In vitro large screening of bioactive compounds of botanical origin for antifungal activity against two mycotoxigenic fungi of wheat: *Aspergillus westerdijkiae* and *Fusarium graminearum*

Fleurat-Lessard, F.*#, Bergougnoux, M., Debourdeau, G., Barreau, C.
INRA Research Unit 1264 Mycology and Food Safety, 71 avenue Edouard Bourlaux, CS 20032, 33882 Villenave d'Ornon Cedex, France

*Corresponding author, Email: francis.fleurat-lessard@bordeaux.inra.fr
#Presenting author, Email: francis.fleurat-lessard@bordeaux.inra.fr

DOI: 10.14455/DOA.res.2014.170

Abstract

The antifungal activity of botanical compounds known for this property was tested on two mycotoxigenic fungi contaminating wheat: *Aspergillus westerdijkiae* and *Fusarium graminearum*. The reduction of fungal growth was checked through *in vitro* screening tests on fungi grown on liquid culture medium in 96- or 24-well microtitration plates. The potential of fungal growth reduction was tested for five phenolic acids, 17 terpenic compounds and eight other substances targetting oxidative metabolic chain in mitochondria. The compounds were introduced in the culture medium at a dose range from 1 $\mu L.L^{-1}$ to 1 $mL.L^{-1}$. Fungal biomass production was assessed after 4 d incubation at 25°C through spectrophotometric measurements, either absorbance measurement or fluorescence readings through fungal cell viability test with Alamar blue reagent. Mycotoxin production was determined after 10 d or 21 d culture for *F. graminearum* and *A. westerdijkiae* respectively, by HPLC-DAD. The LC_{50} and LC_{99} (= MIC) were determined for the most active compounds. The phenolics had very different activities on the two fungal species: *F. graminearum* was found unsensitive to chlorogenic acid and highly sensitive to trans-cinnamic acid, meanwhile it was the opposite with *A. westerdijkiae*. *Aspergillus westerdijkiae* was observed more sensitive than *F. graminearum* to terpene compounds. Unpurified clove oil extract and linalool induced a highly significant growth reduction on both fungi. Among the third group of compounds, allyl- and methyl-isothiocyanate shown a remarkable antifungal activity on both fungi. There was not observed any increase in mycotoxin production neither on treated cultures of *F. graminearum* nor of *A. westerdijkiae*. Fluorimetric measurements improve the accuracy of IC_{50} and MIC assessment. The Alamar blue assay with fluorometric reading was shown a reliable rapid method to screen for antifungal activity of new candidate biomolecules for biocontrol of mycotoxigenic molds infecting cereal crops or stored grain.

Keywords: bioactive compounds, botanicals, antifungal activity, *in vitro* test, *Aspergillus westerdijkiae*, *Fusarium graminearum*, mycotoxin production

1. Introduction

There is a very large range of plant secondary metabolites with biological or ecological roles (Regnault-Roger and Hamraoui, 1995; Regnault-Roger, 2008). Thus, numerous terpenes, phenolics, sulfur compounds, alcaloids exhibit recognized antimicrobial, antifungal, antioxidant or insecticidal activities, sometimes equivalent to synthetic insecticides. However, the distribution of bioactive botanical compounds in plant extracts or essential oils (EO) is highly variable with respect to cultivated species, geographical origin, climatic and soil environment, plant conditioning after the harvest, extraction process, etc. (Hussain et al.,

2008, 2010; Hediouni et al., 2012). This huge variability of bio-active compounds in plant extracts of EO content limits their use as plant or seeds protectants against insect pests and mctoxins. Yet, the antifugal activity of EOs distributed in a few botanical families (*Lamiaceae*, *Myrtaceae*, *Rutaceae*, *Apiaceae*), has been extensively studied on phytopathogenic fungi attacking cereals (Amini et al., 2012; Tabassum and Vidyasagar, 2013) and was also tested as mycotoxin production suppressor for *Fusarium* spp. (Desjardins et al., 1988; Velluti et al., 2004; Yaguchi et al., 22009; Samie and Nefefe, 2012; Aly et al., 2014). Thus, numerous EOs exhibit significant antifungal activity on mycotoxigenic fungi infecting wheat kernels, either in the field (e.g. Fusarium spp.) (Krish et al., 2011; Tabassum and Vidyasagar, 2013) or during storage (e.g. *Aspergillus* spp.) (Fleurat-Lessard et al., 2010). However, the industrial development of bioactive single compounds identified in EO composition as fungicide and mycotoxin inhibitors has not been developed, mainly because unfavorable economic cost and drastic regulatory constraints limit the registration of EOs (Isman, 2008).

The level of antifungal activity of compounds isolated from bioactive EOs is traditionally studied by different kinds of screening tests such as broth dilution test or micro-atmosphere test, depending on their mode of action (Bouddine et al., 2012; Kocić-Tanackov and Dimić, 2013; Gacem et al., 2013). We recently identified some compounds with a high impact on *Aspergillus westerdijkiae* Frisvad and Samson and *Fusarium graminearum* Schwabe mycelial growth in classical *in vitro* tests (Cardiet et al., 2012). Recent studies on structure-activity relationships of EO bioactive constituents generally focused on several biochemical targets for antifungal compounds such as the ergosterol pathway, respiratory chain, and chitin biosynthesis in pathogenic fungi (Voda et al., 2004; Rakotonirainy and Lavédrine, 2004; Pauli, 2005; Lee et al., 2008; Sokovic et al., 2009; Faleiro, 2011; Borrego et al., 2011). Consequently, high speed detection of EO constituents with antifungal activity requires the multiplication of comparative tests. But bioassays on agar medium in Petri dish culture have many drawbacks and do not allow a rapid and simultaneous screening of a large number of candidate biomolecules.

Among these inconveniences, we identified fungal culture growing time, replicates heterogeneity, lack of accuracy in measuring the diameter of growth area as issues that may lead to imprecise assessment of fungal growth inhibition index. Hence, according to this situation, the objectives of our study were as follows: 1) The development of a rapid method enabling screening of a large range of biomolecules for their antifungal activity in controlled conditions e.g. through cultivation of test fungi on liquid culture medium in microtitration plates allowing semi-automatic measurements of different criteria linked to fungal growth reductions (Debourdeau et al., 2012), 2) The application of this rapid bioassay procedure to determine potential of a large range of biomolecules to exhibit an efficient antifungal activity. To meet this second objective, the antifungal candidate biomolecules were selected in different groups of bio-active substances (antifungal, antimicrobial, antioxidant, insecticidal molecules) naturally produced by plants: i/ alkylisothiocyanates from *Brassicaceae* (fungal cell respiration inhibitors); ii/ several kinds of terpenes causing cellular membrane permeability alteration and energy metabolism disturbance (Hyldgaard et al., 2012; Maurya et al., 2013; Almeida Freires et al., 2014); iii/ miscellaneous compounds known as potential inhibitors of fungal cell respiration, 3) The assessment of IC_{50} and MIC dose for the most active antifungal biomolecules in order to identify the most promising compounds that might be used for bioprotection of cereal grain to protect against the risks of fungal spoilage in pre- or post-harvest situations, and 4) Ensure that treatment of mycotoxigenic test fungi culture by the most potent antifungal biomolecules did not increase the production of mycotoxins when applied at sub-lethal doses.

