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Original Research Article

Impact of small chemical elicitors on the production of volatile metabolites by endophytic fungi *Fusarium* sp and *Phomopsis* sp from Cameroonian medicinal plants

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Abstract: This study was designed to study the effect of small organic chemicals on the production of volatile metabolites by *Fusarium* sp and *Phomopsis* sp. Fungi were cultured for 6 days at 25°C in static condition in potato dextrose broth (PDB), PDB supplemented with 1µM of 5-Azacytidine, 1% DMSO, 1% Ethanol, 1%Methanol, 1% Acetone, 1% Acetonitrile, 1% 1-butanol and 1% Chloroform respectively. The ethyl acetate extracts prepared were analyzed by Gas chromatography coupled with Mass spectrometry (GC-MS). The results suggest that the production of volatile metabolites by *Phomopsis* sp and *Fusarium* sp is significantly affected by the chemicals used as elicitors. The production of Heptaethylene glycol; Z-10-Methyl-11-tetradecen-1-ol propionate; 1-butanol. 1-Di (tert-butyl) silyloxy-2-phenylethane; 1, 2-Butanediol and Linolenin, 1-mono by *Phomopsis* sp were induced by chemicals. While, Tetraacetyl-d-xylonic nitrile; 1-hexadecanol; 2-methyl-, Tetradecane, 2,6,10-trimethyl-; Octadecanal; 2-bromo-,(2-phenyl-1,3-dioxolan-4-yl)methyl 9octadecenoate, cis-; Methyl glycolate, benzyldimethylsilyl ether; 4-Octadecenal; Methyltartronic acid; 1,2-butanediol; Hexa-hydro-farnesol; 5-octadecenal; Hexaethylene glycol; Butyl 9, 12-octadecadienoate, Butyl 9-octadecenoate; Stearin 1, 3-di; Mono-2-ethylhexyl adipate, 4-methoxy-3-(2-methoxy-6-oxo-1-cyclohexen-1-yl) phenyl acetate and Heptacosane were produced by *Fusarium* sp only when grown in presence of elicitors. These results suggests the capability these chemicals to stimulate the production of new secondary metabolites by *Fusarium* sp and *Phomopsis* sp.
Keywords: *Fusarium* sp, *Phomopsis* sp, Small organic chemicals,GC-MS.

INTRODUCTION

Problems related to human health such as the development of drug resistance in pathogenic bacteria, fungal infections and life threatening virus claim for new therapeutic agents for effective treatment of diseases in human, plants and animals that are currently unmet [1,2]. Endophytic fungi are vast and largely untapped resources of novel and structurally diverse metabolites [3]. However, metabolite biosynthesis in microbes is tightly controlled by regulatory mechanisms which often limit the discovery of novel metabolites. Thus, a plethora of secondary metabolites encoded in the fungi genomes remain undiscovered [4-6].

Many approaches have been proposed to stimulate the production of new metabolites *invitro*. An effective screening process can be achieved through systematic manipulation of culture conditions for a small number of promising organisms. In fact, culture conditions have a major impact on the growth of microbes and the production of microbial products [4]. In addition, recent studies have indicated that various low molecular weight compounds are able to stimulate novel secondary metabolites in fungi [7-10]. In fact, Cueto *et al*. [11] successfully stimulate the production of a new chlorinated benzophenone antibiotic, pestalone, by the marine fungus *Pestalotia* in presence of 1% ethanol. William *et al*. [7] elicited the *de novo* production of several oxylipins in *C. cladosporioides* in presence of 5-azacytidine. In addition, Guo *et al*. [10] were able to stimulate the production new metabolites by *Eupenicillium* sp in presence of acetone. Therefore, this study was designed to investigate the impact the 5-Azacytidine, DMSO, Ethanol, Methanol, Acetone, Acetonitrile, 1-butanol and Chloroform on the production of volatile metabolites by *Fusarium* sp and *Phomopsis* sp endophytic fungi isolated from *C. odorata* and *T. mantaly* respectively, two medicinal

plants used in Cameroon in the treatment of several diseases [12, 13].

METHODS

Sources of endophytic fungi

Endophytic fungi used in this study were *Fusarium* sp isolated from bark of *C. odorata* (51244/HNC) and *Phomopsis* sp isolated from leaves of *T. mantaly* (42250/HNC). Isolates were identified by sequencing of their ITS1-5.8S-ITS2 region as described by Sánchez Márquez *et al.* [14]. The antimicrobial and antiradical potentials of these endophytic fungi have been reported [15].

