 250 µg/kg for feed material, except for pigs and poultry [4].

The stability of mycotoxins during routine food and feed processing made clear the issues of mycotoxins and mycotoxicosis. This urged the scientists to search for a safer, environmentally friendly and broad-spectrum approach to counter the issues of mycotoxins and mycotoxicosis. So far, preventive strategies at pre-harvest (crop rotation, sowing date, resistant varieties etc.) and post-harvest (segregation, proper storage etc.) are in practice with variable success [5]. However, once the toxigenic fungi have infected the cereals and resulted in the accumulation of mycotoxins in it, the decontamination strategies then can be employed [6]. These procedures include chemical treatments (acids, basis, ozone, ammonia), physical treatments (adsorption, cooking, roasting, frying, baking and UV irradiation) and biological treatments (metabolic conversion, fermentation, enzymatic degradation) aiming to reduce the mycotoxins to safe limits [7–9]. In each case, along with other factors, the nature of target mycotoxin(s) and the pH of the medium determine the success of the employed technique [10].

Organic control using biological agents against mycotoxins is considered a safer option and is now getting popularity in the food industry [11]. In fact, the application of yeasts (cells and their volatiles) and yeast derivative have great potential to minimize the economic losses caused by mycotoxigenic fungi. Several yeast species have been identified to have effective fungal biocontrol activities where they can be utilized against toxigenic fungi to inhibit their growth and mycotoxins synthesis [12,13]. The biocontrol activities of antagonistic yeast against toxigenic fungi involve several mechanisms including space and nutrient competition, parasitism, biofilm formation, production of antifungal compounds and release of oxidants in the environment [14]. However, the fungal growth inhibition does not always predict the retardation of mycotoxins synthesis. Apart from this, some yeast species are known to have great potentials of binding mycotoxins on their cell wall surface [15,16], and others can degrade parent mycotoxin to less or non-toxic metabolites [17,18]. Above all the beneficial effects, the majority of yeasts species hold 'Generally Considered As Safe (GRAS)' status. In the present study, using three genera of toxigenic fungi, we aimed to investigate the effects of a low fermenting yeast (*Lachancea thermotolerans*) on their growth, mycotoxins synthesis and mycotoxins decontamination. Additionally, the volatile organic compounds released by yeast were tested for their preservation potential against phytopathogenic *F. oxysporum* infection in tomatoes.

2. Results and Discussion

2.1. Biocontrol Activity of Yeast VOCs against Toxigenic Fungi

At day 3 of post-exposure to yeast VOCs, *P. verrucosum* developed to a maximum colony diameter of 6.3 mm as compared to 9.3 mm of unexposed fungi. This showed an average 32% lesser development in fungal colony size as compared to the control (Figures 1 and 2). With increasing the exposure time, the size of VOCs exposed fungus colony did not increase significantly, as compared to that of unexposed fungi. This led to increasing differences in colony diameter, as it was 9.5 mm and 10.3 mm on day 5 and 7, respectively, as compared to 15.3 and 18.3 mm in control. Colony size reduction was 37.91% (day 5) and 43.72% (day 7) as compared to control (unexposed fungi). Although not by the VOCs, the yeast isolated from the dry-cured hams in Italy, showed a similar inhibitory effect on the growth of *P. nordicum* [19]. These biocontrol activities of the yeast are assumed to be due to the release of toxic VOCs, as there was no physical contact between yeast and fungi.

In the present study, yeast VOCs also resulted in a significant decrease in *A. parasiticus* colony size, as compared to unexposed fungi. At day 3, 5 and 7 of exposure to yeast VOCs, colony diameter of *A. parasiticus* was 13.5 mm, 29 mm and 45 mm as compared to 18 mm, 34 mm and 48 mm in the control (unexposed to VOC), respectively. This decrease in colony diameter of VOCs exposed fungi, compared with the unexposed control was 25%, 15% and 6% at day 3, 5 and 7, respectively. These results demonstrated that increasing the yeast's VOCs exposure duration to *A. parasiticus* results in the decrease in the severity of fungal growth inhibition, which might be the result of some resistant/tolerant phenomena in the fungus itself. The decrease in the colony size of *A. parasiticus* is likely to be associated with the production of 2-phenylethanol by yeast, which results in an alteration in the expression of the genes responsible for the fungal growth, as has been observed against *A. flavus* [20].

