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Stimulation of the production of new volatile and non-volatile metabolites by endophytic *Aspergillus niger* using small organic chemicals

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Abstract

This study was designed to observe the effect of small organic chemicals on the production of volatile and non-volatile metabolites by an endophytic strain of *Aspergillus niger* isolated from the roots of *Terminalia catappa* Linn. (Tropical-almond, Combretaceae). The fungus was cultured for 6 days at 25°C in static condition in potato dextrose broth (PDB), supplemented with 1% acetone, 1% DMSO, 1% ethanol, and 1µM 5-azacytidine. The ethyl acetate extracts were analyzed by high performance liquid chromatography (HPLC) and gas chromatography coupled with mass spectrometry (GC-MS). Results of HPLC analysis showed increased content of many compounds in PDB culture supplemented with 5-azacytidine, while supplementation with acetone led to a new compound at retention time (RT≈35.87 min), as well as DMSO, and ethanol (RT≈38.05 min). The GC-MS analysis of the ethyl acetate extract of untreated *A.niger* showed the presence of 6 volatile metabolites of which oxalic acid, isobutyl propyl ester was the most abundant compound (60.79%). The chromatographic profile of extracts from *A. niger* cultured with acetone, DMSO, ethanol and 5-azacytidine showed 12, 16, 14 and 13 volatile compounds respectively. Cyclohexanecarboxaldehyde,3,3-dimethyl-5-oxo- was the most abundant compound representing 58.21%, 40.12% and 64.38% in acetone, DMSO and ethanol supplemented cultures extracts respectively. For the 5-azacytidine treated fungus, 9-octadecenoic acid (Z)- (26.91%) and tetradecanoic acid, 12-methyl-, methylester (21.35%) were the most abundant. In addition, a new unidentified compound was detected in the extract of 5-azacytidine treated fungus. This study highlights the potential of small organic chemicals to increase the yield and to stimulate the production of new secondary metabolites by *A. niger*.

Keywords – *Aspergillus niger* – endophytic fungi – GC-MS – HPLC – small organic chemicals – *Terminalia catappa*

Introduction

Natural products are an unsurpassed source of bioactive compounds and constitute a relevant economic resource for the pharmaceutical, cosmetic and food industry (Baker et al. 2000). Medicinal plants provide a unique environment for microorganisms and have been recognized as a repository of endophytes with novel metabolites (Strobel et al. 2004). Endophytic fungi provide a wide variety of structurally unique, and bioactive natural products representing a huge reservoir which offers an

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enormous potential for exploitation for medicinal, agricultural and industrial uses (Tan & Zou 2001, Schulz et al. 2002, Strobel & Daisy 2003, Zhang et al. 2006).

Aspergilli represent a large number of species which are a rich source of secondary metabolites (Schneider et al. 2008); they can harbour 30–50 distinct polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) gene clusters per species (Payne et al. 2006). They are known to produce antimicrobial secondary metabolites like helvolic acid, monomethylsulochrin, ergosterol and 3β-hydroxy-5ά ergosterol (Gao et al. 2007), cytotoxin, brefeldin A (Wang et al. 2002), tensyuic acid, nigerazine B, tensidol A, and ochratoxin (Nielsen et al. 2009, Al-Shaibani et al. 2013). Many studies have described the antimicrobial potential of endophytic *Aspergillus* spp. against a panel of clinically significant human pathogens (Maria et al. 2005, Tayung & Jha 2007, Kumar et al. 2010, Toghueo et al. 2016).

However, Aspergilli possess a greater number of gene clusters encoding for the production of secondary metabolites than the number of natural products that have been isolated from these same organisms (Fisch et al. 2009, Scherlach & Hertweck 2009, Zerikly & Challis 2009). These transcriptionally suppressed gene clusters, are collectively referred to as silent biosynthetic pathways, generally unexpressed under a variety of laboratory culture conditions. In fact, Fisch et al. (2009) reported that in *Aspergillus niger* fewer than 30% of its biosynthetic gene clusters are transcribed under a variety of *in vitro* culture conditions. These silent biosynthetic pathways are anticipated to be a rich source of chemically diverse compounds (e.g. novel organic moieties, multifaceted stereochemical features, unique heteroatom incorporation, etc.) with outstanding potential for generating novel therapeutic leads.