2. Materials and Methods

2.1. Test fungi

Two species of toxigenic fungi causing contamination of cereal grain with a risk of production of mycotoxins were selected as target fungi according to their different ecological and physiological characteristics. The first one, *F. graminearum*, is a hygrophilic species that can grow only on green plants in the field. *Fusarium graminearum* produces noxious mycotoxins, mainly trichothecenes of B group (DON, NIV and/or oxygenated congeners). The strain INRA 349 was used according to its high production of TCTB in *in vitro* liquid culture medium. Conversely, *A. westerdijkiae*, the second one, is an ubiquist filamentous fungus common on plants, in soils, on adultered food, and also in the habitat atmosphere. It is a xerotolerant fungus that may develop on grain kernels after harvest in high moisture content conditions (Fleurat-Lessard et al., 2010). It produces a carcinogenic mycotoxin: ochratoxin A. In this study we used a strain of *A. westerdijkiae* producing significant amounts of OTA mycotoxin and used in toxicological studies at INRA UMR Toxalim (Toulouse, France). *Fusarium graminearum* culture was carried out on a simplified liquid culture medium (SM) routinely use in the laboratory, composed from glucose, KH_2PO_4, K_2HPO4, $MgSO_4$, $(NH_4)_2SO_4$, and oligo-elements. *Aspergillus westerdijkiae* cultures were carried out on SAM medium (Bacha, 2009) composed from glucose, K_2HPO4, NH_4NO_3, KCl, and MgSO4, with pH finally adjusted at 6.5 by HCl 6N. In a first screening with 96-well microplates, each well was filled with 100 µL of culture medium in sterile conditions before inoculation by one of the two fungi. In the second study, restricted to a limited number compounds to be tested, 24-well microplates were used with each well receiving 2 mL of culture medium.

2.2. Selected candidate antifungal biomolecules

A first screening was done with three groups of biomolecules known for their biological activities on noxious organisms: i/ phenolic antioxident compounds naturally found in cereal kernels (Fig. 1); ii/ terpenoid substances isolated from essential oils, known for insecticidal or antifungal or antimicrobial activity (Fig. 2); iii/ miscellaneous natural compounds known for overall deleterious effect on cellular respiration pathway (Fig. 3).

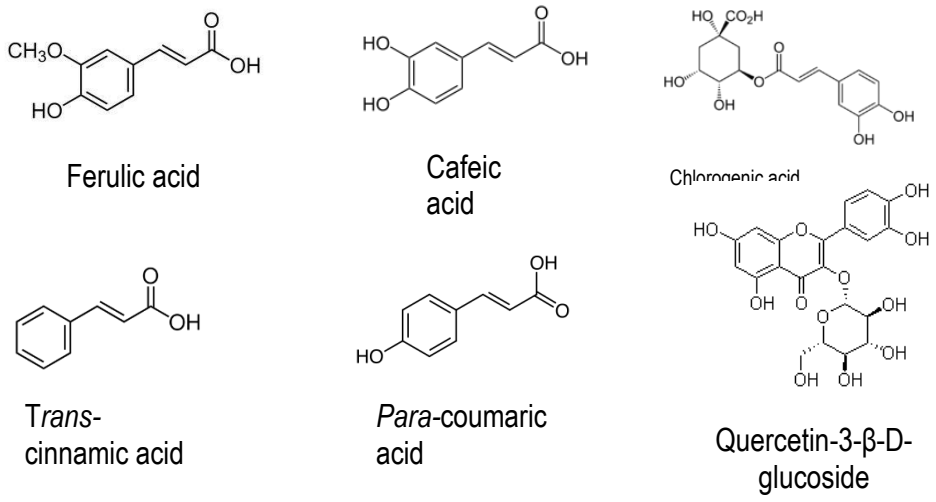

Figure 1 Phenolics substances selected as candidate antifungal biomolecules.

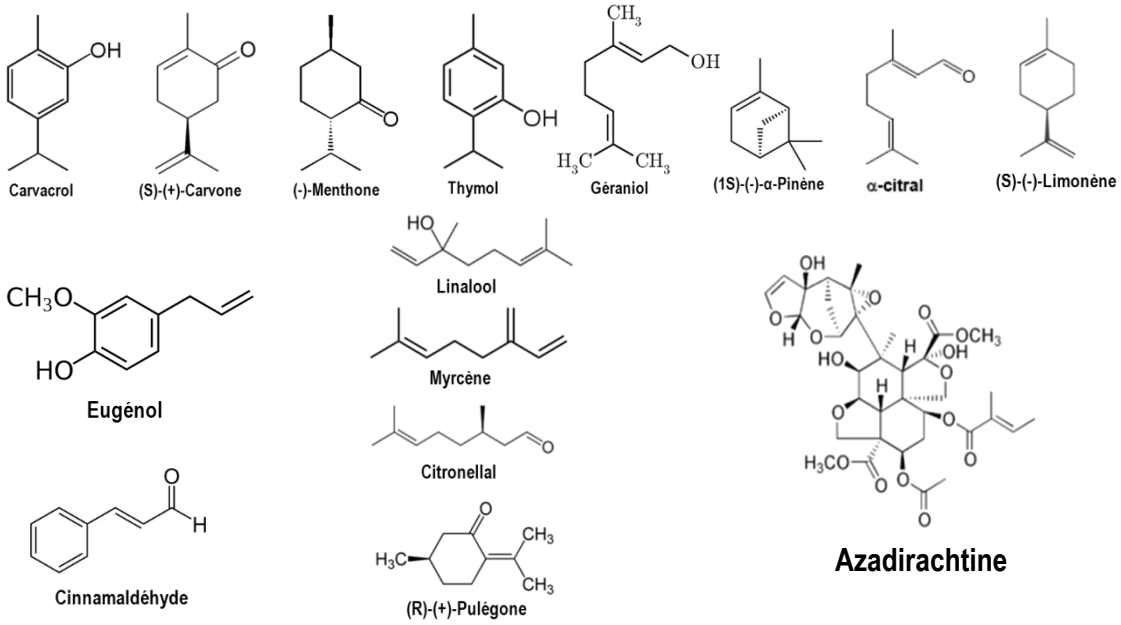

Figure 2 Terpenic substances selected as candidate antifungal biomolecules.

