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Activity of essential oils from *Syzygium aromaticum* and *Rosmarinus officinalis* against growth and ochratoxin A production by *Aspergillus tubingensis* and *Aspergillus luchuensis* from Moroccan grapes

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Summary. Essential oils have been used since ancient times in traditional medicine and agri-food science to preserve food, and to combat human diseases. Essential oils (EOs) from clove and rosemary, obtained by hydro-distillation, were analyzed by GC/MS and evaluated for their antifungal activity against strains of *Aspergillus tubingensis* and *A. luchuensis* and their effect on ochratoxin A (OTA) production by these fungi. The major constituent of clove EO was eugenol (86.4%), and of rosemary EO was eucalyptol (35.9%). Mycelium growth inhibition assays showed that the EOs had dose-depend inhibition effects, which reached 36.6% for rosemary EO, and 100% for clove EO, at EO concentration of 200 µL L^{-1}. These reductions were different for different *Aspergillus* strains, the essential oil type, and the strain/oil type interaction. Analyses of OTA in the culture medium extracts of *Aspergillus* strains was carried out using HPLC-FLD, and was confirmed by LC-MS/MS for positive controls of two OTA-producing strains of *A. tubingensis*. In general, OTA was reduced (from 45 to 100% reduction), except for two strains of *A. tubingensis*, where OTA production was stimulated.

Key words. Antifungal effect, mycotoxins, biological control, clove, rosemary.

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INTRODUCTION

Aspergillus species are widely recognized as food contaminants, which may create severe economic losses, and constitute health risk for consumers due to their ability to produce mycotoxins. Ochratoxin A (OTA) is the one of the most frequent toxic secondary metabolites produced by several fungal species belonging to *Aspergillus* (e.g. *A. ochraceus, A. carbonarius, A. niger, A. alliaceus, A. tubingensis, A. luchuensis*) and *Penicillium* (e.g *P. verrucosum, P. nordicum, P. expansum*). This mycotoxin is hazardous to human health since it can be neurotoxic, carcinogenic, nephrotoxic, hepatotoxic, teratogenic and immunosuppressive (Murphy *et al*., 2006; IARC, 2009; Coronel *et al*., 2010). OTA has been classified as a possible carcinogen for humans (group 2B) by the International Agency for Research in Cancer (IARC, 1993). This mycotoxin has been frequently detected in a wide range of agricultural and food products, including cereals and derived products (Hajjaji *et al*., 2006; Gamza *et al*., 2015), cocoa (Copetti *et al*., 2010), grapes, grape and apple juices (Battilani *et al*., 2006; Leong *et al*., 2006; Selouane *et al*., 2009), and spices (Zinedine *et al*., 2007; Wan Ainiza *et al*., 2015).

To reduce potential hazards from mycotoxin-producing fungi in crops and stored agricultural products, control of mycotoxin-producing fungi using conventional fungicides is often advocated. However, the use of antifungal chemicals is progressively becoming restricted by food safety regulations, as it could lead to development of resistant strains and occurrence of residues in food, feed, crops and the environment. These fungicides may also stimulate the production of mycotoxins (Audenaert *et al*., 2010; Zouhair *et al*., 2014). Therefore, research for alternative antifungal substances is important to control the growth of spoilage fungi and risks of OTA.

Essential oils (EOs) are possible natural alternatives to synthetic fungicides. Several aromatic and medicinal plants have received attention, and have been studied for control of growth of toxigenic fungi to reduce production of mycotoxins (Lappa *et al*., 2017; Mateo *et al*., 2017; Lasram *et al*., 2019; Oliveira *et al*., 2020). EOs are complex mixtures of volatile organic compounds, mainly terpenoids, aromatic, and aliphatic components, all characterized by low molecular weights (Bassole and Juliani, 2012). The Food and Drugs Administration (FDA) classified theses oils "Generally Recognized as Safe" (GRAS) substances (Edris, 2007). Several biological activities have been attributed to EOs, including antibacterial (Risaliti *et al*., 2019) and antifungal properties (Bomfim *et al*., 2015; Santamarina *et al*., 2016). EOs contain major and minor components, and mixtures are more biologically active than major mixed constituents (Gill *et al*., 2002; Mourey and Canillac, 2002), which suggests that the minor components may have potentiating influences or synergistic effects.

Antifungal activity of clove and rosemary essential oils against mycotoxin-producing fungi has been reported (Rasooli *et al*., 2008; Bouddine *et al*., 2012; Bomfim *et al*., 2015; Boukaew *et al*., 2017). El Khoury *et al*. (2016) found that rosemary EO inhibited production of OTA by *A. carbonarius*. In addition, the antifungal activity of the major compounds (eugenol and eucalyptol) of these EOs has also been reported (Morcia *et al*., 2012; Jahanshiri *et al*., 2015; Caceres *et al*., 2016; Nazzaro *et al*., 2017).