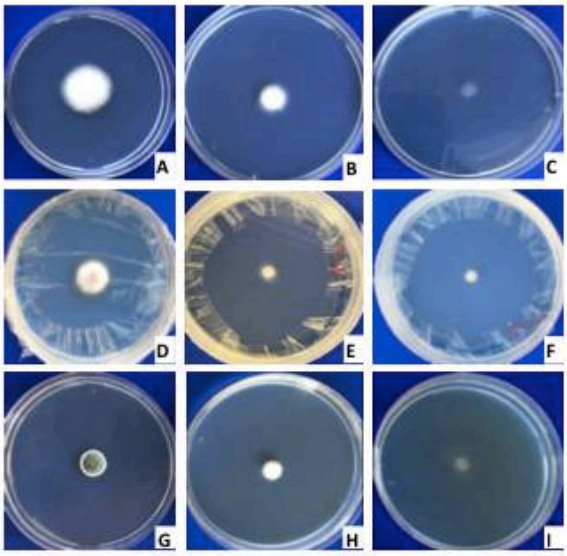

Figure 1. Biocontrol activity of yeast VOCs on *Aspergillus parasiticus* (**A–C**), *Fusarium graminearum* (**D–F**) and *Penicillium verrucosum* (**G–I**). Colony morphology of *A. parasiticus*; (**A**) not exposed to yeast VOCs; (**B**) exposed to VOCs from 100 yeast CFUs; (**C**), exposed to VOCs from 200 yeast CFUs. Colony morphology of *F. graminearum*; (**D**) not exposed to yeast VOCs; (**E**) exposed to VOCs from 100 yeast CFUs; (**F**), exposed to VOCs from 200 yeast CFUs. Colony morphology of *P. verrucosum*; (**G**) not exposed to yeast VOCs; (**H**) exposed to VOCs from 100 yeast CFUs; (**I**), exposed to VOCs from 200 yeast CFUs. In Figure 1 (**D–F**), presence of tapes at the margins shows how the sealing of two base plates opposite to each other was done.

At day 3, 5 and 7, in response to yeast VOCs, *F. graminearum* showed an average colony diameter of 16 mm, 34 mm and 50 mm, which was significantly lower than 29 mm, 49 mm and 64 mm, respectively, as observed in (unexposed) control fungi. This decrease in colony size was 46%, 31% and 23% respectively at day 3, 5 and 7, as compared to unexposed fungi (Figure 2). In line with a present study, in field trials antagonistic yeast (*Cryptococcus nodaensis*) resulted in 50–60% reduction in *Fusarium* head blight in wheat caused by *F. graminearum* [21]. The fungal growth inhibition activities are likely to be associated with the yeast VOCs having 2-phenylethanol, that is a major anti-fungal compound [22,23].

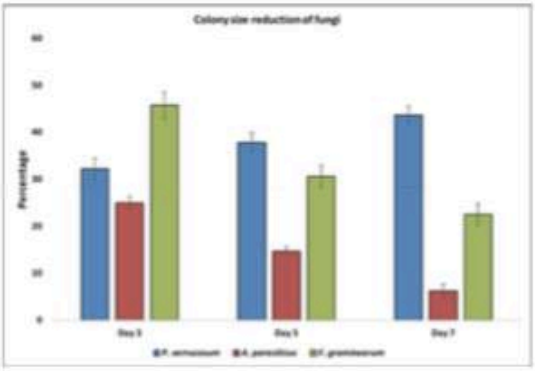

Figure 2. Fungal growth inhibition measured as colony size reduction (%) as compared to control at day 3, 5 and 7, of exposure to yeast VOCs. Spores of selected fungi were inoculated at the center of PDA plates and were sealed against already growing yeast colonies on YPDA.

2.2. Effect of Yeast VOCs on the Mycotoxins Synthesis

The production of mycotoxins by toxigenic fungi, that comes as a result of the reduction of the active fungal growth, leads to the upregulation of the toxigenic genes, as studied in *A. flavus* [23]. In the present study, exposure to yeast VOCs, resulted in a significant reduction in aflatoxins (AFs) synthesis by *A. parasiticus*. In the colonized media plugs, obtained from the yeast VOCs exposed fungi, significantly lower AFs contents (1.43 µg/kg) were noted, as compared to those obtained from unexposed fungi (8.01 µg/kg). This accounted an overall 82% decrease in AFs synthesis by yeast VOCs exposed fungi, compared to the control. A 96% lower AFs synthesis was noted in a yeast VOCs exposed *A. parasiticus* fungal colony, developed from a single spore, as compared to unexposed fungal spore colony (Figure 3). This demonstrated a precise approach to record the effect of yeast VOCs on mono-spore fungal mass. In a similar approach, the addition of 50 µL/L of gaseous allyl isothiocyanate in the fungal growth media resulted in the inhibition of *Aspergillus parasiticus* growth at 3.17 log CFU/g as compared to the control [24]. In line with the present study, inoculation of a baker's yeast in the liquid media having *A. flavus* spore suspension resulted in 15.4 to 41.6% decreased AFB1 synthesis [25]. The suppression of mycotoxins synthesis by the toxigenic fungi, exposed to yeasts, principally happens to be the function of yeast metabolites, mainly 2-phenylethanol, which inhibits fungus growth and/or suppresses the genes involved in mycotoxins synthesis [21].

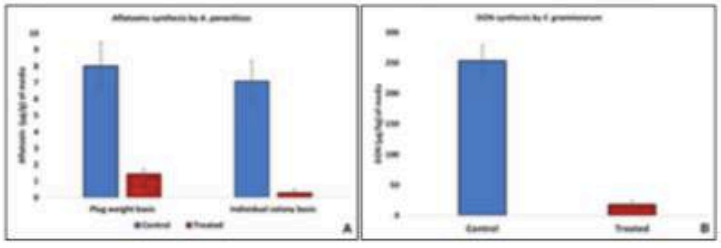

Figure 3. Inhibition of mycotoxins synthesis by toxigenic *A. parasiticus* and *F. graminearum* exposed to yeast VOCs; (**A**) comparison of aflatoxins synthesis between VOCs exposed and unexposed *A. parasiticus* on plug weight and individual colony basis; (**B**) Comparison of DON synthesis between yeast VOCs exposed and unexposed *F. graminearum*.

In case of *F. graminearum* exposed to yeast VOCs, in the colonized media plugs, the DON contents were 18.29 µg/kg as compared to the control (unexposed to VOCs), where it was 254 µg/kg. This showed an overall 93% lower DON synthesis in exposed fungi compared to the unexposed. In line with the findings of a present study, *Saccharomyces cerevisiae* did not only significantly inhibit the growth of toxigenic *F. graminearum*, but also resulted in the decrease of DON and ZEN synthesis [26].