Many strategies have been applied to stimulate the expression of these silent pathways and to promote secondary metabolite biosynthesis. These strategies include to vary medium and growth conditions (Bode et al. 2002), exchange of native gene promoters with constitutive or inducible promoters or overexpression of transcription factors (Brakhage & Schroeckh 2011), co-cultivation with one or more microorganisms (Combès et al. 2012, Marmann et al. 2014), fermentation in the presence of non-ionic adsorption resins (De la Cruz et al. 2012), and addition of small-molecule elicitors such as epigenetic modifiers (William et al. 2008) or organic solvents (Pettit 2011). Therefore, the aim of this study was to investigate the effects of different small organic chemicals on the production of volatile and non-volatile compounds by an endophytic strain of *Aspergillus niger* isolated from the roots of *Terminalia catappa*.

Materials and Methods

Isolation and morphologic identification of endophytic fungi

A root sample of *T. catappa* (National Herbarium Voucher Specimen number 51244/HNC) was collected on the University of Yaoundé 1 main campus (September, 2014). Small root pieces, measuring about 2 mm were surface disinfected with 70% ethanol for 5min, followed by treatment with a 1% active chlorine solution for 15 min, 2 min in 70% ethanol, and a final rinse with sterile distilled water (3 times). Fifteen disinfected root pieces were plated on potato dextrose agar (PDA) containing chloramphenicol (200 mg/l) and kept in the dark at 25°C. After mycelium emerged from plant tissues into the agar, mycelial fragments were transferred to fresh PDA plates as previously described (Sánchez Márquez et al. 2007) and maintained under natural light at room temperature. Endophytic fungi were identified up to genus level based on colony characters, growth and structure of mycelium, and conidia as described by Klich (2002).

Molecular characterization

The identification of the endophytic fungus was made using the nucleotide sequence of the ITS1-5.8S rRNA-ITS2 region. DNA was extracted from small mycelial fragments scraped from the surface of the culture plates using a commercial kit (RedExtract-N-Amp Plant PCR, Sigma Aldrich). The ITS1-5.8S rRNA-ITS2 region was amplified by PCR using primers ITS4 and ITS5 (White et al. 1990). Amplicons were sequenced with primer ITS4 (Sánchez Márquez et al. 2007).

To identify ITS sequences similar to the one obtained from the fungal isolates, the FASTA algorithms were used to screen the EMBL/Genbank database of fungal nucleotide sequences. To visualize the diverse fungal taxa identified by means of molecular characters, a sequence similarity dendrogram was made. Isolate and reference strain (CBS, CICC strains) sequences were aligned using the program ClustalX 2.1 with the default settings, and the dendrogram was made with MEGA 6.06 software using the neighbor-joining method with Kimura 2-parameter distances. Groups of sequences at close proximity within the same branch of the dendrogram were individually aligned with ClustalX to determine their percentage of similarity. Sequences with a similarity greater than 99% were considered to belong to the same species (Sánchez Márquez et al. 2007).

Endophyte culture in medium supplemented with small organic compounds

Aspergillus niger strain TCR was first cultivated on potato dextrose agar. Mycelia pieces of 1x1 mm from a culture were used to inoculate 20 mL of potato dextrose broth (potatoes in infusion 200g/L, dextrose 20g/L, pH 5.1±0.2) (PD; HiMedia) in 100 mL erlenmeyer flasks supplemented with different organic chemicals: 1µM of 5-azacytidine (Sigma Aldrich); 1% dimethyl sulfoxide (DMSO) (Sigma Aldrich); 1% ethanol (HPLC grade, Merck); and 1% acetone (HPLC grade, Merck), and a control without any supplementation. Liquid cultures were grown for 6 days in static condition at room temperature before extraction.

Extraction from fungal cultures

To each culture, 20 mL of ethyl acetate were added and shaken and kept overnight at room temperature. This mixture was then transferred to a separatory funnel, and the organic phase collected. This process was repeated thrice, resulting in a total volume of 60 ml per sample. The ethyl acetate was evaporated at 40°C in a Labconco RapidVap parallel evaporation system. The residue was dissolved in 0.2-0.4 mL of methanol, transferred to pre-weighed microfuge tubes and evaporated to dryness. The dry residue was dissolved in methanol (3 mg/mL) before compositional analysis by HPLC and GC-MS.

Analysis of non-volatile metabolites profile by high performance liquid chromatography (HPLC)

Analysis of non-volatile metabolites was performed on a Agilent 1260 series HPLC system equipped with autosampler and diode array detector (DAD) using a Synergi 4µ Polar-RP 80A column (250 mm × 4.6 mm) (Shimadzu Company). The mobile phase consisted of methanol and water, increasing linearly from 5% methanol at the time of injection to 100% at 90 min. The flow rate was 1 mL/min and column temperature was 40°C. Chromatograms were recorded at 214 nm (σ-σ* transitions shown by several aliphatic molecules) and 254 nm (π-π* transition shown by aromatic molecules).