The aim of the present work was to assess effects EOs from *Syzygium aromaticum* (clove) and *Rosmarinus officinalis* (rosemary) on growth and OTA production for eight strains of *A. tubingensis* and *A. luchuensis*.

MATERIALS AND METHODS

Fungus strains and culture conditions

Seven strains of *A. tubingensis* (MUCL54478, MUCL54481, MUCL54482, MUCL54483, MUCL54484, MUCL54485 and MUCL54486) and one strain of *A. luchuensis* (MUCL54477) were used in this study. These strains were previously isolated from grapes produced in Morocco. All strains were identified (Qjidaa *et al*., 2014), and deposited at BCCM/MUCL (Mycothèque de l'Université Catholique de Louvain, Belgium). The strains (suspensions of conidia) were maintained in 25% glycerol + 0.01% Tween 80 at -20°C. Prior to the study, OTA production of the eight strains was confirmed *in vitro* on Czapek Yeast Autolysate agar (CYA). The identification of the OTA peak in chromatograms was confirmed by methylation according to Zimmerli and Dick, (1995). Inocula for the experiments were prepared by growing each strain on CYA at 25°C for 7 d. Suspensions of conidia ($\approx 10^5$ conidia mL^{-1}) were prepared in sterile distilled water containing 0.5% Tween 80. A Thoma chamber was used to determine the final conidium concentrations.

Essential oil isolation

Dried *S. aromaticum* or dried leaves of *R. officinalis* (100 g) were hydro-distillated (3 h) using a Clevenger-type apparatus. EOs were stored at 4°C in the dark before analysis.

Antifungal tests

In vitro experiments to evaluate the efficacy of EOs against *A. tubingensis* and *A. luchuensis* were carried out using a CYA medium (0.99a_w). Conidia of *A. tubingensis* or *A. luchuensis* were each exposed to a series of increasing concentrations of the tested antifungal agents (Remmal *et al.*, 1993). The EO was emulsified with a 0.2% agar solution to disperse the constituent compounds. Volumes of this dilution were added to sterilized CYA medium and cooled to 45–50°C. The final essential oil concentrations used were 25, 50, 100 and 200 µL L^{-1}. Petri plates were prepared with the different amended media, and experimental controls containing the culture medium alone were also prepared. The plates were each single-point inoculated with 10 µL of a suspension of the appropriate conidia (≈ 10^5 conidia), and the plates were then incubated at 25°C. All the experiments were carried out in triplicate. The Petri plates were examined daily, and the diameter of resulting colonies was measured in two perpendicular directions during a period of 7 d. Linear regression of colony radius (mm) against time (d, from the day the colony was 5 mm diam.) was used to determine growth rates (mm d^{-1}). Lag phase for growth was considered as the number of days elapsed between inoculation and the time when fungal growth became evident (colony reached 5 mm diam.). Petri plates were also used for OTA extractions and determination.

Essential oils analyses

Gas chromatography-mass spectrometry (GC/MS) analyses were carried out using a Thermo Finnigan gas chromatograph directly coupled to a mass spectrometer system (model TRACE GC ULTRA S/N 20062969; Polaris Q S/N 210729). Retention Indices and the confirmation of MS identified compounds were carried out using a gas chromatograph with flame-ionization detection (GC-FID) Thermos Finnigan Trace GC 2000, under the same conditions as used for GC/MS analyses. Compounds were separated using a TR-5 capillary column (60 m × 0.32 mm, 0.25 µm film thickness). The Oven temperature was maintained at 40°C for 2 min, the programmed to 280°C at 5°C min^{-1}, and the final temperature kept for 10 min. The splitless injector was maintained at 220°C and opened after 0.85 min. The flow rate of carrier gas (helium) was 1 mL min^{-1}, and the volume of injected specimen was one µL of diluted oil in hexane. Electron impact mass spectra were recorded at 70 eV. The ion source temperature was 200°C, the interface line temperature was 300°C, and the scan mass range m/z was of 40-650. The same method was previously used by Bennouna *et al.* (2018).

Extraction and OTA analyses

Ochratoxin A was extracted using the method of Bragulat *et al.* (2001). Briefly, three agar plugs (diam. = 7 mm) were removed from the inner, middle, and outer areas of each fungus colony. The plugs were weighed and then dispensed into 3 mL vials before adding 1 mL of methanol to each vial. The vials were shaken for 5 sec with an autovortex and then incubated at 25°C for 60 min. The extracts were centrifuged three times for 10 min at 13,000 rpm. The supernatant was then filter-sterilized through a PVDF hydrophilic filter (0.22 µm) and then analyzed using HPLC technology (Agilent Technologies) with fluorescence detection (excitation 333 nm, emission 460 nm; calibration with OTA standard (Sigma Aldrich). The separation of metabolites was carried out on a C18 RP column (Zorbax SB, 4.6 × 250 mm × 5 µm particle size). The mobile phase (acetonitrile-water-acetic acid; 99:99:2, v/v/v) was pumped at 0.7 mL min^{-1}, and the injection volume reached 20 µL. Run time for samples was 30 min, with OTA being detected at about 11 min. OTA was identified by its retention time (11-11.6 min), according to the OTA standard.