2.3. Effect of No. of Yeast CFU on Fungal Growth and Mycotoxins Synthesis

A curve was observed in *F. graminearum* colony size and DON synthesis in response to exposure to VOCs from increasing numbers of yeast CFUs. VOCs released from as low as 7 yeast CFUs showed an antagonist effect on *F. graminearum* colony size and DON synthesis. A gradual decrease in fungal colony diameter was noted with increasing yeast VOCs up to 247. Increasing the number of yeast CFUs from 250 and onwards, resulted in an increase in fungus colony diameter. A similar trend in the DON synthesis was observed in response to increasing yeast CFUs. The exposure of fungi to VOCs released from 7 yeast CFUs and up till 200, exhibited a gradual decrease in DON synthesis. The VOCs generated from 201 to 398 yeast CFUs, resulted in a complete inhibition of DON synthesis by toxigenic *F. graminearum*. Increasing the yeast CFUs from 402 again resulted in DON synthesis by *F. graminearum* (Figure 4). In the published literature, no such report exists regarding the kinetics of fungal growth and toxin synthesis in response to increasing the amount/number of yeast cells/VOCS. However, it is known that yeast or their VOCs inhibit the growth and mycotoxins synthesis of *F. graminearum* [21,26]. In the first study of its kind, we are able to conclude that to achieve maximum antagonistic effect on fungal growth and DON synthesis, 200–250 yeast CFUs produce optimum VOCs, in 90 mm petri plate experimental settings.

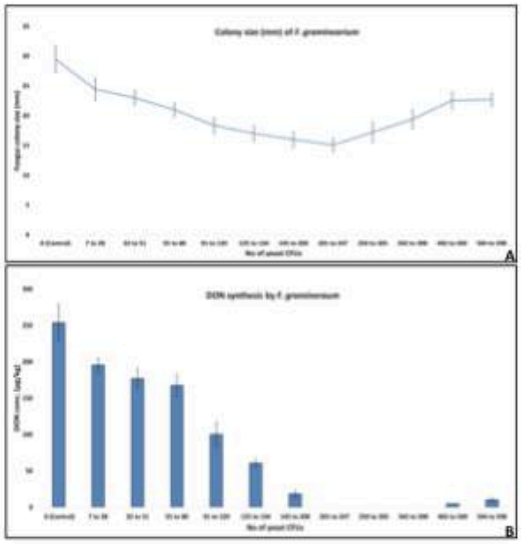

Figure 4. Kinetics of colony size and DON synthesis by *F. graminearum* in response to exposure to increasing numbers of yeast CFUs. (**A**) The colony size of fungi showing a decreasing trend upon exposure to yeast VOCs released from 7 to 250 yeast CFUs, afterwards the effect was reversed; (**B**) Mycotoxins synthesis by fungi showing a decreasing trend upon exposure to yeast VOCs released from 7 to 250 yeast CFUs, afterwards the effect was reversed, an almost similar trend to colony size observed in (**A**).

2.4. Role of Nutrients Availability to Yeast on Its Antagonistic Activities against F. graminearum

The findings noted in Section 2.3, guided us to explore the role of nutrients availability to antagonistic yeast on its antifungal activities. Three concentrations of the Yeast Extract Peptone Agar (YPDA); 1×, 0.5× and 0.1× were used to inoculate yeast cells. A significant reduction in the colony diameter of *F. graminearum* was noted upon exposure to yeast VOCs from 1× and 0.5× YPDA, while the effect was the opposite when yeast was grown on 0.1× YPDA. However, an inverse relation between nutrients availability to antagonistic yeast and DON synthesis by the toxigenic fungi was noted (Table 1).

Table 1. Effect of nutrient availability to yeast on its antagonistic activities.

Treatment	Colony Diameter (mm)	DON Synthesis (µg/kg)	Colony Diameter and DON Synthesis by Fungi Not Exposed to Yeast VOCs	
Control (No yeast)	30 ± 1.0^{a}	311.48 ± 33.78^{a}	Colony diameter (mm)	DON synthesis (µg/kg)
1X YPDA	8 ± 0.6^{c}	218.09 ± 29.52^{b}	30 ± 0.7	331.00 ± 41.09
0.5X YPDA	20 ± 0.8^{b}	203.04 ± 8.69^{b}	32 ± 0.6	340.44 ± 35.00
0.1X YPDA	28 ± 0.7^{a}	164.83 ± 19.22^{c}	29 ± 0.6	312.32 ± 30.33

Yeast cells were inoculated on three different media concentrations, and plates from each media, showing almost the same number of yeast CFU were selected for sealing against germinating *F. graminearum* spores. The effect of VOCs released by yeast were tested on fungal growth and mycotoxin synthesis. The values, in each column, followed by different superscript letters are statistically different from each other at ^{a} $p \leq 0.05$, ^{b} $p \leq 0.01$ or ^{c} $p \leq 0.001$.