Analysis of volatiles metabolite profile by gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis was carried out on a GC-MS-QP2010 Ultra system (Shimadzu Company) comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer instrument using the following conditions: Column Rtx-5MS (30 m × 0.25 mm id × 0.25 µm film thickness, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70eV; carrier gas (helium 99.999%) at a flow rate 1ml/min; injection volume of 1µL; injector temperature 260°C; ion-source temperature 280 °C. The oven temperature was maintained at 110°C (isothermal for 2min), and then programmed at 10°C/min increment to 200 °C, then 5 °C/min to 280°C, and finally maintained for 9min at isothermal 280°C. Mass spectra were taken at 70eV, a scan interval of 0.5s and fragments from 40 to 550Da.

Identification of bioactive compounds

Interpretation of GC-MS mass-spectra was conducted using the database of the National Institute of Standards and Technology (NIST) having more than 62,000 patterns. Spectra of unknown components were compared with those of known components of the NIST library. The name, and molecular formula of the compounds identified were ascertained.

Results

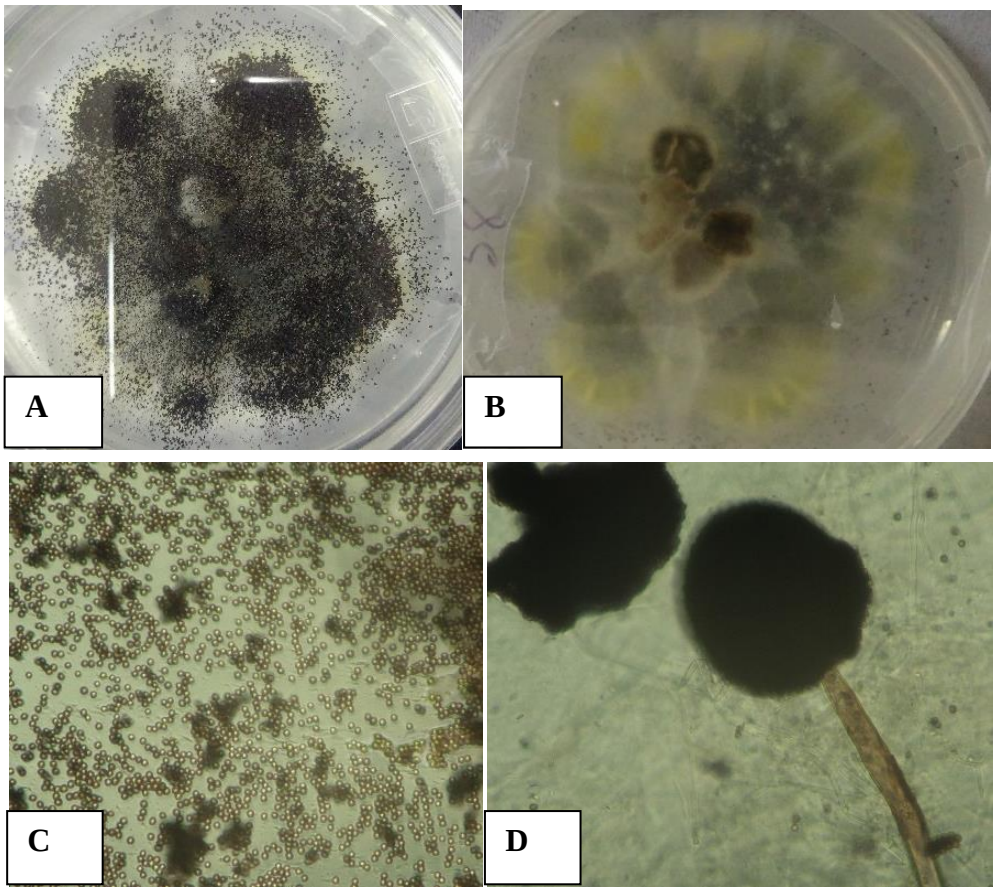

Fig. 1 – Colony of *Aspergillus* sp on PDA after 7 days. Colonies consist of a compact black. **A**. or yellow basal. **B**. felt covered by a dense layer of dark-brown to black conidial heads. **C**. Conidial dark brown. **D**. Septate and hyaline hyphae, and a dark conidiophore globose vesicle.