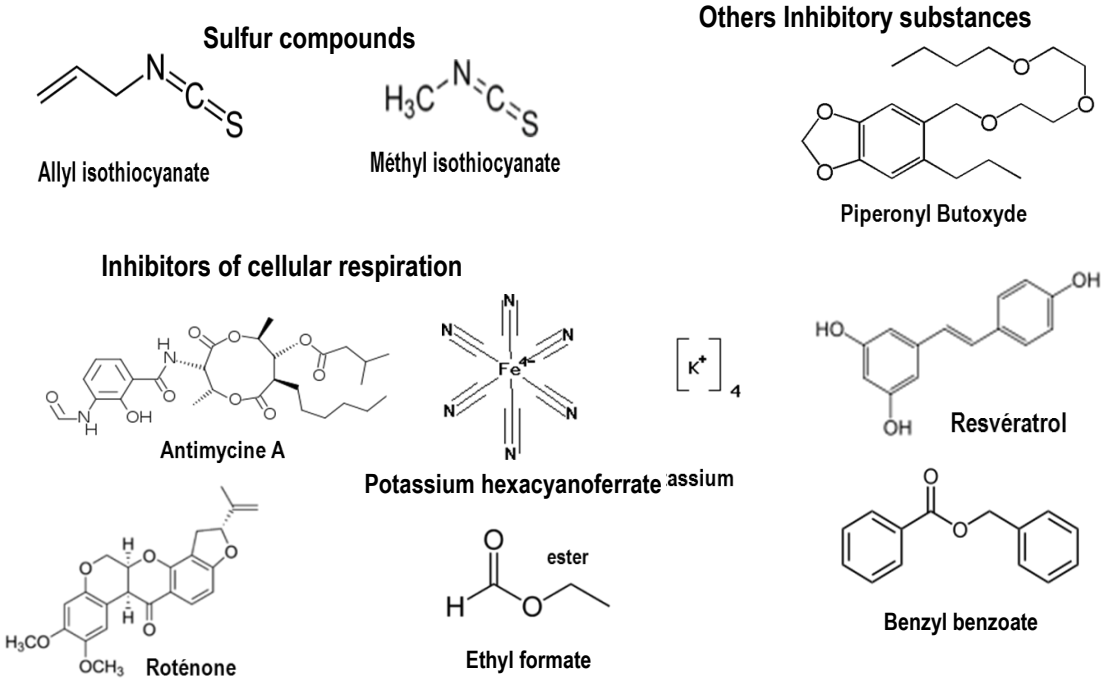

Figure 3 Miscellaneous compounds selected as candidate antifungal biomolecules.

A second series of tests was undertaken with the more active antifungal compounds identified from the first screening: linalool, citral, allylisothiocyanate (AITC) and methylisothiocyanante (MITC), for an accurate determination of inhibitory concentration 50% (IC_{50}) and the minimal inhibitory concentration (MIC), assimilated to IC_{99}.

Bio-active compounds application method

In a preliminary screening aiming at the selection of an appropriate dose range for each compound, each substance was dissolved in ethanol. The test compounds were delivered to inoculated culture medium 48 h after inoculation by the fungus. Each micro-plate well received 10 µL of the appropriate dilution in ethanol to obtain concentrations in liquid culture medium ranging from 0.1 $\mu L.L^{-1}$ to 10^4 $\mu L.L^{-1}$ in decimal progression. Ten µL of pure ethanol was applied either in inoculated control (with fungus inoculum) or in blank control (without fungus inoculum). In a second series of tests, the dose range was adapted to a more narrow range, taking into account the results obtained from the preliminary screening. Immediately after the tested compounds were introduced into the culture medium wells, microplates were sealed by a special adhesive film before incubation which limited culture media evaporation and avoided interference between tested substances or doses. The second series of tests was performed in the same way from fugal cultures in 24-well microtitration plates in liquid medium (other steps unchanged).

2.3. Determination of antifungal activity from fungal culture in microtitration plates

The antifungal properties of the selected candidate biomolecules were evaluated trough the reduction of mycelial growth observed after a 4-day period of incubation after introduction of test compounds into the culture medium (96- or 24-well plates for each screening, respectively). The inoculation with each fungal species was done with 10^4 spores L^{-1} diluted in liquid culture medium. After 48 h fungal growth at 25°C, the microtitration plates were treated with appropriate doses of antifungal candidate compounds. Then, each series of microplates treated with the same compound cultures was isolated in an individual 1.5-L hermetic glass jar during a 4-d incubation period in total darkness at 25°C before assessment of the mycelial growth and comparison of fungal growth with those in inoculated control without any test compound. In the first series of tests in 96-well plates, seven wells (replicates) were used for each compound and concentration (including zero-dose series, *i.e.* the inoculated control series). The eighth well served as non- inoculated control to detect an eventual external contamination (if any was found the whole test was repeated). In the second series aiming at the determination of IC_{50} and MIC for the most active antifungal compounds there were only four replicates (one vertical row of the 24-well plate). The antifungal activity was measured through differential measurements between treated and untreated series.

For the first study carried out on a large number of compounds, two indicators were used: i/a direct measure of absorbance at 630 nm immediately at the end of incubation period; ii/ the measure of the change in color of the Alamar blue reagent at 595 nm four hours after the introduction of the reagent in each plate well. AlamarBlue® assay (alamarBlue™ Cell viability Assay Reagent, Thermo Scientific, USA) is a test for cellular viability appreciation using resazurine reagent, which is a hydrosoluble dye, non toxic, non fluorescent, which pass across cellular membranes. In fungi liquid culture, resazurin is acting as an electron acceptor from mitochondrial cytochromes leading to the transition to a reduced state, resofurin. Resofurin colour is pink and highly fluorescent. The intensity of color change is directly related to the amount of living cell in the liquid culture medium. The intensity of the color change can be semi-automatically red by spectrophotometry or spectroflurofluorometry allowing high-speed screening of a large number of test plates (Fig. 4). For the first screening series, simple spectrophotometric reading of Alamar blue reagent colour change was used (microplate reader Bio-TEK®instruments ELX 800, Germany), while fluorometric readings were used in the second series of tests to obtain optimal accuracy. The culture medium was

buffered by adding MOPS/MES buffers (Sigma-Aldrich, Germany) in this order before adding Alamar blue reagent to avoid immediate colour change of culture media used (Bacha, 2009). The quantification of fungal cell proliferation by alamar blue assay and fluorometric readings were performed by spectrofluorometer (Infinitive® 200 Pro, Tecan, Switzerland) at excitation wavelength 530 nm and emission wavelength 590 nm.

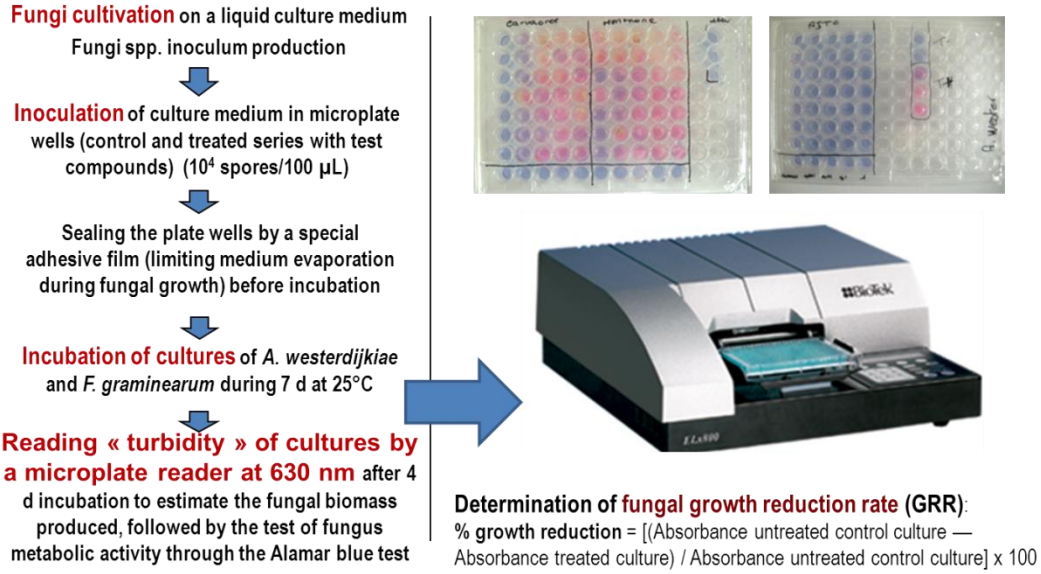

Figure 4 Principles of Alamar blue assay used for the assessment of fungal cell viability after treatment of culture medium by candidate antifungal biomolecules (absorbance readings).

