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EXTRACTION AND IDENTIFICATION OF ANTIFUNGAL COMPOUNDS FROM *BACILLUS SUBTILIS*

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ABSTRACT

In our previous study *Bacillus subtilis* is considered to be the most effective biocontrol agents than the other tested *Basillus* isolates on controlling pathogenic fungal growth of faba bean. The maximum growth of *B. subtilis* was found on trypticase soy broth (TSB) medium at pH 7.0 for 48h. under shaking condition. The active antifungal compounds recorded by Thin Layer Chromatography (TLC) and Gas Chromatography- (Mass Spectrometry (GC-MS) technique) indicated that the detected volatile compounds included Alkyls, Esters, Organic acid, Alkane and Phenyl compounds. Present results demonstrated that *Bacillus subtilis* was rich resources of bioactive volatiles and play an important role in reducing diseases levels.

Key word: Antifungal activity, *Bacillus subtilis*, soil-born and foliar diseases, TLC, GC-MS, VOCs

INTRODUCTION

Faba bean is a subjected to attack by several diseases such as soil-borne diseases. Damping–off, wilt disease caused by *Fusarium oxysporum* is considered the most important disease which causing great losses in annual seed yield (Abou-Zeid *et al*., 1999). Chemical treatments were more effective than other strategies for controlling plant diseases. In general, however they cause to be a environmental problems pollution and fungicide resistance. Biological control proved useful alternative to chemical. Recently it was found that, *Bacillus subtilis* produces a variety of effective volatile antifungal compounds to control of foliar and soil-borne of faba bean diseases (Xu *et al.,*2007 and Zou *et al*., 2007). Therefore, the objective of this study,

was the extraction and identification of volatile antifungal compounds from *Bacillus subtilis* by GC-MS was carried out.

MATERIALS AND METHODS

Effect of different growth broth media on *Bacillus subtillis* growth

Influence of different broth media on the growth and production of antifungal compounds of *Bacillus subtillis* has been detected by growing it under shaking condition at 37ºC for 48 h. The tested broth media were nutrient broth (NB), sucrose broth (SB), trypticas dextrose broth (TDB) and trypticas soya broth (TSB) (Kajimura and Kaneda, 1996). The growth was measured spectrophotometerically at 620nm.

Extraction of antifungal volatile organic compounds (VOCs) from cultural filtrates of *Bacillus subtilis*

As maximum antibiotic production was observed from *Bacillus subtilis* growing in (TSB) medium, the filtrate was centrifuged at 10,000 rpm for 20 min to separate the bacterial cells. The supernatant was acidified to pH 2.5 with concentrated HCl and added ammonium sulphate (40% w/v) up to saturation to precipitate the antifungal volatile organic compounds (VOCs). Different solvents were used for extraction of antifungal compounds from cell free supernatant, the solvents used were n-hexane, ethyl acetate, petroleum ether (60-80ºC) and chloroform to determine the best solvent for extraction of antifungal VOCs. This solvent, n-hexane, ethyl acetate, chloroform and petroleum ether (1:1 ratio) was added to the supernatant, and then separated using separating funnel. All the solvent extracts were assayed for their antifungal activity directly onto thin layer chromatography plats TLCs (Weiwei *et al.,* 2008).

Separation and purification of antifungal VOCs

The solvent containing VOCs was evaporated under vacuum at 40ºC and tested for number of components present by using Thin Layer Chromatography (E. Merk, AG, and Darmstadt, Germany). About, 40 - 50 µl of the organic solvent extract was spotted on the TLC plate and a system consisted of chloroform: methanol: petroleum ether (60: 20: 20) was used as a mobile phase. The plats were developed and bands were visualized with UV light (365 nm). R_f of each fraction were calculated (Kumar *et al.,* 2009).

Detecting the antifungal activity of VOCs on TLCs plat (bioautography)

Antifungal activity of the active volatile organic components which extracted by previous organic solvent was bio-assayed directly onto TLCs plat against the most common causal pathogen of wilt diseases for faba bean *Fusarium oxysporum*. The plate was air-dried and sprayed by Rose Bengal and set for to be solid form and then sprayed separately with spore suspension of *Fusarium oxysporum*. The sprayed plate was placed in a closed humid tray and incubated at 25°C for 72 hours. Antifungal activity of the bacterial filtrate components was indicated by the absence of mycelial growth around the migrated compound on the plate (Mohanlall and Odhav 2009). The active fractions which cause the absence of mycelia around the migrated compound on the plate was scrapped and tested for identified chemical constituents by using GC-MS chromatography as follows.