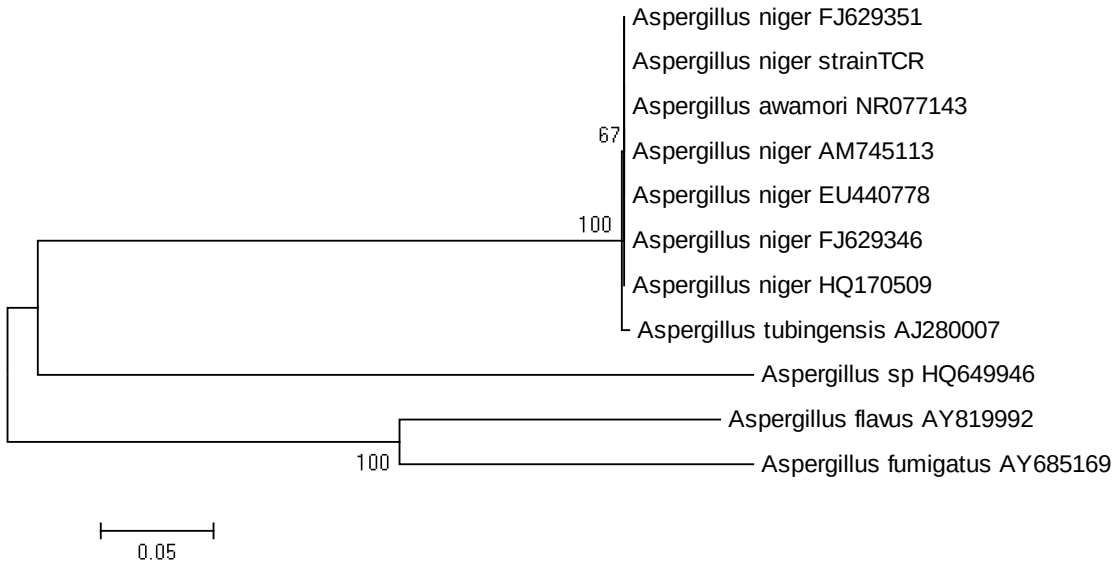

Fig. 2 – Phylogenetic tree showing relationship of *Aspergillus niger* strain TCR with other related fungal species. ITS1-5.8S-ITS2 sequence analyzed by neighbor-joining tree made using p-distance for nucleotides and the pairwise gap deletion option. Numbers at branch nodes are bootstrap values, indicating support based on 500 replications.

Morphological characteristics of the fungal isolate

Colonies growing on PDA at 25± 2°C were initially white and then turned quickly into black with conidial production. The reverse was pale yellow with radial fissures in the agar. Hyphae were septate and hyaline, while the conidial heads were large, globose, and dark brown, becoming radiate and tending to split into several loose columns with time. The conidial head was biseriate. Conidiophores were long, smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle. Metulae and phialides covered the entire vesicle. Conidia were dark brown to black, very rough-walled, and globose to subglobose (Fig 1).

Molecular characterization of the fungal isolate

The isolate was identified using ITS regions of rDNA and the search for sequence similarity in BLAST nucleotide using FASTA algorithms. The isolate of *Aspergillus* sp gave 99% similarity with *Aspergillus niger* FJ629351 and was therefore identified as *Aspergillus niger* (Fig 2). The phylogenetic analysis was based on the data from *A. niger*, together with those that presented similarity in the NCBI database, and other sequences belonging to different species, with the objective of confirming that the isolates were grouped with the families and the closest BLAST identity. The phylogenetic analysis is presented in Fig 2.

Comparative HPLC profiles of non-volatile metabolites from *A. niger*

Fig 3 shows the non-volatile metabolite profile obtained by HPLC analysis of ethyl acetate extracts of *A. niger* cultures at 25°C in static condition, in absence of organic chemicals (fig 3A) and in presence of acetone (fig 3B), DMSO (fig 3C), ethanol (fig 3D) and 5-azacytidine (fig 3E).

The chromatograms showed a variation in production of metabolites due to exposure to different organic chemicals. In Fig 3, the intensity of compound 1 (RT≈2.9 min) was found to be highly increased in the presence of DMSO, while in presence of ethanol and acetone it decreased. The production of compounds 2 (RT≈29.4min) and 3 (RT≈46.4min) was inhibited in the presence of DMSO and 5-azacytidine respectively. In comparison to untreated fungus, the production of the group of compound 4 (RT≈55.6min) increased with 5-azacytidine, and the production of compound 5 (RT≈61.1min) also increased with DMSO and 5-azacytidine. The presence of DMSO inhibited the production of compound 6 (mAU<<50000) (RT≈69.3min) while acetone (mAU≈50000) and ethanol (mAU≈140000) reduced its production. The presence of DMSO also impaired the production of compound 7 (RT≈75.32min), and the group of compounds 8 (RT≈82.39min) and 9 (RT≈85-92min). Contrarily, the culture with 5-azacytidine significantly stimulated their production by *A.niger*. The production of compound 10 (RT≈95.5min) was not affected by the culture additives. In general, the presence of 5-azacytidine in PDB increased the production of many compounds, while one new compound was detected in PDB supplemented with acetone (RT≈35.87), DMSO, and ethanol (RT≈38.05 min).