Determination of fungal growth reduction rate (GRR)

1. Index of growth reduction from spectrophotometric measurements:
Growth reduction rate (GRR %) = [(Absorbance untreated control culture — Absorbance treated culture) / Absorbance untreated control culture] x 100

2. Index of growth reduction from fluorescence measurements was calculated as follows:
Growth reduction rate (GRR %) =100-[((FT-FSC)/(FC-FSC)) x 100]
FT: fluorescence in « treated » wells (10 µL of selected compound)
FSC: Fluorescence in blank (not inoculated)
FC: Fluorescence in control series (EtOH 10 µL)

2.4. Analysis of mycotoxin production by treated fungi at infra-lethal dose

After the measurements of fungal growth, the mycelial biomass was separated from the liquid medium by centrifugation, before freeze-drying and weighing. Then, mycotoxins have been extracted and analyzed from the clarified culture medium. The solvent was evaporated under nitrogen. The dried extract was re-dissolved into methanol/water (50/50 v/v) and TCT B content was determined by classical reverse-phase HPLC analysis (H-P ChemStation Series 1100, Agilent Technologies, Palo Alto USA) on column Kinetex 2.6 U XB-C18 (4,6 x 150 mm), 5 µm, 40°C, and DAD. Only the signal at 230 nm was used for the quantification of TCTB content by reference to an internal standard added to injected samples. Ochratoxin A was analyzed after extraction from acidified liquid culture medium by $CHCl_3$ solvent. After evaporation of the solvent under nitrogen, the extract was re-dissolved in pure methanol and OTA concentration was quantified by HPLC analysis (same equipment as above) with column Zorbax Eclipse XDB-C8 (4,6 x 15 mm), 5 µm, 30°C, equipped with an UV detector. Only the signal at 330 nm was kept for the quantification of OTA concentration in injected

samples. In the first assay, after 10 d incubation following the introduction of test compounds in fungal cultures, the extraction of TCTB from *F. graminearum* in organic phase of liquid culture medium was performed for the 11 most bio-active compounds (among 32) by ethyl acetate solvent after pooling the content of three wells (among 7) for each modality (compound*dose) to obtain 2 replicates. In the second restricted study, the production of TCT B and OTA was determined on separated cultures spiked with a dose of the bio-active compounds close to IC_{50}. The procedure of mycotoxin analysis remained unchanged. In this last trial, the time of incubation of *F. graminearum* and *A. westerdijkiae* cultures after inoculation was prolonged up to 10 d and 21 d, respectively, in order to maximize their respective mycotoxin production (if any). The concentration of mycotoxins in culture medium was inferred to the mass of mycelium dry biomass produced.

2.5. Statistical analyses and model used for IC_{50} and MIC assessment

The dose-effect relationship and the significant differences between doses (same compound), or between different compounds at a fixed dose (e.g. inhibitory concentration 50% (IC_{50})) were analysed by an ANOVA after the conversion of the rate of fungal growth reduction (GRR) by an angular transformation (Asin √% GRR). The means (from 7 replicates) were compared and distributed in similarity groups by Bonferroni's test. Data processing was carried out with XLStat software (Addinsoft France, Paris). Data obtained in the first screening are not presented in detail there. In the second assay, the determination of IC_{50} and IC_{99} (considered as equivalent to MIC) were performed by fitting the observed data (4 replicates per assay condition) to the logistic regression model with four parameters. The XLStat software "dose-activity" curve modelling enabled to determine IC_{50} and MIC values with upper and lower limits of confidence intervals with error probability ≤ 0.05.

3. Results and Discussion

3.1. Phenolic acids

Phenolic acids exhibited variable inhibitory effects on *F. graminearum* growth: Trans-cinnamic and cafeic acids induced a reduction of *F. graminearum* growth rate of more than 81.5% from a concentration of 3 $mM.L^{-1}$ in liquid culture medium (simplified culture medium SM). P-coumaric and ferulic acids were poor inhibitors of *F. graminearum* growth rate: only the highest dose of 5 $mM.L^{-1}$ in liquid culture medium induced more than 90% inhibition. Chlorogenic acid had no effect on this fungus strain even at a high dosage (Fig. 5). Tests on *A. westerdijkiae* revealed that this species was more tolerant to phenolic acids than *F. graminearum* (at 10 $mM.L^{-1}$, the maximum growth reduction rate was 86.5% with chlorogenic acid at 10 $mM.L^{-1}$) (Fig. 6).

Surprisingly, chlorogenic acid was the more active compound against *A. westerdijkiae* when it showed no activity on *F. graminearum*.

3.2. Terpenoid compounds

Individual terpenoid compounds (EO constituents) exhibited low inhibitory effects on *F. graminearum* growth when applied at a low dosage. Citral, linalool and clove oils were active from the dose 0.1 $mL.L^{-1}$. Only the highest dose 10 $mL.L^{-1}$ was effective on fungal growth for a majority of the range of tested terpenoid compounds (Fig. 7). *Aspergillus westerdijkiae* growth was affected by S-carvone, citral, geraniol and clove oils at doses from 0.1 to 1 $mL.L^{-1}$. Very high growth reduction rates were observed at a dose of 1 $mL.L^{-1}$ for the following

terpenoid compounds: eugenol, S-carvone, citral, geraniol, and clove oils (Fig. 8). Citral and clove oils were active on the two fungi at a low dosage.

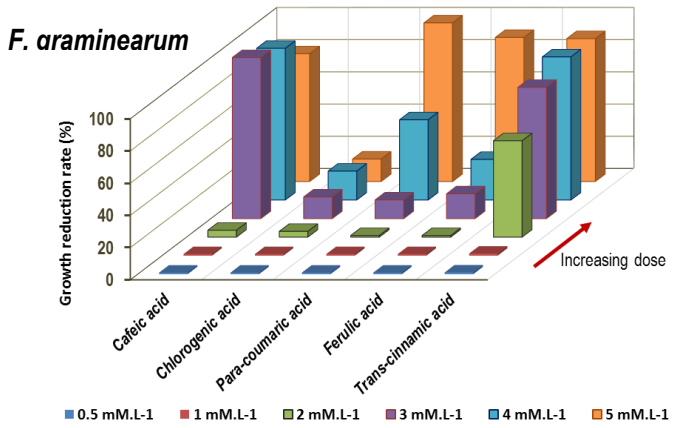

Figure 5 Antifungal activities of some phenolic acid compounds on *F. gaminearum* grown in liquid culture medium (Alamar blue assay with spectrophotometric readings).