Detection of antifungal activity for VOCs on PDA plate

The pathogenic fungus *F. oxysporum* 5 days old cultures were inoculated at PDA plate (9cm) and the active fraction which caused the absence of mycelia around the migrated compound on the plate was scrapped and putted in three cork boreal were used as replicates, and the control were inoculates by the pathogen alone. The inoculated plates were incubated at 25^{o}C until the mycelial growth cover the medium surface in control treatment.

Identification of chemical constituents in active antifungal VOCs by using GC-MS chromatography

The GC-MS analysis for derivative extracted compound by using BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) +TMCS (trimethylchlorosilane) as derivative kits was performed on Agilent 6890 N gas chromatography instrument coupled with an Agilent MS-5975 inert XL mass selective detector and an Agilent auto sampler 7683-B injector (Agilent Technologies, Little Fall, NY, USA). A capillary column HP-5MS (5% phenyl methylsiloxane) with dimension of 30 m x 0.25 mm I'd x 0.25 µm film thickness (Agilent Technologies, Palo Alto, CA, USA) was used for the separation and identification of VOCs by biotechnology laboratory. The initial oven temperature was 40°C, held for 2 min, ramped at 6°C min^{-1} to 180°C and ramped at 10°C min^{-1} to 250°C and held for 3 min. The ions were

detected in the range 30-350m/z. The mass spectra of the unknown compounds were compared with Chem Station 6890 Scale Mode software with two libraries (NIST & Wily) which provide best information about the identification of active compound which separated from TLCs (Liu *et al.*, 2008).

RESULTS AND DISCUSSION

It is obvious from Table (1) that *Bacillus subtilis* showed maximum growth under shaking condition on trypticase soy broth (TSB) medium, while nutrient broth (NB) appeared to be a poor medium for the growth of *B. subtilis*. These results closely resemble those reported by (Kumar *et al.,* 2009) who found that maximum growth and antimicrobial production of *B. subtilis* grown on TSB medium at pH 7 was observed at 620 nm.

Table 1. Effect of different broth media on *Bacillus subtilis* growth recorded spectrophotometrically at 620 nm.

Medium	Bacterial growth (Turbidity)
Nutrient broth	**0.70**
Sucrose broth	**1.75**
Trypticase soy broth	**2.6**
Trypticase dextrose broth	**2.2**

Different solvents were used for extraction of antimicrobial compounds from cultural filtrates of *B. subtilis* spotted on TLC plat (Fig.1) and tested for their antifungal activities. Among these solvents ethyl acetate extract represented the most suitable agent for extraction of antifungal compound among the other tested solvents (Fig.2). On the contrary Kumar *et al.* (2009) who reported that among different organic solvent used for the extraction of antimicrobial volatile organic compounds from cultural filtrates of *B. subtilis*, chloroform was the best solvent for extraction of antifungal compounds against *Trichophyton* species.

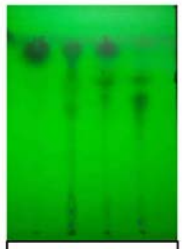

Fig.1

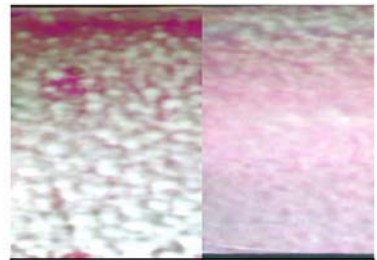

Fig2a. Ethyl acetate | Fig2b. Control

(Fig1) :TLC plate with different bands of bacterial compounds extracted by different solvents (1)n-hexane, (2)ethyl acetate, (3)chloroform and (4)petroleum ether
(Fig 2a) Bioautograph of the effect of TLC plat bioactive fraction
(Fig 2b) TLC plate without bioactive fraction.

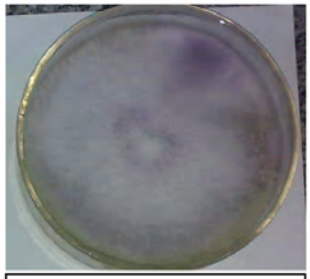

Fig 3b. Control

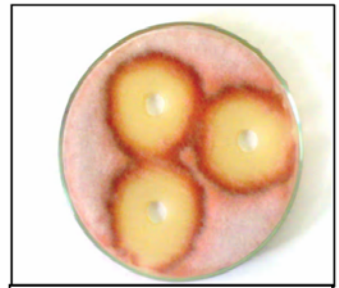

Fig 3a. Ethyl acetate

Fig (3) Effect of bioactive band of *B. subtilis* on the growth of *F. oxysporium.*

The active antifungal band which was separated by TLC plat showed R_f value 0.65. This band showed the highest antifungal activity against *F. oxysporum*. This band was scrapped and tested for their antifungal activity on potatoes dextrose agar (PDA) plate media inoculated with *F.oxisporum*. This antifungal compounds reduced mycelial growth by 85%. (Fig. 3a) compared with control (Fig. 3b).