Results from the compositional analysis of extracts (table 1) showed that the small organic chemicals stimulated the production of new volatile metabolites compared to untreated *A. niger* cultures. In fact, the chemical profile of the extract from untreated *A.niger* showed the presence of 6 volatile metabolites while the culture supplementation with acetone, DMSO, ethanol and 5-azacytidine induced the production of 12, 16, 14 and 13 volatile compounds respectively. Oxalic acid, isobutyl propyl ester (60.79%) was the most abundant compound in the untreated sample while, when cultured with acetone, DMSO and ethanol, the most abundant compound was rather Cyclohexanecarboxaldehyde,3,3-dimethyl-5-oxo- representing 58.21%, 40.12% and 64.38% respectively. The culture with 5-azacytidine produced two compounds with significant contents, namely 9-octadecenoic acid (Z)- (26.91%) and tetradecanoic acid,12-methyl-, methylester (21.35%) and also stimulated the production of one new and unidentified volatile compound (RT=26.683min).

Compound $C_{21}H_{21}N_3O_5$ was identified in untreated *A. niger* extract (0.29%) and also when treated with DMSO (0.09%) and ethanol (0.04%) making it a molecular marker of the endophyte. Compound $C_{20}H_{28}F_3NO_3$ was also identified in extract of untreated (6.42%) and acetone treated fungus (0.37%). Disulfide, dipropyl was identified in extracts from of *A. niger* cultured with acetone (0.20%),