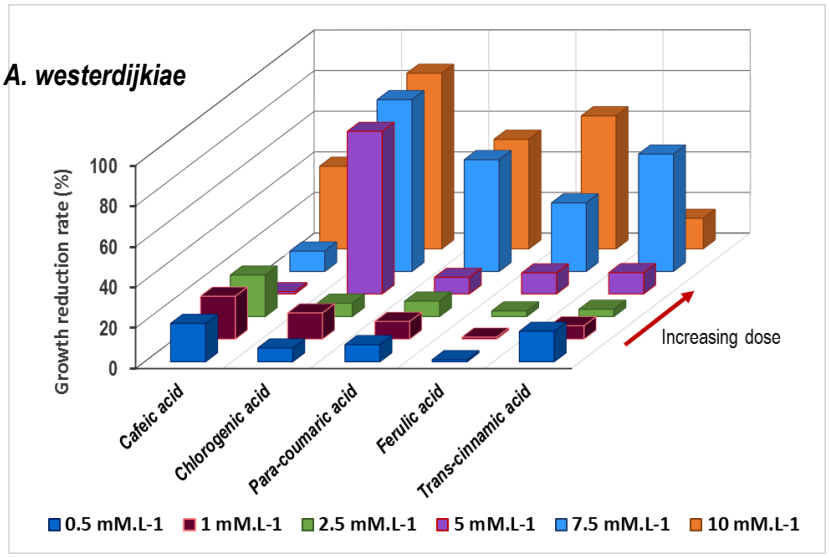

Figure 6 Antifungal activities of some phenolic compounds on *A. westerdijkiae* grown in liquid culture medium (Alamar blue assay with spectrophotometric measurements).

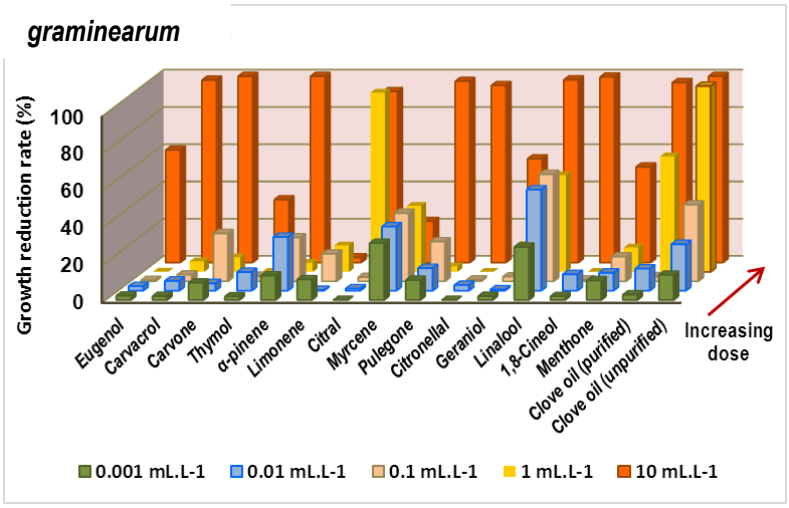

Figure 7 Growth reduction rate induced by terpenoid compounds on *F. graminearum* cultures grown on liquid medium (Alamar blue assay with spectrophotometric reading).

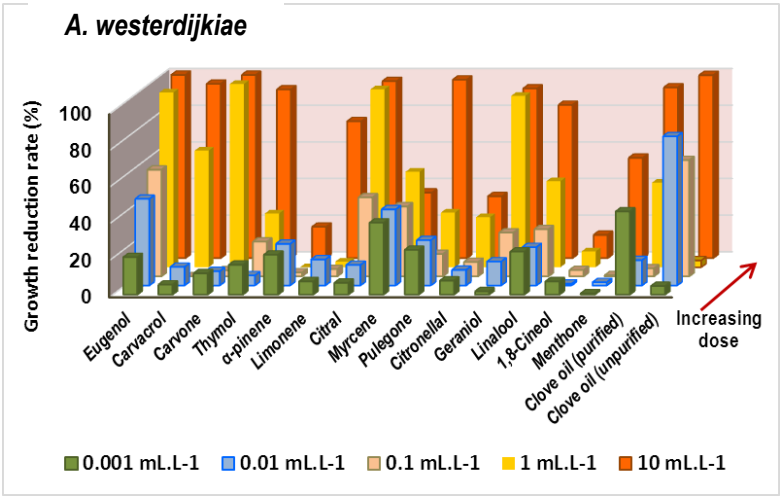

Figure 8 Growth reduction rate induced by terpenoid compounds on *A. westerdijkiae* cultures grown on liquid medium (Alamar blue assay with spectrophotometric reading).

3.3. Miscellaneous substances that may inhibit cellular respiration

F. graminearum and *A. westerdijkiae* were shown both very sensitive to AITC, MITC and trans-cinnamaldehyde. Two inhibitors of mitochondrial respiration, potassium hydroxycyanoferrate (KHCF) and antimycin A, reduce the growth of *F. graminearum* at a high dose range (0.5 – 10 $mM.L^{-1}$). Rotenone was without any observed effect on mycelial growth. Piperonyle butoxide (inhibitor of mono-oxigenase (e.g. cytochrome P-450)) and benzyl benzoate (antifungal compound) induced a significant reduction of fungal growth which was dose-related (Fig. 9, 10).

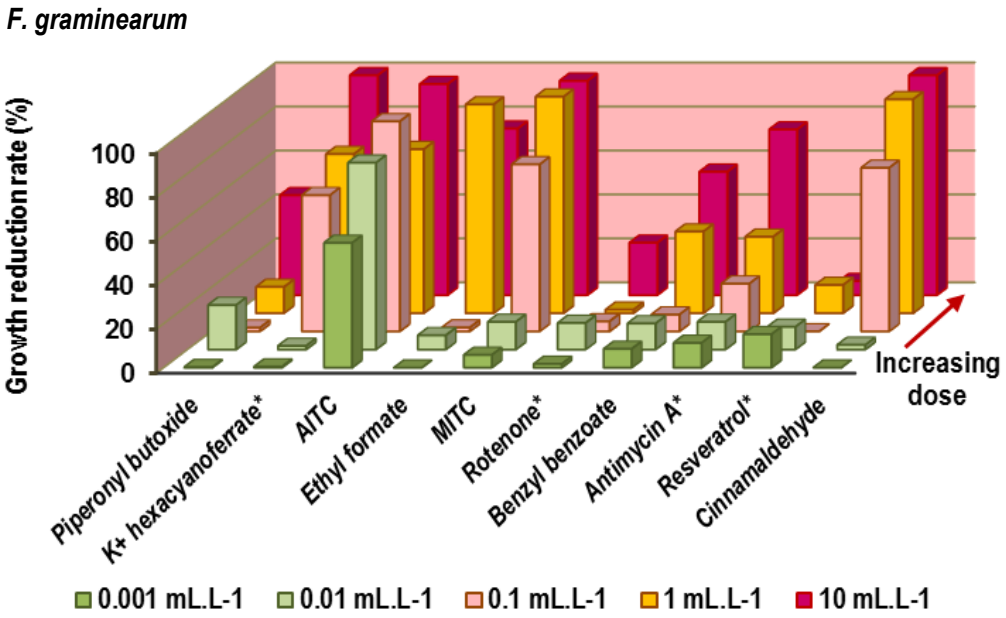

Figure 9 Growth reduction rate induced by cellular respiration inhibitors on *F. graminearum* cultures grown on liquid medium (Alamar blue assay with spectrophotometric reading).