Analysis of VOCs extracted by ethyl acetate from cell free filtrate of *B. subtilis*, grown on TSB medium, by (Mass Spectrometry (GC-MS) resulted at least 32 compounds. Data are given in Table (2). It seems that volatile organic compounds produced by *B. subtilis* contain more than one kind of bioactive compounds including alkyls (16 compound), esters (3 compounds), organic acid (1 compound), and alkane (12 compounds). These different compounds determine the different antagonistic natures of bacterial metabolites, (Fig 4).

Therefore, such antagonistic volatiles producing bacterium is potentially effective biocontrol agent against soil-borne disease pathogen such as *Fusarium oxysporum*, these data are in agreement with that reported by Weiwei *et al*., (2008) who recorded that volatiles compound from *Bacillus subtilis* inhibited mostly the growth of *B. cinerea* (75% inhibition,), whereas the inhibition against *R. solani* and *F. oxysporum* was 46% inhibition. It seems that these volatiles play a significant role in reducing the pathogenic fungal infection ability.

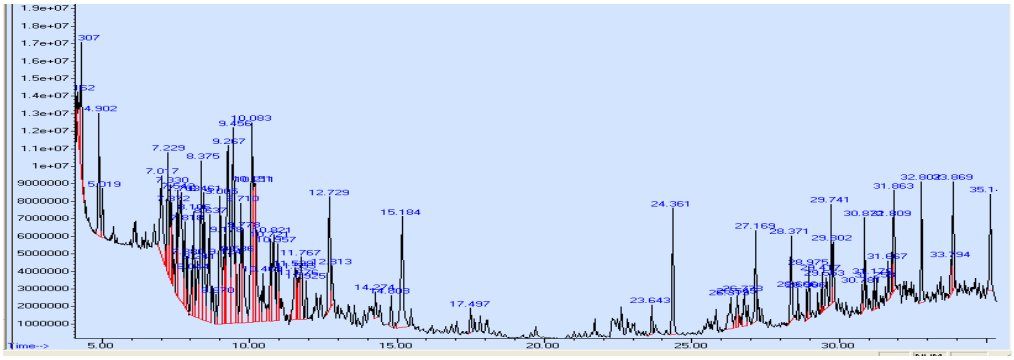

Fig (4). GC profiles of *B. subtilis* volatile.

The volatile organic compounds included fatty acids known as long chain Hexadecanoic acid (C_{16}) and Octadecanoic acid (C_{18}) which is considered to be more biologically active than shorter hydrocarbon side chains Dodecanoic acid (C_{12}). These results are in harmony with those reported by Weiwei *et al*. (2008) who reported that Mycosubtilins with C_{16} or C_{17} fatty acid chains are considered to be more biologically active than the iturins, which have shorter hydrocarbon side chains (C_{14} and C_{15}). Meanwhil, fungitoxicity increases with the number of carbon atoms in the fatty acid chain; *i.e*., C_{17} homologues are 20-fold more active than the C_{14} forms. On the other hand, Touré *et al.,* (2004) suggested that the length of the fatty acid chain varies from C_{13} to C_{16} for surfactins, from C_{14} to C_{17} for iturins, and from C_{14} to C_{18} in the case of fengycins. Different homologous compounds for each lipopeptide family are thus usually co produced. Iturins and fengycins display strong antifungal activity and inhibit the growth of a wide range of plant pathogens. Also, data presented in Table (2) revealed that the fragmentation of the major compound was found to be rather similar to that of alkyl compounds with quality match (QM) of 90 - 99%. This compound was found to consist about 51.67% as a relative percentage amount. Also, alkane compounds

represent about 38.18% as a relative percentage amount with (QM) of 91-99%. However this compound is known to possess an antimicrobial activity especially the fractions of organic acid with (QM) 93. Focused on volatile organic compounds (VOCs) are small molecules that characteristically have a high vapor pressure and easily volatilize with ideal infochemicals because they can act over a wide range of distances and their spheres of activity will extend from proximal interactions to greater distances via diffusion in air, including in soil pores (Wheatley, 2002).