A non-significant effect on the colony diameters and a significant reduction in the DON synthesis alongside with decreasing the nutrient availability to antagonistic yeast, is conceivably thought to be due to down-regulation of mycotoxins biosynthetic genes with no effect on the genes responsible for fungal growth. The effect of the yeast's (*Pichia anomala*) VOCs upon growth- and toxigenic gene expression in *A. flavus* showed a many-fold down regulation [20]. However, the findings of the present study are in contrast with those of Reference [21], who by using *Cryptococcus* isolates, reported 50–60% lesser severity of *Fusarium* head blight caused by *F. graminearum*. These differences in VOCs exposed fungal growth and mycotoxins synthesis indicate yeast- and fungus- species-specific antagonistic mechanisms. Further exploration in these directions may provide a valuable explanation of the mechanism involved.

2.5. Mycotoxins Binding onto Yeast Cell Wall (YCW)

Application of yeast cells and their derivatives as mycotoxins decontaminating agents in food and feed industry have been widely reported [27–29]. The efficacy of yeast-based mycotoxins binding agents is potentially related to the nature of targeted mycotoxins, their polarity, the pH of the medium, contact duration and the nature of the yeast itself [30]. Considering these variables enhance the mycotoxins binding spectrums, most of the commercially available mycotoxins binding products contain mixture of yeast, bacterial enzymes and some clay. Adding live or inactivated yeast cells in the buffer solution, already contaminated with various levels of OTA, resulted in a significant removal of the toxin from the solutions. At an initial contamination level of 0.9 µg/kg, live yeast cells were able to reduce OTA at 63% and 67% in the supernatant of buffer solution of pH 5 and pH 7, respectively. However, in the pellet of pH 5 and pH 7 buffer solutions, 29% and 30% of the OTA was detected. The differences in the OTA contents (those removed from the supernatant and found in the pellet) may be caused by the degradation of the toxin by live yeast or its enzymes. The adsorption of OTA on the YCW has been reported to be a function of pH of the media as well as thickness of the YCW itself [10,26].

At higher OTA contamination levels (1.8 µg/kg), live yeast cells showed mycotoxin adsorption/reduction in the supernatant at 75% and 71% in the buffer solution of pH 5 and pH 7, respectively. However, unexpectedly, in the pellet of these solutions, 32% (pH 5 buffer) and 49% (pH 7 buffer) of the total OTA was found. In the case of inactivated yeast, the amount of OTA removed

from the supernatant of buffer solutions was higher, and its significant quantity was detected in the pellets of corresponding buffer solutions. The reduction of OTA in the supernatant of buffer solutions of pH 5 and 7 were 71% and 82%, respectively (Figure 5). Almost all amount of OTA removed from the supernatant was found in the pellet (68% and 80% respectively in pellet of pH 5 and pH 7 buffer). By exploring the interaction between some mycotoxins and yeast cell wall components, it was identified that there exists weak hydrogen and van der Waals bonds making the interaction more "adsorption" rather than "binding" [31]. The interaction of OTA and YCW involves polar as well as non-polar (hydrophobic amino acids of YCW and aromatic ring of OTA) bindings followed by rearrangement of the water in the solvent [32].

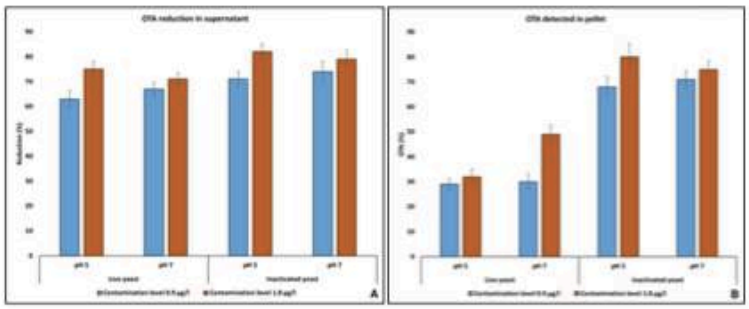

Figure 5. Adsorption of OTA by live and inactivated yeast cells; (**A**) percentage of OTA removed from the supernatant of the buffer solutions; (**B**) percentage of the OTA detected in the pellet.

In the case of AFs, the adsorption of toxins on YCW was lower as compared to OTA. At pH 5, at contamination levels of 0.2 µg/kg and 0.4 µg/kg, the adsorption of AFs on live yeast cells was 16% and 34%, respectively. In the buffer solutions contaminated with AFs at 0.2 and 0.4 µg/kg, the addition of inactivated yeast cells resulted in 20% and 29% removal of the toxins. At pH 7, adsorption of AFs was much lower (up to 10%) by the live and inactivated yeast cells. In line with the present study, lower adsorption of AFB1 at pH 7, compared to pH 5, and overall lesser adsorption (10%) was noted by adding 5 mg of YCW power in buffer solution [10]. In another study, the adsorption of AFB1 on YCW was lower as compared to OTA and ZEA [33], as has been observed in the present study.

In a similar experimental setup, live yeast cells showed a significant lower DON adsorption (17%) from a buffer solution of pH 5 when compared to that at pH 7, which was 52%. However, in the pellet of live cells, DON was not detected at all, which can be explained by the fact that it might have been degraded into metabolites by live yeast cells. More detailed studies are needed to explore the possible mechanism of DON removal by live yeast cells. By the inactivated yeast, less significant removal of DON from the supernatant was observed, while significant concentration of the removed toxin was detected in the pellet (data not shown). In line with the present study, adsorption of DON on yeast was lesser (12.6%) as compared to other mycotoxins, like ZEN (66.7%), fumonisins (67%) and T-2 toxin (33%) [30].