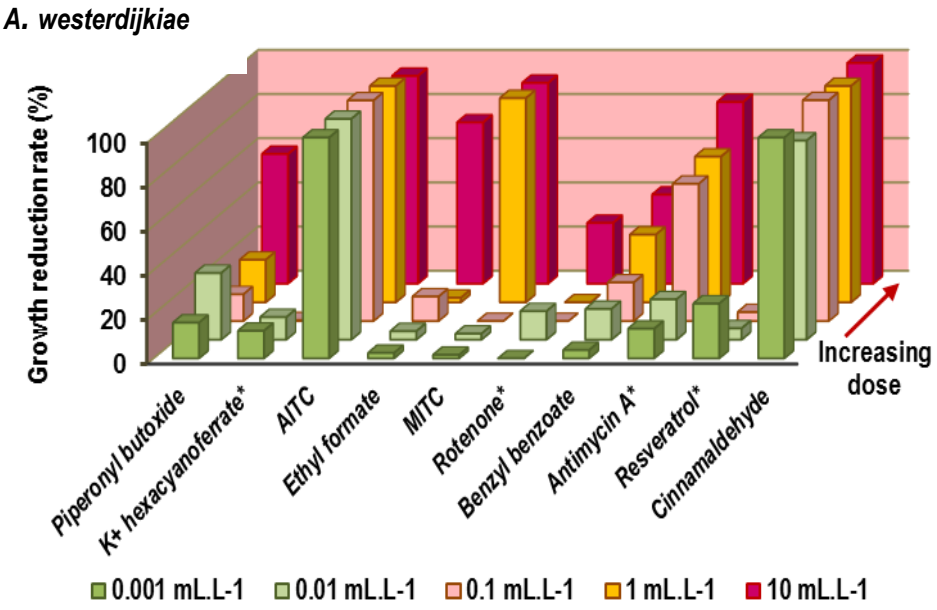

Figure 10 Growth reduction rate induced by cellular respiration inhibitors on *A. westerdijkiae* cultures grown on liquid medium (Alamar blue assay with spectrophotometric reading).

3.4. IC_{50} and MIC of selected antifungal biomolecules for *F. graminearum and A. westerdijkiae*

Alkyl-isothiocyanates, AITC and MITC, exhibited very high antifungal activity on the two fungal species. The recorded MIC value for these two compounds was 4 to 5 times less than MIC value of the two terpenoid compounds, citral and linalool (Table 1). *A. westerdijkiae* had MIC values significantly less than the ones of *F. graminearum*, indicating a higher susceptibility of *A. westerdijkiae* to test biomolecules than *F. graminearum*. The advantage of

alkyl-isothiocyanates over oxygenated terpenoids may be related to their high vapour pressure and a fumigant effect revealed in a previous study (Cardiet et al., 2012).

Table 1 IC_{50} and MIC value of growth reduction rate for *F. graminearum* and *A. westerdijkiae* cultivated in vitro after treatment by selected efficient antifungal biomolecules.

		IC_{50} (µL.L^{-1})			MIC (µL.L^{-1})		
Fungus sp. and strain	**Compound**	**Value**	**Low F.L.**	**High F.L.**	**Value**	**Low F.L.**	**High F.L.**
F. graminearum INRA 349 strain	Citral	**190.5**	178.8	202.6	**496.1**	467.6	529.6
	Linalool	**358.3**	nd	nd	**768.4**	nd	nd
	AITC	**27.9**	24.5	31.1	**99.1**	94.1	104.9
	MITC	**39.6**	36.6	42.3	**110.1**	104.8	116.4
A. Westerdijkiae INRA Toxalim strain	Citral	**67.9**	61.5	74.2	**179.6**	168.3	192.9
	Linalool	**389.0**	nd	nd	**1074.1**	nd	nd
	AITC	**25.7**	22.7	28.4	**51.4**	48.0	55.5
	MITC	23.9	21.4	26.3	49.6	46.7	52.9

3.5. Effect of antifungal biomolecules on mycotoxigenesis

After the first screening on 32 biomolecules, 11 selected compounds from this first large screening having the higher activity on *F. graminearum* (strain INRA 349) growth were « treated » by a non-lethal dose of antifungal compounds clearly identified beforehand. In these new conditions, it was observed that *F. graminearum* produced about 50% biomass compared to a control (untreated) culture (Fig. 11A). The production of trichothecenes B (DON + A-DON) related to the amount of dry fungal biomass production was at least divided by six compared to the production in control (untreated) culture. In most of the series, although it was observed mycelial significant production, mycotoxins production remained nil (Fig. 11B). All the measures of TCTB or OTA concentration in the treated cultures were weighed by the corresponding biomass produced in the same time. The concentration was presented as the content in mycotoxins per biomass production (µg.mg^{-1} fungal biomass). In our experimental conditions, it was shown that for *F. graminearum*, GRR was accompanied in all cases by a reduction of mycotoxin biosynthesis in large proportions. No "boosting" effect of infra-lethal dose of bio-active compounds in culture medium was observed.

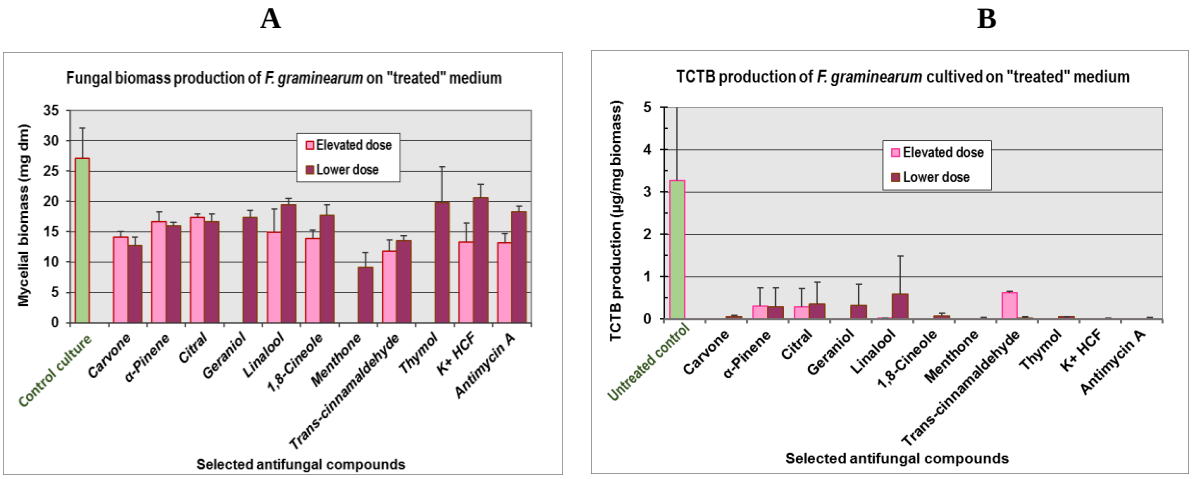

Figure 11 Fungal biomass production (1) and TCTB concentration (2) in *F. graminearum* culture medium after treatment by non-lethal dose of a range of antifungal compounds.