Culture of fungi with chemical elicitors and extraction

Fusarium sp and *Phomopsis* sp were cultured in Potato dextrose broth supplemented respectively with 1µM of 5-Azacytidine (Sigma Aldrich), 1% DMSO (Sigma Aldrich), 1% Ethanol (HPLC grade, Merck), 1%Methanol (HPLC grade, Merck), 1% Acetone (HPLC grade, Merck), 1% Acetonitrile (HPLC grade, Merck), 1% 1-butanol (HPLC grade, Merck) and 1% Chloroform (HPLC grade, Merck) in static condition for 6 days. Medium supplemented with each of these chemicals was incubated in the same condition. Two flasks were used for each chemical.

To each culture, 20 mL of ethyl acetate were added. The cultures were 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. Ethyl acetate was evaporated at 40°C in a Labconco RapidVap parallel evaporation system. The residue was dissolved in 0.2 to 0.4 mL of methanol, transferred to pre-weighed microfuge tubes and subjected to evaporation to dryness. The weight of each elute was determined (Table 1) before making stock solutions (2mg/ml) in Methanol.

Analysis of extracts by Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis was carried out on a Agilent 7890 A GC Sysytem &7000 Triple quad GC MS System (Shimadzu Company) system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer instrument employing the fellowing conditions: Colunm varian HP-5MS 5% Phenyl Methyl Silox 325 °C: 30 m (L) x 250 µm (D) x 0.25 µm (T), operating in electron impact mode at 70eV, helium (99.999%) was used as carrier gas at a flow rate 1.3ml/min and a injection volume of 1µL was employed, injector temperature 260°C, ion-source temperature 280°C. The oven temperature was programmed at 110°C (isothermal for 2min), with an increase of 10°C/min, to 200C/min, then 5°C/min to 280°C/min, ending with a 9min isothermal at 280°C, Mass spectra were taken at 70eV, a scan interval of 300 mill second and fragments from 20 to 600M/Z.

Identification of compounds

Interpretation on GC-MS spectrum was conducted using the database of the National Institute Standard 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 retention time of the compounds identified were ascertained.

RESULTS AND DISCUSSION

Effect of elicitors on dry mass of extracts

The presence of chemicals in the cultured medium affect the growth and production of metabolites by these fungi (Table 1). The dry mass ranged from 0.49 to 3.32 mg and from 0.3 to 2.04mg for *Fusarium* sp and *Phomopsis* sp respectively. The cultured of fungi with 5-azacitydine have shown to increase the dry mass of *Fusarium* sp and *Phomopsis* sp by 2.29 and 1.29 times respectively.

Table-1: Effect of organic chemicals on dry mass of extracts produced by *Fusarium* sp and *Phomopsis* sp (mg)

Elicitors	Dry mass of extracts (mg)	
	Fusarium sp	*Phomopsis* sp
Untreated fungus	1.45±0.01	0.82±0.07
DMSO	0.54±0.04	0.98±0.11
Ethanol	0.92±0.03	0.85±0.09
Methanol	1.76±0.04	0.94±0.09
Acetone	0.49±0.03	0.70±0.10
Acetonitrile	0.82±0.00	0.76±0.07
1-Butanol	1.31±0.00	1.06±0.06
Chloroform	0.68±0.05	0.30±0.08
5-Azacytidine	3.32±0.00	2.04±0.00

Data are represented as mean values ± standard deviations of the assay preformed in duplicate

Table-2: Metabolites identified in ethyl acetate extracts from *Phomopsis* spcultured absence and in presence of Acetone, Acetonitrile, Chloroform, 1-butanol and 5-azacytidine

Compounds Identified	RT	Extract of non-treated fungus	Acetone	Acetonitrile	Chloroform	1-Butanol	5-Azacytidine
Isopropyl lactate	2.315	+	+	+	+	+	+
Heptaethylene glycol	2.390	-	-	-	-	+	-
1,2-Butanediol	2.460	-	+	-	+	-	-
1-Di (tert-butyl)silyloxy-2-phenylethane	3.580	-	-	-	+	-	-
Dimethyl Sulphoxide	6.630	+	-	+	-	-	+
Dodecane	16.675	+	+	+	-	-	+
Phenol, 2,4-bis (1,1-dimethylethyl)-	24.851	+	+	+	+	+	+
Folic acid	31.242	+	-	-	-	-	-
3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol	32.203	+	+	+	+	+	+
Z-10-Methyl-11-tetradecen-1-ol propionate	33.106	-	-	-	-	+	-
13-heptadecyn-1-ol	33.107	+	-	-	-	-	-
Linolenin, 1-mono	36.410	-	+	+	+	-	+