2.6. VOCs Inhibits F. oxysporum Infection in Tomato

Surface inoculated tomatoes with *F. oxysporum* showed development of fungal infection, which spread to wider surface areas (Figure 6). The infection rate was scored on the basis of visible mycelium mass on the tomatoes' surface area and it showed an average contamination of 76% (Figure 6).

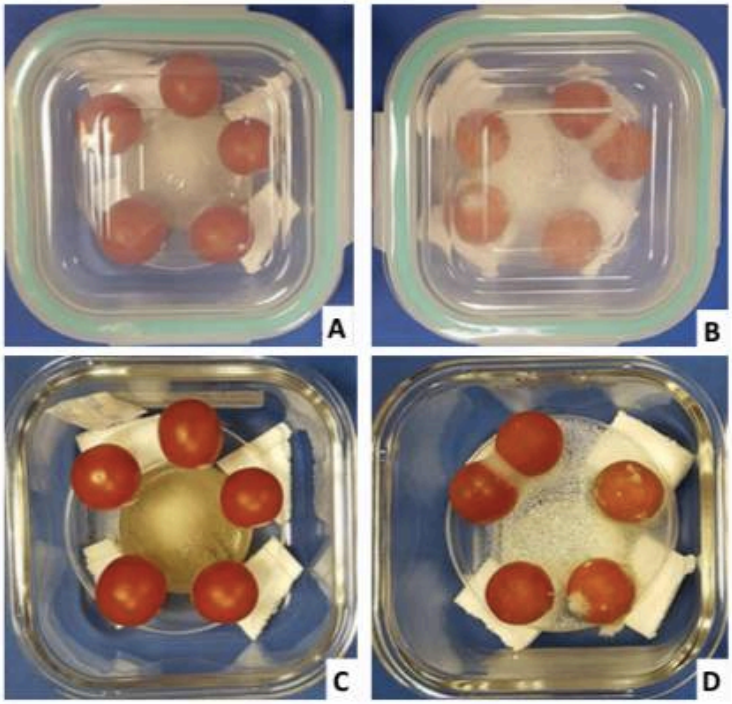

Figure 6. In vitro biocontrol activity of yeast VOCs against *F. oxysporum* infection on tomato; (**A**) tomato inoculated with *F. oxysporum* on their surface and exposed to yeast VOCs, from a streak inoculated 60 mm Petri dish with live yeast cells; (**B**) tomatoes inoculated with F. oxysporum, but not exposed to yeast VOCs. Tomatoes after 34 days of incubation at 26 °C; (**C**) tomato incubated in the presence of yeast VOCs showing no fungal growth; (**D**) tomato incubated without VOCs showing extensive fungal growth on the surface.

On the other hand, tomatoes having inoculated fungal spores on their surface and exposed to yeast VOCs showed complete absence of infection till the end of the experiment (day 34 post- infection). These findings proved that VOCs were able to completely inhibit the fungal growth and germination, and hold the potential to protect the vegetables (tomatoes) from fungal infection for a longer time. In lines with present study, yeast cells sprayed on detached grape barriers were able to be protected from the fungal infection by *A. carbonarius* [22].

3. Conclusions

Biocontrol activity of low fermenting yeast (*Lachancea thermotolerans*) was tested against three fungi, each belonging to a different genus. The VOCs produced by antagonistic yeast inhibited the growth and sporulation of all three fungi as compared to the control. AF synthesis by *A. parasiticus* and DON synthesis by *F. graminearum* were significantly reduced by yeast VOCs. The effect of yeast VOCs on *F. graminearum* growth and DON synthesis was noticed from as low as 7 yeast CFUs, and showed a direct relation up to ~200 CFUs and then after a stationary phase (~450 CFUs) the

effect was reversed. Reducing the availabilities of nutrients (as monitored by using different media dilutions) resulted in a non-significant effect on the growth of toxigenic *F. graminearum*; however, DON synthesis was significantly reduced. Live and inactivated yeast cells were able to remove 71% and 82% of OTA from the buffer solutions, respectively. Finally, in the most applied part of this work, using tomatoes artificially inoculated with *F. oxysporum* spores for 34 days it was demonstrated that yeast VOCs could inhibit the growth of *F. oxysporum* spores completely; while in the control exposed to no yeast cells, the infection rate scored on the basis of visible mycelium mass on tomatoes surface showed an average contamination of 76%. Therefore, it can be postulated that low-fermenting yeast (*Lachancea thermotolerans*) holds a strong potential as a biocontrol, decontaminant and preservative agent against significant toxigenic fungi and their mycotoxins.

4. Materials and Methods

4.1. Chemicals, Supplies and Biological Strains

Aflatoxins (B1, B2, G1, G2), OTA and DON powders were obtained from Romer Labs. ELISA kits for aflatoxins (RIDASCREEN® Aflatoxin Total, Art No. R4701), OTA (RIDASCREEN® Ochratoxin A, Art No. R1311) and DON (RIDASCREEN DON, Art No. R5906) were purchased from R-Biopharm AG, Darmstadt, Germany. The buffer solutions of pH 5 (acetate buffer) and pH 7 (phosphate buffer) were prepared according to the protocol described by Faucet-Marquis et al. [10]. ELISA plate reader (Multiskan FC, Thermo Scientific, Waltham, MA, USA) installed with Skanlt software (Version 4.1. Thermo Scientific, MA, USA, 2015) was used to obtain the absorbance of mycotoxins in ELISA plates. A calibration curve was generated by using absorbance data of known mycotoxins' standards solutions (5–6), and the absorbance values of unknown samples were added to the calibration curve to calculate the amount of toxins in our samples. For this purpose, a software (Z9996 RIDA®-SOFT Win, R-Biopharm, Darmstadt, Germany) was used. Low fermenting yeast (*Lachancea thermotolerans* 751) was obtained from Dipartimento di Agraria, Universita di Sassari, Italy. *A. parasiticus*, *P. verrucosum* and *F. graminearum* were isolated from animal feed samples obtained from an animal feed market located in Doha, Qatar. Morphological examination was followed by PCR based identification of these isolates. Mycotoxins synthesis potential of the isolates was confirmed by the presence of key genes involved in biosynthetic pathways followed by in vitro analysis of the production of mycotoxins on laboratory media [34].