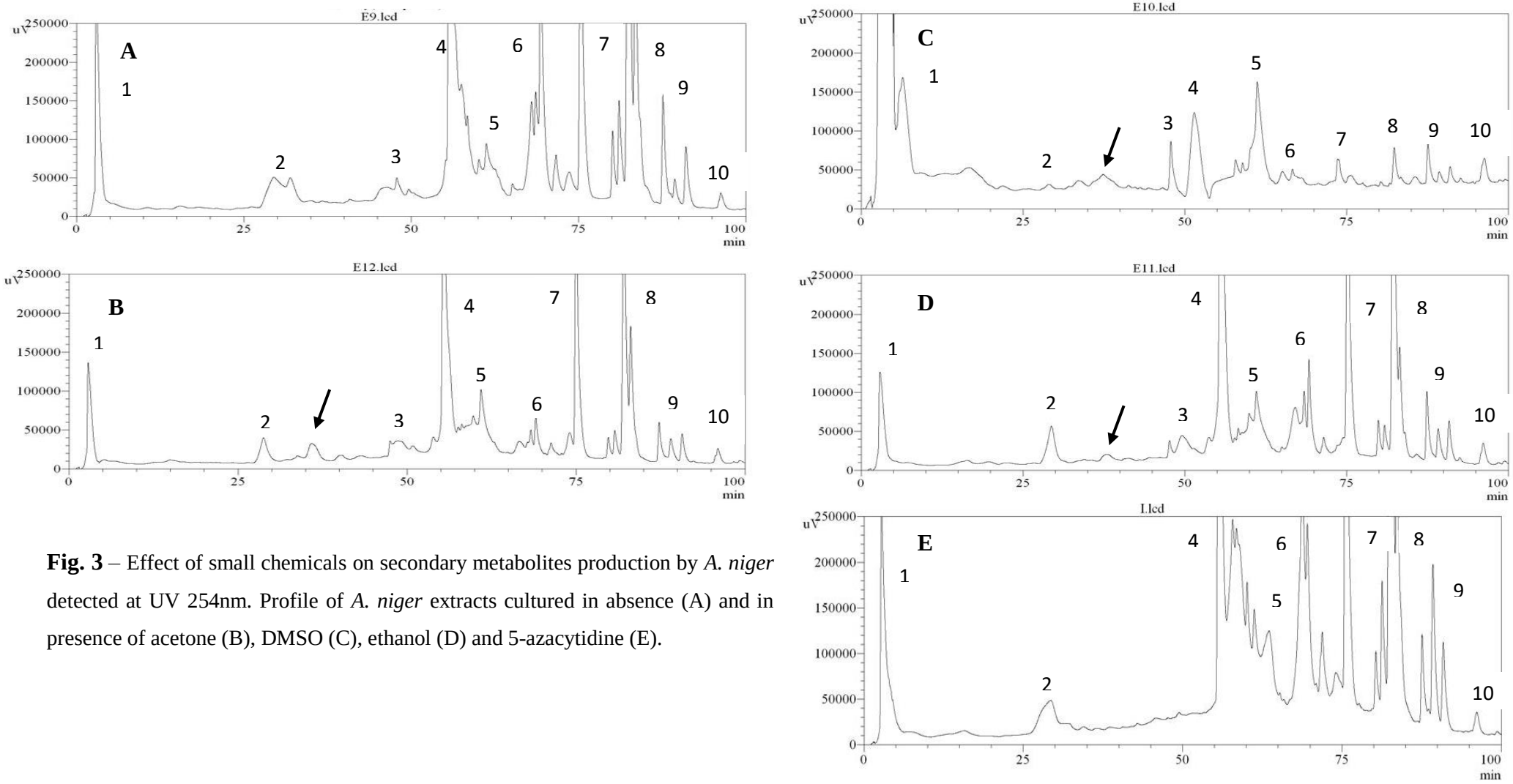

Fig. 3 – Effect of small chemicals on secondary metabolites production by *A. niger* detected at UV 254nm. Profile of *A. niger* extracts cultured in absence (A) and in presence of acetone (B), DMSO (C), ethanol (D) and 5-azacytidine (E).

Table 1 Volatile metabolites identified by GC-MS in the ethyl acetate extract of *A. niger* cultured without and in the presence of acetone, DMSO, ethanol and 5-Azacytidine.

Chemical name	**Chemical formula**	**[1]RT (min)**	**[2]Abundance of compounds identified in each extract (%)**				
			Untreated fungus	Acetone	DMSO	Ethanol	5-azacytidine
Trichloro[2.2.1.02,6]heptane, 3-bromo-	C_7H_9Br	6.147		0.43			
Propyldithyopropane	$C_6H_{14}S_2$	7.390			0.23		
3-isopropyl[3]manxine-6,9-diol	$C_{17}H_{25}NO_2$	7.397				0.37	3.67
Ethenone	C_2H_2O	7.523					2.82
Phenol, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{14}O$	9.742		9.94			
2-(((carbobenzyloxy)amino)methyl)-4-benzyl-5-((carbomethoxy)-amino)oxazole	$C_{21}H_{21}N_3O_5$	10.293	0.29		0.09	0.04	
2,2-diphenyl-2,2'-BI-1,3-dioxolane	$C_{18}H_{18}O_4$	10.299		0.38			
Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-,[1S-(1α,2β,4β)]-	$C_{15}H_{24}$	11.548		0.34			
1-tert-butyl-4-methyl-3-piperidinone	$C_{10}H_{19}NO$	12.268		2.10			
Cyclododecanecarbonitrile,1-(3-hydroxybutyl)-2-oxo,	$C_{17}H_{29}NO_2$	12.270			4.30		
1,2,4-cyclopentanetrione,3-butyl-	$C_9H_{12}O_3$	12.290				5.20	
2H-3,9A-methano-1-benzoxepin, octahydro-2,2,5A,9-tetramethyl-,[3R-(3α,5Aα,9α,9Aα)]-	$C_{15}H_{26}O$	14.277			0.33		5.49
1-(propyldisulfanyl)propane	$C_6H_{14}S_2$	14.637			0.26		
1-methylcyclohex-1-en-4-carboxylic acid	$C_8H_{12}O_2$	14.874		15.31			
1-oxetan-2-one,4,4-diethyl-3-methylene-	$C_8H_{12}O_2$	14.877			18.63		
Δ,1α-cyclohexaneacetic acid	$C_8H_{12}O_2$	14.923				21.82	
2-propenyl (2,2-difluorocyclopropryl)methyl ether	$C_7H_{10}F_2O$	15.759	1.19				
Cyclohexanecarboxaldehyde,3,3-dimethyl-5-oxo-	$C_9H_{14}O_2$	15.827		58.21	40.12	64.38	
1-penten-1-one,2-methyl-	$C_6H_{10}O$	16.430			1.53		
Disulfide, dipropyl	$C_6H_{14}S_2$	17.076		0.20	0.29	0.08	
3-hexen-2-one, 5-methyl-	$C_7H_{12}O$	17.107			12.19		
Pentanoic acid, 2-methyl	$C_6H_{12}O_2$	17.115	7.46				
Tetradecanoic acid, 12-methyl-, methylester	$C_{16}H_{32}O_2$	17.123				1.20	21.35
L-proline, N-valeryl-, undecyl ester	$C_{21}H_{39}NO_3$	17.363			0.75	0.21	3.63