RT: Retention time;-: Not present; +:Present

Table-3: Metabolites identified in ethyl acetate extracts from *Fusarium* spcultured absence and in presence of Acetone, DMSO, Ethanol, Methanol, 1-butanol and 5-azacytidine

Compounds Identified	RT	Extract of non-treated fungus	Acetone	DMSO	Ethanol	Methanol	1-butanol	5-Azacytidine
Isopropyl lactate	2.315	+	+	+	-	+	+	-
Methyltartronic acid	2.329	-	-	-	+	-	-	-
Heptaethylene glycol	2.394	-	+	-	+	-	-	-
Hexaethylene glycol	2.390	-	-	-	-	+	-	-
1,2-butanediol	2.465	-	-	-	+	-	-	-
Methyl glycolate, benzyldimethylsilyl ether	2.891	-	-	+	-	-	-	-
Tetraacetyl-d-xylonic nitrile	2.928	-	+	-	-	-	-	-
Dimethyl sulfoxide	6.255	+	-	-	-	-	-	+
Dodecane	16.666	+	+	+	+	+	+	+
Tetradecamethylcycloheptasiloxane	23.848	-	+	-	-	-	-	-
3-furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	24.031	+	-	-	+	-	-	-
Phenol, 2,4-bis (1,1-dimethylethyl)-	24.889	+	+	+	+	+	+	+
Hexa-hydro-farnesol	26.805	-	-	-	+	-	-	-
1-hexadecanol, 2-methyl-	26.809	-	+	-	-	-	-	-

Phenol, 2,4-bis (1,1-dimethylethyl)-	26.814	-	-	-	-	-	-	-
2-hexadecanol	26.819	-	+	+	-	+	-	-
Tetradecane, 2, 6,10-trimethyl-	26.987	-	+	-	-	-	-	-
5-octadecenal	31.232	-	-	-	-	+	-	-
3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol	32.198	+	-	-	-	+	-	+
Octadecanal, 2-bromo-	33.097	-	+	-	-	-	-	-
13-heptadecyn-1-ol	33.098	+	-	-	-	+	-	+
1-oxa-spiro[4,5]deca-6,9-diene-2,8-dione, 7,9-di-tert-butyl	33.519	-	+	-	-	+	-	-
4-Octadecenal	35.020	-	-	+	-	-	-	-
Cis-13-eicosenoic acid	35.028	-	+	-	+	-	-	-
(2-phenyl-1,3-dioxolan-4-yl)methyl 9-octadecenoate, cis-	35.398	-	+	-	-	-	-	-
Linolenin, 1-mono	36.401	+	-	-	+	+	+	+
Butyl 9,12-octadecadienoate	39.081	-	-	-	-	-	+	-
Butyl 9-octadecenoate	39.138	-	-	-	-	-	+	-
Stearin, 1,3-di	39.391	-	-	-	-	-	+	-
Mono-2-ethylhexyl adipate	39.395	-	-	-	-	-	-	+
1-monolinoleoylglycerol trimethylsilyl ether	39.405	+	-	-	-	-	-	-
Diisooctyl phtalate	40.637	-	+	-	-	+	+	-
4-methoxy-3-(2-methoxy-6-oxo-1-cyclohexen-1-yl) phenyl acetate	40.764	-	-	-	-	-	-	+
Heptacosane	45.365	-	-	-	-	-	-	+

RT: Retention time;-: Not present; +: Present

Effect of elicitors on production of metabolites

The analysis of the ethyl acetate extracts from endophytic fungi *Fusarium* sp and *Phomopsis* sp cultured in presence and in absence of small organic chemicals was performed using gas chromatography coupled with mass spectrometry (GC-MS) to evaluate the impact of these chemicals on the production of volatile metabolites. The detected volatile components according to their retention time (RI) are given in table 2 and 3. The number of compounds identified was highly dependent to the extract. In general, 12 and 34 components were identified from *Phomopsis* sp and *Fusarium* sp extracts respectively.