Quantification of OTA was achieved by measuring peak areas according to a linear standard curve, and the limit of detection was 0.2 ppb. All analyses were repeated three times. OTA production by ochratoxigenic fungus strains was confirmed using an UPLC-MS/MS system (Waters) coupled with a triple-quadrupole (Xevo TQ-S micro) mass spectrometer (MS). The OTA was separated using an UPLC C18 column (2.1 µm particle size, 1.7 × 100 mm) with a C18 pre-column (2.1 µm particle size, 1.7 × 20 mm). Both the column and guard column were maintained at 45°C. The flow rate was 0.4 mL min^{-1} and injection volume of 2 µL. The mobile phase consisted of a gradient (Table 1) achieved with the following mobile phase: phase A) water/0.1% formic acid/5 mM ammonium formate, and phase B) methanol/0.1% formic acid/5 mM ammonium formate.

Table 1. Gradient profile of LC-MS/MS.

Time (min)	Phase A	Phase B
0	98	2
1	98	2
13	1	99
14	98	2
17	98	2

The mass spectrometry analysis ESI-MS/MS was carried out using the positive electrospray ionization mode (ESI+) in a multiple reaction monitoring mode (MRM) at capillary voltage of 3.5 kV, cone voltage of 8 V, source temperature of 150°C, and desolvation temperature of 500°C. Argon was used as desolvation with a flow rate of 1000 L h^{-1}. Full scan mode was employed in the mass range of 30-1250 Da. The transition of the most abundant product transition ion (m/z 404.2 < 239.1) and the second least abundant transition (target or confirmatory) ion (m/z 404.2 < 358.2) were selected for quantification and identification. MassLynx (V4.1) software (Waters Corporation) was used for the data acquisition.

Statistical analyses

Linear regressions of colony radius against time (d) was used to determine the growth rates (mm d^{-1}) under each set of conditions, were obtained with the program Microsoft Excel version 2013. Analysis of variance for the different growth data sets was carried out using IBM SPSS Statistics, version 20.

RESULTS

Essential oil composition

Hydro-distillation of clove and rosemary tissues yielded an average, respectively, of 3 mL and 2 mL for 100 g of the dry plant material. Based on the results of the GC/MS and GC/FID analyses, different compounds were identified in the oils (Table 2). The most abundant chemical components were eugenol (86.4%), eugenyl acetate (8.9%) and a-humulene (1.1%) for clove EO, and eucalyptol (35.9%), camphor (21.2%) and o-cymene (4.8%) for rosemary EO.

Essential oil antifungal effects on fungus lag phases and growth rates

The lag phases observed in CYA cultures supplemented with clove EO were always greater than for rosemary EO, and this under all doses tested. A concentration of 200 µL L^{-1} of clove oil completely inhibited growth of two ochratoxigenic strains of *A. tubingensis* (MUCL54482 and MUCL54484) and two non-ochratoxingenic strains *A. tubingensis* MUCL54486 and *A. luchuensis* MUCL54477. In contrast, the lag phases for the control cultures were 1 d, and for the cultures supplemented with clove EO were about 2 to 3 d for 25 to 50 µL L^{-1}, and 4 to 6 d for 100 to 200 µL L^{-1}. However, the lag phases after rosemary EO treatments were similar for all doses tested (approx. 2 d).

Statistical analyses (Table 3) revealed that factors of the EOs, EO concentrations of EOs, fungus strains, as well as their interactions, had significant effects ($P \leq 0.001$) on the antifungal activity. In control plates, mycelium growth had already reached the edges of the Petri dishes after 7 d of incubation, and the mean growth rate was 7.4 mm d^{-1}. Clove EO at different concentrations (0-200 µL L^{-1}) decreased the radial growth rate of all the tested strains (Figure 1). Clove EO applied at 25 µL L^{-1} gave mean growth rates between 6.4 and 7.3 mm d^{-1}, and mean growth rate reductions varied between 1.2

Table 2. Chemical composition of rosemary and clove essential oils.