Chemical name	**Chemical formula**	**[1]RT (min)**	**[2]Abundance of compounds identified in each extract (%)**				
			Untreated fungus	Acetone	DMSO	Ethanol	5-azacytidine
Tridecanoic acid	$C_{13}H_{26}O_2$	17.465		1.23		0.63	
Tetradecanoic acid	$C_{14}H_{28}O_2$	17.487			4.30		
9-octadecenoic acid (Z)-	$C_{18}H_{34}O_2$	17.493					26.91
5,6-dichlorohexene	$C_6H_{10}Cl_2$	18.800				0.20	4.87
1-undecyne	$C_{11}H_{20}$	18.803			2.74		
Cyclopentanecarboxaldehyde,2-methyl-5-(1-methylethenyl)-,[1S-(1α,2β,5β)]-	$C_{10}H_{16}O$	18.850			1.78		
(5S,8R)-5-isopropyl-8-methyl-2-methylene-3,9-decadien-1-ol	$C_{15}H_{26}O$	18.856				0.28	
(3R,3AS,9AR,9BR)-octahydro-3-methyl-1H-furo[3'4':4,5]-isoxazolo[2,3-A]pyridin-1-one	$C_{10}H_{15}NO_3$	18.859					6.05
Acetic acid, methylester	$C_3H_6O_2$	19.060					1.46
Cycloheptene,5-methyl-	C_8H_{14}	19.163		1.00			
1-undecyne	$C_{11}H_{20}$	19.197				1.36	
(Z,Z)-heptadeca-8,11-dien-1-yl bromide	$C_{17}H_{31}Br$	19.203			9.46		
2-propenyl (2,2-difluorocyclopropyl) methyl ether	$C_7H_{10}F_2O$	19.237					2.11
2-furoic acid, but-3-yn-yl ester	$C_9H_8O_3$	20.380	23.86				11.99
2-(2-furyl)-1-phenylpropane	$C_{13}H_{14}O$	20.403			2.97		
N-(4-isopropylphenyl)-2-furamide	$C_{14}H_{15}NO_2$	20.417		10.49		3.99	
Unknown compound	***	26.683					0.83
3-[6-(N-benzyltrifluoromethansulfonamido)hexyl]cyclohexanone	$C_{20}H_{28}F_3NO_3S$	26.721	6.42	0.37			
1-(cyclohexylamino)-4,5,6,7,8,9-hexahydro-10H-azepino[3,2,1]indol-2-one	$C_{18}H_{26}N_2O$	26.736				0.23	
Benzocoumarin,1,2,3,8-tetramethoxy-	$C_{17}H_{16}O_6$	26.747					8.81
Oxalic acid, isobutyl propyl ester	$C_9H_{16}O_4$	28.610	60.79				

Compounds were identified through comparison of their spectra with those of known components of the NIST library; [1]Retention time; [2]Relative abundance of extract components was automatically generated from electronic integration of individual pics of the chromatogram relative to the total pics area; ***Unknown compound.

DMSO (0.29%) and ethanol (0.08%). L-proline,N-valeryl-,undecylester was identified in extracts from *A. niger* treated with DMSO (0.75%), ethanol (0.21%) and 5-azacytidine (3.63%). Tridecanoic acid and N-(4-isopropylphenyl)-2-furamide were identified in extracts from *A. niger* treated with acetone and ethanol while 5,6-dichlorohexene and 3-isopropyl[3]manxine-6,9-diol were identified in extracts from fungus treated with ethanol and 5-azacytidine. The 2-furoic acid, but-3-yn-yl ester, the most abundant compound (23.86%) in extract of the non treated fungus, was also identified in the extract with 5-azacytidine (11.99%). 1-undecyne was identified in extracts with DMSO (2.74%) and ethanol (1.36%). Overall, 3 compounds ($C_7H_{10}F_2O$, $C_6H_{12}O_2$ and $C_9H_{16}O_4$) were only identified in extract without organic supplements. Compounds C_7H_9Br, $C_{10}H_{14}O$, $C_{18}H_{18}O_4$, $C_{15}H_{24}$, $C_{10}H_{19}NO$, $C_8H_{12}O_2$ and C_8H_{14} were specifically stimulated in the presence of acetone. The same observation was made with compounds $C_6H_{14}S_2$, $C_{15}H_{26}O$, $C_6H_{10}O$, $C_7H_{12}O$, $C_{10}H_{16}O$, $C_{17}H_{31}Br$ and $C_{13}H_{14}O$ identified only when cultured with DMSO. Compounds $C_9H_{12}O_3$, $C_8H_{12}O_2$, $C_{16}H_{32}O_2$, $C_{15}H_{26}O$ and $C_{18}H_{26}N_2O$ were specifically produced by *A. niger* in presence ethanol while, C_2H_2O, $C_{15}H_{26}O$, $C_{16}H_{32}O_2$, $C_{18}H_{34}O_2$, $C_{10}H_{15}NO_3$, $C_3H_6O_2$, $C_7H_{10}F_2O$, $C_{17}H_{16}O_6$ and the unknown compound were stimulated only in the presence of 5-azacytidine (Table 1).