In the first screening, *A. westerdijkiae* did not produce quantifiable amounts of OTA when grown on liquid culture medium during 10 d. In the second study with the four most efficient antifungal compounds, at infra-lethal dose still enabling mycelial growth: citral, linalool, AITC and MITC, we demonstrated that: i/ These tested compounds has proven to be effective antifungal molecules with activity both on *F. graminearum* and *A. westerdijkiae* according to the high reduction of biomass production during extended periods of culture (10 d for *F. graminearum* and 21 d for *A. westerdijkiae*); ii/ These molecules reduced the production of mycotoxins by the two fungi at a very low level, close to the limit of quantification (Fig. 12).

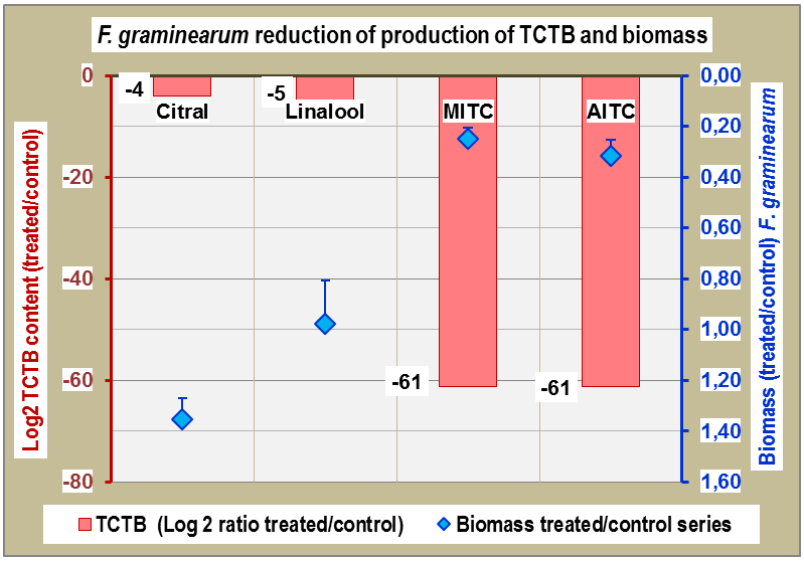

Figure 12 Inhibition of TCTB production and reduction of biomass produced by *F. graminearum* treated by four antifungal biomolecules (at infra-lethal dose).

The rapid bioassay we developed to screen EOs and other plant extracts individual constituents for their antifungal activity from *in vitro* culture against two fungi grown in liquid medium give results closely correlated with the ones of conventional tests performed

on solid culture medium of the same fungi done in a previous study (Cardiet et al., 2012). Some of the molecules showing the highest antifungal activity against *F. graminearum* and *A. westerdijkiae* were already known as effective against other molds or yeasts: citral against *Candida albicans* (Alvino Leite et al., 2014); clove oil (eugenol) against *A. niger* (Bouddine et al., 2012); geranial and neral against *Phytophtora cactorum* (Lee et al., 2008); linalool against cellulolytic molds of paper books (Rakotonirainy and Lavédrine, 2005). However, any study proposed a screening method allowing the simultaneous comparison of a large range of candidate antifungal molecules, with accurate and reproducible tests.

This bioassay with standardized lecture of results by spectrometric and fluorometric reading may allow to prospect very rapidly not only for antifungal potential of individual biomolecules, but also for synergism in combinations of associated compounds with different mode of action or dose ratio. Additionally, we demonstrated that there was no negative impact of "good" antifungal compounds on mycotoxigenesis with the two test fungi. It was confirmed that compounds with high vapour pressure, AITC and MITC, have a promising potential as antifungal agents, being active on fungal growth and on spore germination as well (Cardiet et al., 2012). This high vapor pressure is probably confering to these molecules a very high power of fungal cell membrane crossing and a rapid interaction with their biochemical target in the mitochondrion (leading to cell respiration inhibition). The Alamar blue assay was shown very accurate when supported by fluorescence readings that minimize the interference of the presence of fungal biomass in the microtitration plates during the lecture. The determination of the growth reduction rate was more accurate than with simple spectrophotometric (or turbidity) measurements, with more importance of the presence of mycelium that may interfer with optical wavelengths intensity measurements.

4. Conclusions and future prospects

The rapid bioassay we refined in this study allowed to screen a large number of antifungal candidate biomolecules and to accurately determine IC_{50} and MIC for each of them. From this bioassay, we showed in a first screening using AlamarBlue assay protocol and spectrophotometric readings that among phenolic acids, trans-cinnamic acid and cafeic acid induced the most effective mycelial growth limitation for *F. graminearum* cultures (MIC in the range 2.5 – 4 $mM.L^{-1}$) and chlorogenic acid for *A. westerdijkiae* cultures. Among terpenoid compounds, eugenol, carvone, citral, and raw clove oil exhibited the highest antifungal activity (MIC for *A. westerdijkiae*, respectively 5, 4.5, 8, and 5 $\mu L.L^{-1}$). Among miscellaneous substances, piperonyl butoxide, AITC, and MITC showed the highest antifungal activity (MIC assessed respectively at 16, 3, and 6 $\mu L.L^{-1}$ for *F. graminearum*). With the four substances with the highest antifungal effect: citral, linalool, AITC and MITC, the addition of these substances in the culture medium did not induce any increase in mycotoxin production by the two fungi species.

Future studies could examine the potential of the most active detected antifungal compounds on a larger range of species and strains of noxious fungi, either phytopathogenic (*Penicillium, Rhizoctonia, Pythium, Botrytis, Alternaria,* etc.) or pathogenic for human and/or animal health (*Aspergillus, Byssochlamys, Penicillium, Trichophyton*, etc.). The main difficulty to overcome before an eventual development for use as plant protection specialties is associated to the need to develop a controlled-release formulation specifically design to regulate the diffusion of these volatile substances proven to be effective against *Fusarium graminearum* and *Aspergillus westerdijkiae.*

Acknowledgements

We are grateful to B. Fuzeau†, M.N. Bonin-Verdal, V. Atanasova-Penichon, A.-L. Boutigny, and J. Merhej who contributed to different parts of this study, especially in experimental design and analytical works. We thank O. Puel (UMR Toxalim, Toulouse, France) for kindly affording *A. westerdijkiae* strain used in this study. This work was part of the integrated research and development project "EcopreservGrain" granted by "Conseil Régional d'Aquitaine" (Bordeaux, France).