Overall, these results suggest that the production of volatile metabolites by *Phomopsis* sp and *Fusarium* sp is significantly affected by the organic chemicals used as elicitors. This observation was also reported by many authors [8, 16,17]. From table 2, Isopropyl lactate, Phenol 2, 4-bis (1, 1-dimethylethyl)-, and 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol were identified in all the extracts. Phenol, 2, 4-bis (1,1-dimethyl ethyl) was also identified by GCMS in extracts of many fungi including *Monochaetia kansensis*[18] and *Colletotrichum gloeosporioides*[19]. Folic acid and 13-heptadecyn-1-ol were identified only of ethyl acetate extract of *Phomopsis* sp cultured without any chemicals. These results suggests a possible inhibition of their synthesis in presence of all the chemicals used. Moreover, the production of Dimethyl sulphoxide by *Phomopsis* sp was inhibited with the presence of acetone, choloroform, and 1-butanol. Dodecane was also inhibited by the presence of chloroform and 1-butanol. Heptaethylene glycol and Z-10-methyl-11-tetradecen-1-ol propionate were synthesized only when the fungus was grown in presence of 1-butanol. This suggest a possible stimulation of their synthesis by 1-butanol. 1-di (tert-butyl) silyloxy-2-phenylethane was identified only in extract from culture of fungus grown in presence of chloroform.1,2-Butanediol was stimulated by the presence of acetone and chloroform. Linolenin, 1-mono was identified in extracts from acetone, acetonitrile, chloroform and 5-azacytidine treated fungus.

The production of some metabolites by *Fusarium* sp was inhibited in presence of organic chemicals used while others were induced (table 3). In fact, the production of 1 monolinoleoylglycerol trimethylsilyl ether was inhibited in presence of all the chemicals used. The production of Isopropyl lactate was inhibited by the presence of 5-azacytidine and ethanol. Out of 5-azacytidine, all other chemicals used inhibited the production of dimethyl sulfoxide. While, 3-furanacetic acid, 4-hexyl-2, 5-dihydro-2,5-dioxo- was produced only in presence of ethanol. 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol and 13-heptadecyn-1-ol were inhibited in presence of acetone, DMSO, ethanol, ethanol and 1-butanol and the production of Linolenin, 1-mono was inhibited in presence of acetone and DMSO.

Dodecane, Phenol, 2, 4-bis (1,1-dimethylethyl)- were identified in all extracts from *Fusarium* sp (table 3). While, Tetraacetyl-d-xylonic nitrile; 1-hexadecanol; 2-methyl-, Tetradecane, 2,6,10-trimethyl-; Octadecanal; 2-bromo-,(2-phenyl-1,3-dioxolan-4-yl)methyl 9octadecenoate, cis- were specifically produced in presence of acetone. Methyl glycolate, benzyldimethylsilyl ether and 4-Octadecenal were produced only by culturing *Fusarium* sp in presence of DMSO. The presence of ethanol in the culture medium, stimulate the production of Methyltartronic acid; 1,2-butanediol, and Hexa-hydro-farnesol. Methanol stimulated the production of 5-octadecenal and Hexaethylene glycol. While, Butyl 9, 12-octadecadienoate, Butyl 9-octadecenoate and Stearin 1, 3-di were produced only in presence of 1-butanol. The presence 5-azacytidine stimulated the synthesis of Mono-2-ethylhexyl adipate, 4-methoxy-3-(2-methoxy-6-oxo-1-cyclohexen-1-yl) phenyl acetate and Heptacosane. The production of some metabolites was induced in presence of many elicitors. Thus, in presence of acetone and ethanol, Heptaethylene glycol and Cis-13-eicosenoic acid were specifically produced. Acetone and methanol induced the production of 1-oxa-spiro [4, 5] deca-6, 9-diene-2, 8-dione, 7, 9-di-tert-butyl; Diisooctyl phthalate and 2-hexadecanol.

CONCLUSION

This preliminary work was for the exploration of the potential of small organic elicitors to induce to synthesis of new metabolites by *Fusarium* sp and *Phomopsis* sp. The analysis done by GC-MS predicts that these small organic chemicals are capable to stimulate the production of new bioactive compounds by fungi. Further work will be carried out for evaluation of antimicrobial potential of these extracts and large scale culture of fungi in presence of elicitors for isolation the induced metabolites.

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