Retention Index	Compound	Identification	Amount (%)	
			Rosemary	Clove
940	α-pinene	GC, MS	0.12	-
956	camphene	GC, MS	4.15	-
1024	γ-terpinen	GC, MS	0.35	-
1035	o-cymene	GC, MS	4.79	-
1044	eucalyptol	MS	35.88	-
1166	camphor	GC, MS	21.15	-
1186	borneol	GC, MS	4.68	-
1208	α-terpinol	GC, MS	2.90	-
1232	verbenone	MS	0.56	-
1301	bornyl acetate	MS	0.38	-
1390	eugenol	MS	-	86.41
1442	a-humulene	GC, MS	1.24	1.11
1477	germacren D	MS	0.15	0.14
1552	eugenyl acetate	MS	-	8.94
1615	caryophyllene oxide	MS	-	0.12
Total			76.35	96.72

Table 3. ANOVA for effects of essential oils and their concentrations on growth rates of strains of *Aspergillus* section *Nigri*.

Source of variation	Growth study			*P*
	DF[(a)]	MS[(b)]	F	
Strain (S)	7	2,835	14,812	0.000
Concentration (C)	4	131,808	688,548	0.000
Essential oil (EO)	1	65,125	340,206	0.000
S × C	28	0,731	3,817	0.000
S × EO	7	2,546	13,299	0.000
C × EO	4	61,150	319,441	0.000
S × EO × C	28	0,416	2,175	0.001

DF[(a)], degrees of freedom
MS[(b)], mean square

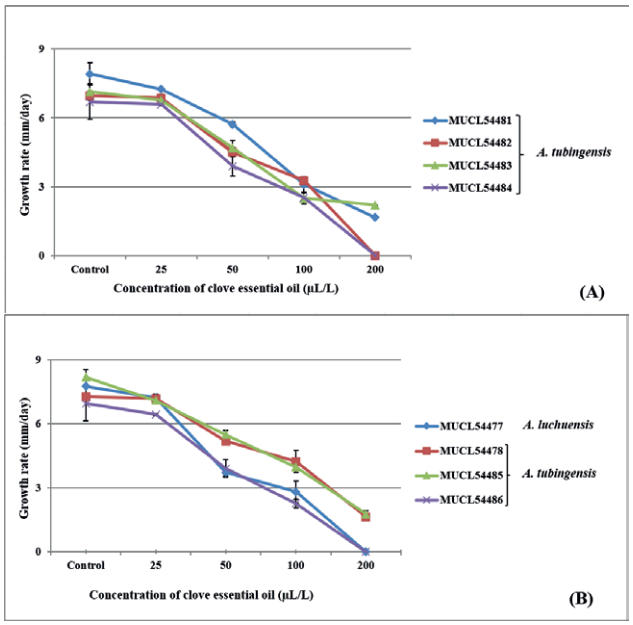

Figure 1. Mean colony growth rates (mm d^{-1}) of *Aspergillus tubingensis* and *A. luchuensis* strains on CYA medium supplemented with different concentrations of clove essential oil (A: OTA producing strains; B: non-OTA producing strains).

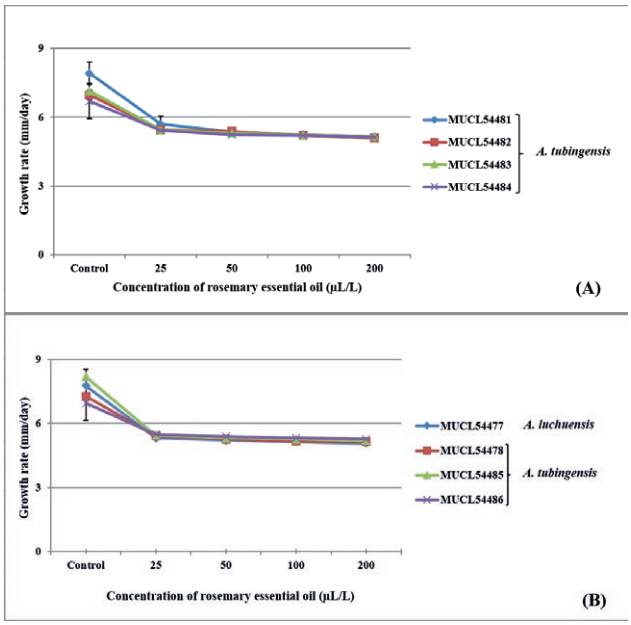

Figure 2. Mean growth rates (mm d^{-1}) of *Aspergillus tubingensis* and *A. luchuensis* on CYA medium supplemented with different concentrations of rosemary essential oil. (A: OTA producing strains; B: non-OTA producing strains).

and 13.3%. Greater concentrations of clove EO, complete (100%) inhibition of half of the tested strains. However, for the other strains, growth rate reductions compared to the control varied between 69.2 and 78.4%, with growth between 1.6 and 2.2 mm d^{-1}. Rosemary EO was less (Figures 2 and 3). At 25 µL L^{-1}, mean fungus growth rates were between 5.3 and 5.7 mm d^{-1} and resulted in reductions from 18.9 to 33.5% compared to the control. At 200 µL L^{-1} of rosemary EO, the mean growth rates were between 5.1 and 5.3 mm d^{-1}, resulting in inhibition between 23.5 to 36.6%. Clove EO therefore displayed greater antifungal potential as a mycelium growth inhibitor than rosemary EO (Figure 3).