4.2. Effect of Yeast VOCs on Growth and Sporulation of Toxigenic Fungi

To ascertain the effect of yeast VOCs on the growth and sporulation of *A. parasiticus*, *P. verrucosum* and *F. graminearum*, yeast and fungal co-culture experiments were performed [22]. The experimental setup prevented the direct contact of yeast with fungal colony. Yeast extract peptone dextrose agar (Yeast extract: 10 g, peptone: 20 g, dextrose: 20 g and agar: 15 g for 1 L of media) plates were streak inoculated with 100 µL of yeast cell suspensions (10^6 cells/mL) and incubated for 48 h at 25 °C. The lid of the plate was replaced by another Petri plate that was point inoculated with 10 µL of either *A. parasiticus* (10^7 spores/mL), *P. verrucosum* (10^6 spores/mL) or *F. graminearum* (10^4 spores/mL). These plates were tightly sealed with parafilm® with additional layers of adhesive tape to block the leakage of VOCs. The control plates of fungi were sealed with YPDA plates without yeast cells. The colony characteristics (size, sporulation and colony morphology) were measured at days 3, 5 and 7 post-sealing.

4.3. Effect of Yeast VOCs on the Mycotoxins Synthesis

In the experiments above (Section 4.2), on day 10 (*Aspergillus parasiticus*) and day 15 (*F. graminearum*), three plugs (6 mm each) from the colonized media were removed with a cork borer and then weighed. Mycotoxins were extracted in 1 mL of 70% methanol and sonicated for 60 min. A total of 500 µL of the extract was transferred to a new vial and was allowed to dry under a stream of

Nitrogen. The dried extract was re-suspended in 500 µL of 10% methanol (for aflatoxins) or distilled water (for DON) before performing ELISA.

4.4. Effect of Number of Yeast Colony Forming Units (CFU) on F. graminearum Growth and DON Synthesis

To record the effect of variable numbers of yeast CFU on the fungal growth and mycotoxin production, 100 µL of serial dilutions of yeast cells suspensions (20 dilutions) were spread inoculated on the YPDA plates. After 48 h of the yeast cells growth, these plates were sealed against *F. graminearum* point inoculated (10 µL from 10^6 spores/mL) PDA plates. The effect of as low as 7 CFUs up to 598 yeast CFUs on the *F. graminearum* growth zone was recorded. Diameters of developing fungal zones were recorded at day 3, and the DON synthesis was confirmed at day 21 of the experiment. For each yeast condition or dilution, at least 3 replicates were performed. The sealing of fungal spores inoculated plates against yeast plates was performed as described above.

4.5. Effect of Nutrient Availability to Yeast on Its Antagonistic Activities

In order to test the role of nutrient availability on the antagonistic spectrum of yeast, three YPDA medium concentrations; 1× (as described in Section 4.2), 0.5× and 0.1× were used. Live yeast cells from overnight culture in YPDB were plated on each medium plate. Three media plates, each from different concentrations of YPDA having almost same number of yeast CFUs, were selected for sealing against *F. graminearum*. At day 3 of post-sealing, the diameter of developing colony was measured whereas, at day 21, DON synthesis was determined as described above.

4.6. In-Vitro Mycotoxins Binding Experiments

For the preparation of inactivated yeast, overnight yeast cells culture in YPDB (yeast extract: 10 g, peptone: 20 g, dextrose: 20 g in 1 L of distilled water) were maintained in a shaking incubator at 26 °C. After autoclaving and centrifugation briefly at 5000× *g*, the supernatant was discarded and the pellet was re-suspended in distilled water. The contents were centrifuged at 5000× *g* for 5 min at 4 °C. The residues were dried at 80 °C for 12 h before grinding them into powder form [35]. A total of 5 mg of YCW or 20 µL of live yeast culture was incubated in 990 µL or 970 µL of each buffer for 5 min, respectively. Mycotoxin solutions (10 µL) were added to each tube to reach the final concentration of 0.9 and 1.8 µg/kg of OTA, 0.2 and 0.4 µg/kg of AF and 40 and 80 µg/kg of DON. Tubes were incubated at 37 °C with end to end continuous shaking for 30 min. Supernatants were separated by centrifugation at 9200× *g* and collected in new tubes. After drying using SpeedVac, the pellets were re-suspended in 0.13 M sodium hydrogen carbonate solution, 10% methanol or distilled water for analysis of each mycotoxins; OTA, AF and DON. ELISA assays were performed for each supernatant and pellet.