Table 2. Total ionic chromatogram (GC–MS) of ethyl acetate extract of *Bacillus subtilis.*

Pk	IUBC Name	RT	Area %	Molecular Structure	Class	Quality match (QM)
1	1- Octadecanoic acid	27.16	1.29	$C_{18}H_{36}$	Organic acid	99
2	Hexadecanoic acid,methyl ester	28.97	0.58	$C_{16}H_{32}O_2$	Ester	99
3	Eicosane	29.80	0.56	$C_{27}H_{56}$	Alkane	99
4	Tetracosane	33.87	1.53	$C_{24}H_{50}$	Alkane	99
5	1-Hexadecene	23.64	0.40	$C_{16}H_{34}$	Alkyl	98
6	Pentadecane,8-heptyl	31.86	1.00	$C_{15}H_{32}$	Alkyl	98
7	Tricosane	31.86	1.48	$C_{23}H_{48}$	Alkane	97
8	Octadecane	28.60	0.29	$C_{18}H_{38}$	Alkane	97
9	Dodecane	15.18	3.13	$C_{12}H_{26}$	Alkane	97
10	Tridecane	17.49	0.40	$C_{13}H_{28}$	Alkane	97
11	2,6- Diisopropylnaphthalene	26.31	0.99	$C_{16}H_{20}$	Alkyl	97
12	Octadecanoic acid , methyl ester	31.18	0.34	$C_{18}H_{36}O_2$	Ester	96
13	1-Docosene	31.81	0.78	$C_{22}H_{44}$	Alkyl	96
14	Z-8-Hexadecene	30.78	0.34	$C_{16}H_{32}$	Alkyl	95
15	Benzen, 1,2,3,trimethyl	9.454	4.98	C_9H_{12}	Alkyl	95
16	Dodecanoic acid,1-methylethylester	24.36	2.08	$C_{12}H_{24}O_2$	Ester	95
17	Cclohexane, methyl	4.310	2.31	C_7H_{14}	Alkyl	94
18	1-Nonadecane	29.73	1.12	$C_{19}H_{40}$	Alkane	94
19	P-xylene	7.543	1.46	C_8H_{10}	Alkyl	94
20	Decane	10.20	2.62	$CH_3(CH2)_8CH_3$	Alkane	94
21	undecane	12.72	2.77	$C_{11}H_{24}$	Alkane	94
22	Nonane (CAS)	7.703	3.92	C_9H_{20}	Alkane	93
23	1-Ethyl-4-methylcyclohexane	7.818	1.46	C_9H_{18}	Alkane	93
24	Cyclohexane, propyl	8.37	3.4	C_9H_{18}	Alkyl	93
25	Benzen,1-ethyl-2-methyl	11.53	1.12	C_9H_{12}	Alkyl	93
26	Decan,3methyl	11.92	0.52	C_9H_{12}	Alkyl	93
27	Pentasiloxane,dodecamethyl	14.81	0.49	$C_{12}H_{36}O_4Si_5$	Alkyl	93
28	Cyclohexane, butyl	10.95	1.02	$C_{10}H_{20}$	Alkyl	91
29	Tetratriacontane	31.26	0.31	$C_{34}H_{70}$	Alkane	91
30	Octane,2,6-dimethyl	8458	2.27	$C_{10}H_{22}$	Alkyl	91
31	Trisiloxane, octamethyl	7.228	1.65	$C_8H_{24}O_2Si_3$	Alkyl	90
32	Benzen,1-ethyl-3- methyl	10.82	2.51	C_9H_{12}	Alkyl	90

Our results indicate the effectiveness of about 32 VOCs, from *B. subtilis*, against some plant pathogenic fungi. This finding should be considered when formulating bicontrol compounds or establishing a biocontrol strategy.

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استخلاص وتعريف المركبات المضادة للفطريات من الباسيلس ستلس

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تستخدم الباسيلسات كطرق بيوتكنولوجية حديثة لمقاومة أمراض الفول البلدي حيث وجد أن أعلى نمو *Bacillus subtilis* قد سجل على بيئة تريبتاز سوى السائلة على أس هيدروجيني 7 لمدة 48. كما تم عزل المركبات المقاومة للفطريات الممرضة من عزلة *Bacillus subtilis* وتم تعريف هذة المركبات عن طريق جهاز GC-MS وتقسيم المركبات إلى (32) مركب وهم (16 مركب الكايل ،3 مركب استر، 12مركب الكان، 1 مركب حمض اورجانيك) تلعب هذة المركبات دور هام لما لها من قدرة كبيرة على مقاومة الكائنات الممرضة المتسببة لأمراض الذبول *F. oxysporum* .لذا وجد أن *subtilis Bacillus*تعتبر مصدر غنى بالمواد الفعالة التي تلعب دور مهم في مقاومة الأمراض.