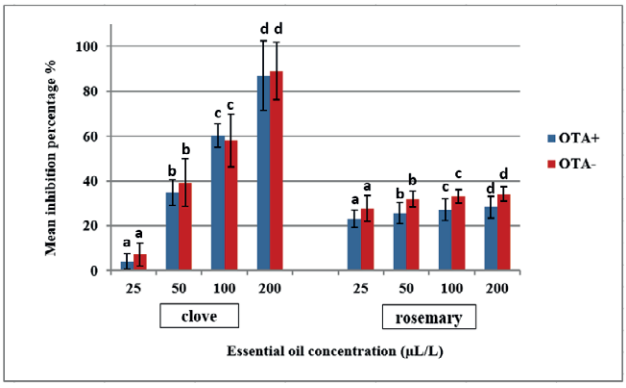

Figure 3. Mean inhibition percentages (%) of *Aspergillus tubingensis* and *A. luchuensis* on CYA medium supplemented with different concentrations of clove or rosemary essential oils. (OTA^+, OTA producing strains; OTA^- non-OTA producing strains). Means within accompanied by the same letters are not significantly different (LSD, $P = 0.05$).

Ochratoxin A production

OTA production was assessed for all treatments of cultures of *A. tubingensis* and *A. luchuensis* using HPLC-FLD (Table 4). No OTA was detected in culture extracts of non OTA producing strains, with or without clove or rosemary EOs, except for the *A. tubingensis* MUCL54478 strain grown in presence of clove EO at 25 µL L^{-1}, where 8.3 µg g^{-1} OTA was detected. For OTA producing strain controls, the OTA amounts produced were between 0.06 to 0.14 µg g^{-1}. The rosemary EO reduced accumulation of OTA for all the strains studied, except for *A. tubingensis* strains MUCL54483 and MUCL54484 at 200 µL L^{-1}, where OTA was increased (means of 0.44 µg g^{-1} for MUCL54483 and 0.22 µg g^{-1} for MUCL54484). Where clove EO was applied at 50, 100 or 200 µL L^{-1}, OTA has not been detected (depending on the strain), but exceeded the control levels of OTA, especially for strain MUCL54484 at 25 or 50 µL

Table 4. Mean OTA amounts produced by different strains of *Aspergillus tubingensis* and *A. luchuensis* on CYA medium.LOD = 0.2 µg kg^{-1}; LOQ = 0.6 µg kg^{-1}.

Essential oils	Doses (µL L^{-1})	OTA (µg g^{-1})[a] (Mean±S.D. [b])							
		OTA non-producing strains				OTA producing strains			
		A. luchuensis	*A. tubingensis*			*A. tubingensis*			
		MUCL54477	MUCL54478	MUCL54485	MUCL54486	MUCL54481	MUCL54482	MUCL54483	MUCL54484
S. aromaticum	Control	ND[c]	ND	ND	ND	0.06±0.09	0.14±0.2	0.08±0.11	0.07±0.09
	25	ND	8.29±2.69	ND	ND	<LOQ [e]	<LOQ	0.12±0.02	0.41±0.11
	50	ND	ND	ND	ND	<LOQ	<LOQ	ND	0.43±0.27
	100	ND	ND	ND	ND	<LOQ	<LOQ	ND	ND
	200	NG[d]	ND	ND	NG	ND	NG	ND	NG
R. officinalis	Control	ND	ND	ND	ND	0.06±0.09	0.14±0.2	0.08±0.11	0.07±0.09
	25	ND	ND	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ
	50	ND	ND	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ
	100	ND	ND	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ
	200	ND	ND	ND	ND	0.1±0.04	0.06±0.01	0.44±0.16	0.22±0.16

[a] means (n=3); [b] S.D: standard deviaton; [c] ND: not detected; [d] NG: no growth; LOD: limit of detection; LOQ[e]: limit of quantification.