4.7. In-Vitro Testing of Yeast VOCs against F. oxysporum Infection in Tomato

Organic tomatoes produced locally in Qatar were purchased from the supermarket. In disinfected glass containers (12.5 × 12.5 × 5.5), five tomatoes of similar weight (8 ± 0.45 g) were placed on a sterile platform. Below the tomatoes, a Petri dish (60 × 15 mm) having a full surface growth of yeast cells was placed in a way to allow the VOCs to disperse throughout the box. On the surface of each tomato, 5 µL of *F. oxysporum* cell suspension (10^4 spores/mL) was placed. After sealing, boxes were shifted to an incubator at 26 °C for 34 days. In the control boxes, YPDA plates non-inoculated with yeast cells were placed. An objective scoring of fungal infection on the surface of each tomato was recorded.

4.8. Statistical Analysis

The obtained data was subjected to analysis of variance test (ANOVA). The mean values of treated groups were compared with the untreated control by using *t*-test. The means were considered

as significant at $p \leq 0.05$. SPSS statistical software (Version 23, IBM, NY, USA, 2017) was used for this purpose.

Author Contributions: S.J. is the supervisor of R.Z. and Z.U.-H.; S.J., R.Z., and Z.U.-H. conceptualized the study. R.Z. and Z.U.-H. performed the experiments. R.Z., Z.U.-H., S.J. and R.A.-T. analyzed the data. Z.U.-H., R.Z. and S.J. wrote and revised the manuscript. V.B. provided the yeast strain and reviewed the article along with S.J.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Milani, J.M. Ecological conditions affecting mycotoxin production in cereals: A review. *Vet. Med.* **2013**, *58*, 405–411. [CrossRef]
2. Tola, M.; Kebede, B. Occurrence, importance and control of mycotoxins: A review. *Cogent Food Agric.* **2016**, *2*. [CrossRef]
3. Bhat, R.; Rai, R.V.; Karim, A.A. Mycotoxins in food and feed: Present status and future concerns. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 57–81. [CrossRef]
4. Mazumder, P.M.; Sasmal, D. Mycotoxins—Limits and regulations. *Anc. Sci. Life* **2001**, *20*, 1–19. [PubMed]
5. Abbas, H.K.; Wilkinson, J.R.; Zablotowicz, R.M.; Accinelli, C.; Abel, C.A.; Bruns, H.A.; Weaver, M.A. Ecology of *Aspergillus flavus*, regulation of aflatoxin production and management strategies to reduce aflatoxin contamination of corn. *Toxin Rev.* **2009**, *2–3*, 142–152. [CrossRef]
6. Pankaj, S.K.; Shi, H.; Keener, K.M. A review of novel physical and chemical decontamination technologies for aflatoxin in food. *Trends Food Sci. Technol.* **2018**, *71*, 73–83. [CrossRef]
7. Shanakhat, H.; Sorrentino, A.; Raiola, A.; Annalisa, R.; Paolo, M.; Silvana, C. Current methods for mycotoxins analysis and innovative strategies for their reduction in cereals: An overview. *J. Sci. Food Agric.* **2018**. [CrossRef] [PubMed]
8. Gonçalves, B.L.; Carolina, F.S.C.C.; Diane, V.N.; Carlos, H.C.; Carlos, A.F.O. Mycotoxins in fruits and fruit-based products: Occurrence and methods for decontamination. *Toxin Rev.* **2018**. [CrossRef]
9. Varga, J.; Kocsubé, S.; Péteri, Z.; Vágvölgyi, C.; Tóth, B. Chemical, physical and biological approaches to prevent ochratoxin induced toxicoses in humans and animals. *Toxins* **2010**, *2*, 1718–1750. [CrossRef] [PubMed]
10. Faucet-Marquis, V.; Joannis-Cassan, C.; Hadjeba-Medjdoub, K.; Ballet, N.; Pfohl-Leszkowicz, A. Development of an in vitro method for the prediction of mycotoxin binding on yeast-based products: Case of aflatoxin B1, zearalenone and ochratoxin A. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 7583–7596. [CrossRef] [PubMed]
11. Tsitsigiannis, D.I.; Dimakopoulou, M.; Antoniou, P.P.; Tjamos, E.C. Biological control strategies of mycotoxigenic fungi and associated mycotoxins in Mediterranean basin crops. *Phytopathol. Mediterr.* **2012**, *51*, 158–174.
12. Kabak, B.; Dobson, A.D.W.; Var, I. Strategies to prevent mycotoxin contamination of food and animal feed: A review. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 593–619. [CrossRef] [PubMed]
13. Palumbo, J.D.; O'Keeffe, T.L.; Abbas, H.K. Microbial interactions with mycotoxigenic fungi and mycotoxins. *Toxin Rev.* **2008**, *27*, 261–285. [CrossRef]
14. Liu, J.; Sui, Y.; Wisniewski, M.; Droby, S.; Liu, Y. Review: Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. *Int. J. Food Microbiol.* **2013**, *167*, 153–160. [CrossRef] [PubMed]
15. Caridi, A. New perspectives in safety and quality enhancement of wine through selection of yeasts based on the parietal adsorption activity. *Int. J. Food Microbiol.* **2007**, *120*, 167–172. [CrossRef] [PubMed]
16. McCormick, S.P. Microbial detoxification of mycotoxins. *J. Chem. Ecol.* **2013**, *39*, 907–918. [CrossRef] [PubMed]
17. Halász, A.; Lásztity, R.; Abonyi, T.; Bata, Á. Decontamination of mycotoxin-containing food and feed by biodegradation. *Food Rev. Int.* **2009**, *25*, 284–298. [CrossRef]