Discussion

Aspergillus niger (Ascomycota) is one of the most pharmaceutical friendly organisms that produce industrially important enzymes as well as bioactive secondary metabolites. It has proven high potential in producing antimicrobial compounds (Magnuson & Lasure 2004, Nielsen et al. 2009). However, new insights into the molecular biology of this fungus have demonstrated that its genetic potential in terms of producing a greater chemical diversity of compounds has been vastly underestimated in the past (Fisch et al. 2009). The culture of microorganisms with small organic chemicals has been proposed to stimulate the silent genes in order to produce new secondary metabolites (Pettit 2011). Therefore, this study was designed to study the effect of acetone, DMSO, ethanol and 5-azacytidine on the production of volatile and non-volatile metabolites by an endophytic strain of *Aspergillus niger* in culture.

The results achieved in this study indicated that the tested small chemicals have significant impact on the qualitative and quantitative diversity of volatile and non-volatile secondary metabolites produced by *A. niger*. These findings corroborate previous reports by many authors who claimed that *A. niger* is an outstanding biosynthetic source of compounds (Fisch et al. 2009, Nielsen et al. 2009, Richter et al. 2014, Al-Shaibani et al. 2013, Siddiquee et al. 2015). The studies by Siddiquee et al (2015) and Hameed et al (2015) also reported rich metabolites diversity though, they were different from those identified in our study given the differences in *A. niger* origin, the formulation of PDB used, culture conditions, the extraction procedure and GC-MS analysis. In our study, the *A. niger* strain was an endophyte isolated from a plant, *Terminalia catappa* where the secondary metabolism is known to be different from that of terrestrial saprobic species (Strobel and Daisy 2003).

The HPLC profile of ethyl acetate extract of fungus cultured in the presence of acetone, showed in addition to the peaks found in control (untreated *A. niger*), one new peak at 35.87 minutes. Similarly, Guo et al (2014) reported that acetone was able to stimulate the production new metabolites by *Eupenicillium* sp. With ethanol as elicitor, one new peak was detected at 38.05 minutes in the HPLC profile while, 13 volatile compounds not found in control were identified with GC-MS analysis. Ethanol was also found by Cueto et al. (2001) to elicit the synthesis of a new chlorinated benzophenone antibiotic, pestalone, by the marine fungus *Pestalotia*. The HPLC profile of the extract from DMSO treatment showed an inhibition of the production of several compounds, and one new peak was detected at 38.05

minutes. On the contrary, the GC-MS profile showed the presence of 16 volatile metabolites of which 15 were not found in the control, suggesting that the presence of DMSO in PDB might have directed the secondary metabolism to the synthesis of volatile instead of non-volatile compounds. The GC-MS profile of extract from culture of *A. niger* with 5-azacytidine showed the presence of 13 compounds with one new compound detected at 26.683 minutes with 0.83% abundance. The 5-azacytidine, a DNA methyltransferase inhibitor, has previously been reported to stimulate the synthesis of new secondary metabolites in fungi (William et al. 2008, Henrikson et al. 2009).

Comparison of the intensities of different compounds detected with HPLC and the abundance of volatile compounds identified with GC-MS indicated that the rate of production of some compounds is elicitor-specific. In fact, the production of 1-undecyne was 1.36% in the presence of ethanol, and more than doubled when supplemented with DMSO (2.74%). Similar compositional variation trends were observed with many other components such as 5,6-dichlorohexene that increased from 0.2% with ethanol to 4.87% with 5-azacytidine; L-proline, N-valeryl-, undecyester that also increased to 3.63% with 5-azacytidine supplementation from 0.75% with DMSO and 0.21% with ethanol and 3-isopropyl[3]manxine-6,9-diol that increased to 3.67% with 5-azacytidine from 0.37% with ethanol. Such variation also concerned Cyclohexanecarboxaldehyde,3,3-dimethyl-5-oxo- that was highly produced in the presence of ethanol (64.38%) in comparison to DMSO (40.12%) and acetone (58.21%).

Conclusion

This study has demonstrated that small organic chemicals such as acetone, ethanol, DMSO, and 5-azacytidine can elicit the production of secondary metabolites by the endophytic *A. niger*. On a general point of view, this finding suggests a possible biotechnological application of these chemicals to increase the yield of specific compounds. This approach has shown promise, and has the potential to be exploited in the search for new bioactive chemical scaffolds against a large array of diseases.

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