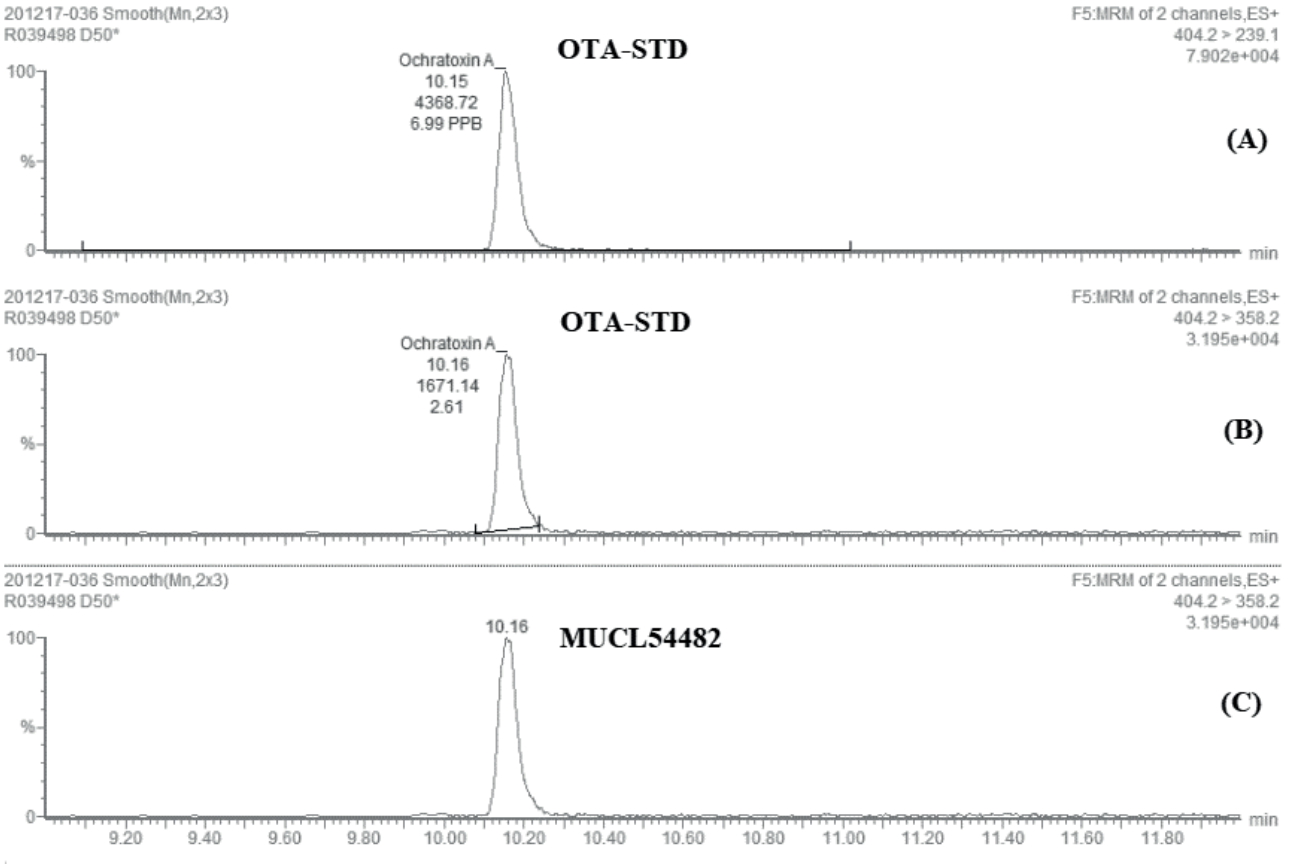

Figure 4. UPLC-MS/MS chromatograms of OTA standard (A and B) and for OTA from *Aspergillus tubingensis* strain MUCL54482 (C).

L^{-1} (respectively, 0.41 and 0.43 µg g^{-1}). Confirmation of OTA production by *A. tubingensis* strains MUCL54482 and MUCL54483 was carried out by UPLC-MS/MS, with production of 1050 µg kg^{-1} by *A. tubingensis* strain MUCL54482 (Figure 4).

DISCUSSION

GC/MS analyses of the clove and rosemary EOs from Moroccan origins showed that eugenol was the major component of Clove EO, and eucalyptol (1,8-cin-

eole) was the major compounds in rosemary EO. For clove EO, eugenol was 86.4% of the total volatile compounds. This agrees with the results of Santamarina *et al*. (2016), who showed that the proportion of eugenol in commercial clove EO was 88.6%. Bozik *et al*. (2017) also reported that eugenol was the major constituent in two commercial clove Eos, with amounts of 81.7 and 77.8%. In addition, Boukaew *et al*. (2017) and Sharma *et al*. (2017) concluded, respectively, that eugenol constituted 62.4 and 75.4% of clove EO volatiles.

Results for rosemary EO in the present study showed that eucalyptol was 35.8% of total volatiles. Risaliti *et al*. (2019) also reported similar results, with a proportion of 48.7%. Farhat *et al*. (2017) reported that the proportion of eucalyptol in Tunisian rosemary EO ranged from 20.8 to 64.7% depending according on the extraction methods used. Analysis of rosemary EO volatiles from Brazil (Takayama *et al*., 2016) and Morocco (Bouyahya *et al*., 2017) showed that the major component was eucalyptol, at 28.5% in Brazil and 23.67% in Morocco. Studies of the chemical composition of EOs from clove and rosemary revealed variability in their chemical profiles dependent on plant growing conditions, phenological stage, sampling time, and extraction and analytical methods used, and even existence of new plant chemotypes as suggested by Kokkini *et al*. (2004) and Sharma and Tripathi (2008). The chemical composition and antibacterial activity of clove and rosemary EOs has been described by several authors. However, studies on antifungal activity of these essential oils against *A. niger* aggregates and their effects on OTA synthesis are scarce.