18. Schatzmayr, G.; Zehner, F.; Täubel, M.; Schatzmayr, D.; Klimitsch, A.; Loibner, A.P.; Binder, E.M. Microbiologicals for deactivating mycotoxins. *Mol. Nutr. Food Res.* **2006**, *50*, 543–551. [CrossRef] [PubMed]
19. Virgili, R.; Simoncini, N.; Toscani, T.; Camardo, L.; Formenti, S.; Battilani, P. Biocontrol of *Penicillium nordicum* growth and ochratoxin a production by native yeasts of dry cured ham. *Toxins* **2012**, *4*, 68–82. [CrossRef] [PubMed]
20. Hua, S.S.T.; Beck, J.J.; Sarreal, S.B.L.; Gee, W. The major volatile compound 2-phenylethanol from the biocontrol yeast, *Pichia anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*. *Mycotoxin Res.* **2014**, *30*, 71–78. [CrossRef] [PubMed]
21. Khan, N.; Schisler, D.; Boehm, M.; Lipps, P.; Slininger, P. Field testing of antagonists of *Fusarium* head blight incited by *Gibberella zeae*. *Biol. Control* **2004**, *29*, 245–255. [CrossRef]
22. Fiori, S.; Urgeghe, P.P.; Hammami, W.; Razzu, S.; Jaoua, S.; Migheli, Q. Biocontrol activity of four non- and low-fermenting yeast strains against *Aspergillus carbonarius* and their ability to remove ochratoxin A from grape juice. *Int. J. Food Microbiol.* **2014**, *17*, 45–50. [CrossRef] [PubMed]
23. Chang, P.K.; Hua, S.S.T.; Sarreal, S.B.L.; Li, R.W. Suppression of aflatoxin biosynthesis in *Aspergillus flavus* by 2-Phenylethanol is associated with stimulated growth and decreased degradation of branched-chain amino acids. *Toxins* **2015**, *7*, 3887–3902. [CrossRef] [PubMed]
24. Nazareth, T.M.; Corrêa, J.A.; Pinto, A.C.; Palma, J.B.; Meca, G.; Bordin, K.; Luciano, F.B. Evaluation of gaseous allyl isothiocyanate against the growth of mycotoxigenic fungi and mycotoxin production in corn stored for 6 months. *J. Sci. Food Agric.* 2018. [CrossRef] [PubMed]
25. Šari, L.; Škrinjar, M.M.; Saka, M.B.; Plavši, D.V.; Abarkapa, I.V. Effect of the baker's yeast on the production of aflatoxin B1 by *Aspergillus flavus*. *Food Prog. Qual. Saf.* **2008**, *4*, 165–168.
26. Armando, M.R.; Pizzolitto, R.P.; Dogi, C.A.; Cristofolini, A.; Merkis, C.; Poloni, V.; Dalcero, A.M.; Cavaglieri, L.R. Adsorption of ochratoxin A and zearalenone by potential probiotic *Saccharomyces cerevisiae* strains and its relation with cell wall thickness. *J. Appl. Microbiol.* **2012**, *113*, 256–264. [CrossRef] [PubMed]
27. Dawson, K.A. Application of yeast and yeast derivatives in poultry industry. *Proc. Aust. Poult. Sci. Symp.* **2011**, *13*, 100–105.
28. Piotrowska, M.; Masek, A. *Saccharomyces cerevisiae* cell wall components as tools for ochratoxin A decontamination. *Toxins* **2015**, *7*, 1151–1162. [CrossRef] [PubMed]
29. Mil, T.D.; Devreese, M.; Baere, S.D.; Ranst, E.V.; Eeckhout, M.; Backer, P.D.; Croubels, S. Characterization of 27 mycotoxin binders and the relation with in vitro zearalenone adsorption at a single concentration. *Toxins* **2015**, *7*, 21–33. [CrossRef] [PubMed]
30. Devegowda, G.; Rayu, M.V.L.N.; Swamy, H.V.L.N. Mycotoxins: Novel solutions for their counteraction. *Feedstuffs* **1998**, *7*, 12–15.
31. Jouany, J.P.; Yiannikouris, A.; Bertin, G. The chemical bonds between mycotoxins and cell wall components of *Saccharomyces cerevisiae* have been identified. *Arch. Zootech.* **2005**, *8*, 26–50.
32. Ringot, D.; Lerzy, B.; Bonhoure, J.P.; Auclair, E.; Oriol, E.; Larondelle, Y. Effect of temperature on in vitro ochratoxin biosorption onto yeast cell wall derivatives. *Process Biochem.* **2005**, *40*, 3008–3016. [CrossRef]
33. Joannis-Cassan, C.; Tozlovanu, M.; Hadjeba-Medjdoub, K.; Ballet, N.; Pfohl-Leszkowicz, A. Binding of zearalenone, aflatoxin B1, and ochratoxin A by yeast-based products: A method for quantification of adsorption performance. *J. Food Prot.* **2011**, *74*, 1175–1185. [CrossRef] [PubMed]
34. Hassan, Z.U.; Al-Thani, R.F.; Migheli, Q.; Jaoua, S. Detection of toxigenic mycobiota and mycotoxins in cereal feed market. *Food Control* **2018**, *84*, 389–394. [CrossRef]
35. Bzducha-Wróbel, A.; Błażejak, S.; Kawarska, A.; Stasiak-Różańska, L.; Gientka, I.; Majewska, E. Evaluation of the efficiency of different disruption methods on yeast cell wall preparation for β-Glucan isolation. *Molecules* **2014**, *19*, 20941–20961. [CrossRef] [PubMed]