References

Almeida Freires, I. de, Mendonça Murata, R., Furletti, V.F., Sartoratto, A., Mathias de Alencar, S., Mara Figueira, G., Aparecida de Oliveira Rodrigues, J., Teixera Duarte, M.C., Rosalen, P.L., 2014. Coriander sativum L. (Coriander) essential oil: antifungal activity and mode of action on Candida spp., and molecular targets affected in human whole-genome expression. Plos One 9, e99086. www.plosone.org

Alvino Leite, M.C., Parente de Brito Bezerra, A., Pereira de Sousa, J., Queiroga Sarmento Guerra, F., Oliveira Lima, E. de, 2014. Evaluation of antifungal activity and mechanisms of action of citral against *Candida albicans*. Evidence-based Complementary and Alternative Medicine, http://dx.doi.org/10.1155/2014/378280 , 9 p.

Aly, S.E., Sabry, B.A., Shaheen, M.S., Hathout, A.S., 2014. Assessment of antimycotoxigenic and antioxidant activity of star anise (Illicium verum) *in vitro*. Journal of the Saudi Arabia Society of Agricultural Sciences (*in press*) http://dx.doi.org/10.1016/j.jssas.2014.05.003

Amini, M., Safaie, N., Salmani, M.J., Shams-Bakhsh, M., 2012. Antifungal activity of three medicinal plant essential oils against some phytopathogenic fungi. Trakia Journal of Sciences 10, 1-8.

Bacha, N., 2009. Caractérisation des polycétones synthases intervenant dans la biosynthèse d'ochratoxine A, d'acide pénicillique, d'asperlactone et d'isoasperlactone chez *Aspergillus westerdijkiae*. PhD thesis, Polytechnics Institute of Toulouse University, France.

Borrego, S., Valdès, O., Vivar, I., Lavin, P., Guiamet, P., BBattistoni, P., Gómez de Saravia, S., Borges, P., 2012. Essential oils of plants as biocides against microorganisms isolated from Cuban and Argentine documentary heritage. ISRN Microbiology 2012, doi: 10.5402/2012/826786, 7 p.

Bouddine, L., LLouaste, B., Achahbar, S., Chami, N., Chami, F., Remmal, A., 2012. Comparative study of the antifungal activity of some essential oils and their major phenolic components against *Aspergillus niger* using three different methods. African Journal of Biotechnology 11, 14083-14087.

Cardiet, G., Fuzeau, B., Barreau, C., Fleurat-Lessard, F., 2012. Contact and fumigant toxicity of some essential oil constituents against a grain insect pest *Sitophilus oryzae* and two fungi, *Aspergillus westerdijkiae* and *Fusarium graminearum.* Journal of Pest Science 85, 351-358.

Debourdeau, G., Boutigny, A.L., Merhej, J., Fleurat-Lessard, F., 2012. Caractérisation de l'activité antifongique de biomolécules d'origine végétale sur les champignons mycotoxinogènes : *Aspergillus westerdijkiae* et *Fusarium graminearum.* Proceedings Journée Nationale « Mycotoxines » Talence, France, INRA-MycSA Bordeaux Publ. (abstract).

Faleiro, M.L., 2011. The mode of bacterial action of essential oils. In Mendés-Vilas (Ed.) Science against microbial pathogens: communicating current research and technological advances. ©Formatex 2011, pp. 1143-1156.

Gacem, M.A., Ould El Hadj Khelil, A., Gacemi, B., Halla, N., Djerbaoui, A.N., Bouderhem, A., Hadef, S., Benreguieg, M., Adli, D.E.H., 2013. Antimlycotoxigenic and antifungal activities of Citrullus colocynthis seeds against *Aspergillus flavus* and *Aspergillus ochraceus* contaminating wheat stored. African Journal of Biotechnology 112, 6222-6231.

Hussain, A.I., Anwar, F., Nigam, P.S., Ashraf, M., Gilani, A.H., 2010. Seasonal variation in content, chemical composition and antimicrobial and cytotoxic activities of essential oils from Mentha species. Journal of Science in Food and Agriculture 90, 1827-1836.

Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Frontiers in Microbiology 3, article 12, doi: 10.3389/fmicb.2012.00012.

Isman, M.B., 2008. Perspective botanical insecticides: for richer, for poorer. Pest Management Science 64, 8-11.

Krisch, J., Tserennadmid, R., Vágvölgyi, C., 2011. Essential oils against yeasts and moulds causing food spoilage. In Mendés-Vilas (Ed.) Science against microbial pathogens: communicating current research and technological advances. ©Formatex 2011, pp. 11335-1142.

Lee, Y.-S., Kim, J., Lee, S.-G., Shin, S.-C., Park, I.-K., 2008. Fumigant antifungal activity of essential oil components from *Acorus gramineus* against three phytopathogenic fungi. Zeitschrift für Naturforschung 63c, 503-506.

Lee, Y.-S., Kim, J., Lee, S.-G., Shin, S.-C., Park, I.-K., 2008. Antifungal activity of *Myrtaceae* essential oils and their components against three phytopathogenic fungi. Flavour and Fragances Journal 23, 23-28.

Maurya, S., Kushwaha, A.K., Singh, G., 2013. Biological significance of spicy essential oils. Advances in Natural Science 6, 84-95.

Mediouni Ben Jemâa, J., Haouel, S., Bouaziz, M., Khouja, M.L., 2012., Seasonal variations in chemical composition and fumigant activity of five *Eucalyptus* essential oils against three moth pests of stored dates in Tunisia. Journal of Stored Products Research 48, 61-67.

Pauli, A., Anticandidal low molecular compounds from higher plants with special reference to compounds from essential oils. Medical Research Review 26, 223-268.

Rakotoniraini, M.S., Lavédrine, B., 2004. Screening for antifungal activity of essential oils and related compounds to control the biocontamination in libraries and archives storage areas. International Biodeterioration & Biodegradation 55, 141-147.

Mahoney, N., Molyneux, R., 2004. Phytochemical inhibition of aflatoxigenicity in *Aspergillus flavus* by constituents of walnut (*Junglans regia*). Journal of Agricultural and Food Chemistry 52, 1882-1889.

Samie, A., Nefefe, T., 2011. Antifungal activities of essential oils from Southern African medicinal plants against five *Fusarium* species. Journal of Medicinal Plants Research 6, 465-478.

Soković, M.D., Vucojević, J., Marin, P.D., Brkić, D.D., Vajs, V., van Griensven, L.J.L.D., 2009. Chemical composition of essential oils of *Thymus* and *Mentha* species and their antifungal activities. Molecules 14, 238-249.

Tabassum, N., Vidyasagar, G.M., 2013. Antifungal investigations on plant essential oils. A review. International Journal of Pharmacy and Pharmaceutical Sciences 5, suppl. 2, 19-28.

Velluti, A., Sanchis, V., Ramos, A.J., Turon, C., Marin, S., 2004. Impact of essential oils on growth rate, zearalenone and deoxynivalenol production by *Fusarium graminearum* under different temperature and water activity conditions in maize grain. Journal of Applied Microbiology 96, 716-724.