In the present study, the lag phases, radial growth and OTA accumulation by strains of *A. tubingensis* and *A. luchuensis* were strongly influenced by clove and rosemary EOs. In general, the EO type, their concentrations, fungus isolate, and interactions between these factors had highly significant effects on radial fungus growth. For clove EO, mean inhibition rates greater than 90% were obtained at 200 µL L^{-1}, showing that this essential oil had effectiveness exceeding that for some fungicides widely used in Morocco (azoxystrobin, benomyl, hexaconazole and pyrimethanil) (Zouhair *et al*., 2014; Laaziz *et al*., 2017). Inhibition rates for clove EO were between 1.2 and 13 3% at the lowest concentration of the EO (25 µL L^{-1}), and were 69.2 to 100% for 200 µL L^{-1}. Passone *et al*. (2012) reported that the application of 500 µL L^{-1} of clove EO inhibited the growth of *Aspergillus* section *Nigri* by 48.8 to 100%, depending on EO concentration, fungus strains and water activity levels. Santamarina *et al*. (2016) reported significant antifungal activity of clove EO against *Fusarium* spp. They demonstrated that for a concentration of 300 mg mL^{-1}, there was a growth reduction of 88% for *F. graminearum*. Boukaew *et al*. (2017) also studied the antifungal effect of clove EO but on *A. flavus* responsible of the deterioration of maize in storage in Thailand. They demonstrated that low concentrations of clove essential oil (1 and 10 µL L^{-1}) induced greater growth inhibition (respectively, 85.7 and 94.0%), and complete growth inhibition (100%) was achieved at EO concentrations of 50 and 100 µL L^{-1}.

This study showed that *A. tubingensis* growth inhibition rates from treatments with rosemary EO ranged from 23.5 to 36.6%, depending on the EO concentration. Bouddine *et al*. (2012) also reported that rosemary EO showed a weak effect on *A. niger*, as assessed using the broth dilution method. Rasooli *et al*. (2008), using the disc diffusion method, showed antifungal activity of rosemary EO at 20 µL, giving 17% inhibition of *A. parasiticus*. Bomfim *et al.,* 2015) showed that the same essential oil inhibited growth of *F. verticillioides* by 29.7% at a concentration of 300 µg mL^{-1}. The weak effect (1.6 to 24.2% at 1 µL mL^{-1}) of rosemary EO on growth inhibition for fungal pathogens was also demonstrated by Matusinsky *et al*. (2015).

Investigation of synergistic or potential effects of different constituent compounds of EOs can increase insights into how EO composition affects antifungal activities. Morcia *et al*. (2012) demonstrated that eugenol, the major component of clove EO, had high activity against *A. tubingensis* and *A. carbonarius*. However, eucalyptol (the major component rosemary EO) was only active at the greatest doses tested (2%). Nazzaro *et al*. (2017) also reported that eucalyptol and eugenol exhibited important antifungal activity. Several studies demonstrated that the lipophilic properties and low molecular weights of essential oils allow them to cross cell membranes, causing irreversible cell wall and cellular organelle damage and affecting inorganic ion equilibria and pH homeostasis (Pawar *et al*., 2006; Helal *et al*., 2007).

High efficacy of EOs in disease control has been reported by several authors. The use of EOs in the packaging of fruits and other commodities was reported by Sivakumar *et al*. (2014, Guerra-Rosas *et al*. (2017), Munhuweyi *et al*. (2017), and Talebi *et al*. (2018). Campos *et al*. (2016) reported that the use of thyme and sage EOs in the headspace of strawberry packages led to decreases fruit contamination. To improve shelf-life without changing the organoleptic characteristics of jujube fruit, Nikkhah and Hashemi (2020) demonstrated that the treatments of the fruit with thyme, cinnamon or rosemary EOs (at, respectively, 0.156, 0.625, or 0.078 g L^{-1}), during 60 d of storage, did not negatively impact on

taste, texture, aroma or overall acceptance. They also suggested that using multiple essential oils at low concentrations may increase the antifungal activity because of synergistic actions. They demonstrated that, the concentration of EOs penetrating the fruit flesh remained under taste detection thresholds. According to the European Food Safety Authority (EFSA, 2012), the Acceptable Daily Intake (ADI) and Acceptable Operator Exposure Level (AOEL) for eugenol are 1.0 mg kg^{-1} body weight d^{-1}. In the present study, total inhibition for clove essential oil was obtained at 200 µL L^{-1} meaning that the use of this essential oil to conserve fruits in storage is likely to be safe for consumers. On the other hand, based on the Maximized Survey-derived Daily Intake (MSDI) approach, EFSA (2013), reported that 1,8-cineole (eucalyptol) presented no safety concern (1200 µg *capita*$^{-1}$ d^{-1})

In parallel to the fungal growth inhibitory effect by the two tested EOs, there were contradictory effects (reduction or stimulation) on OTA biosynthesis caused by the tested *Aspergillus* strains. Although several authors have reported that *A. tubingensis* produces OTA, the ability of this species to produce this toxin is controversial. Studies have shown that strains of *A. tubingensis* were able to produce a metabolite identified by HPLC-FLD as OTA (Medina *et al*., 2005; Perrone *et al*., 2006; Selouane *et al*., 2009; Chiotta *et al*., 2011). Other studies have considered that *A. tubingensis* was not an OTA producer (Abarca *et al*., 2004; Frisvad *et al*., 2011). Pantelides *et al.* (2017) also showed that none of 261 strains of *A. tubingensis* isolated from wine grapes were found to be ochratoxigenic when analyzed by UPLC-MS/MS, while some of these isolates were initially considered to be toxin producers as indicated by HPLC-FLD. In the same way, Storari *et al.* (2012) re-examined *A. tubingensis* strains previously described as ochratoxigenic using LC-MS, and could not detect the toxin, concluding that *A. tubingensis* cannot be considered as an ochratoxigenic species. This agrees with more recent publications (Gil Serna *et al*., 2019 and Tavakol *et al*., 2020). Therefore, data related to OTA production in controls in the present study was validated using an UPLC-MS/MS based method, in order to be sure that the observed peak was not an artifact (Figure 4). The present results showed that the *A. tubingensis* strains used in this study produce OTA, and this is the first report where production of OTA by *A. tubingensis* has been confirmed using the UPLC-MS/MS technique.

The inhibitory effect of rosemary EO on OTA production was also reported by El Khoury *et al.* (2016), who found that this EO inhibited production of OTA by *A. carbonarius.* Bomfim *et al.* (2015) concluded that rosemary EO reduced fumonisin B1 and B2 contamination by *F. verticillioides*. Experiments with *A. parasiticus* clearly illustrate reduction of aflatoxin production from rosemary EO (Rasooli et al., 2008). The use of eugenol also reduced aflatoxin B1 production by *A. parasiticus* and *A. flavus* (Jahanshiri *et al*., 2015; Caceres *et al*., 2016).

Several studies have shown that use of some essential oils as biocontrol agents against fungi lead to stimulation of the mycotoxin biosynthesis. Stimulation of OTA production depended on the strain, nature of the essential oil and the applied dose. Results of the present study showed that sub-lethal concentrations of EOs stimulated production of OTA by *A. tubingensis*. Lappa *et al.* (2017) demonstrated that the treatment of *A. carbonarius* with lemongrass, cinnamon or mandarin essential oils decreased growth rate, but increased OTA production. Stimulation of OTA biosynthesis by essential oils was also reported by Passone *et al.* (2012), who demonstrated that species of *A. niger* aggregate and *A. carbonarius* tended to stimulate OTA production in the presence of 1000 µL L^{-1} of poleo essential oil. In addition, Mateo *et al.* (2017) also reported stimulation of AFB1, AFB2, AFG1 and AFG2 accumulation in some cultures treated with ethylene-vinyl alcohol copolymer films containing oregano, carvacrol and cinnamon. These results are similar to those reported by Bluma and Etcheverry. (2008) for the species *Aspergillus* section *flavi*.

Mycotoxin production may be stimulated when stressful environmental conditions and sub-lethal antifungal agent doses are maintained in culture media during the growth of mycotoxin-producing fungi (Mateo *et al*., 2013; Prakash *et al*., 2015).

In conclusion, the effects of clove and rosemary EOs on fungal growth and OTA production by different *Aspergillus* isolates have shown that these oils had significant antifungal activity. Inhibition rates varied (20 to 100%) for fungus isolate and essential oil dose. In general, the statistical analyses showed that the different concentrations of clove and rosemary EOs, the *Aspergillus* strains, and their interactions, had strong effects on fungus growth. Mean inhibition rates greater than 85% were obtained at 200 µL L^{-1} of clove EO, which exceeds some fungicides (azoxystrobin, benomyl, hexaconazole and pyrimethanil) that have been tested and are widely used in Morocco. Clove EO can be used to control fungal growth and mycotoxin production as a potential alternative to chemical control. However, more research is required to evaluate the *in vivo* efficacy of these EOs, and to understand their effects on the expression of OTA biosynthesis and regulation genes.

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