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Screening of Compounds Secreted by Local Isolates of Phosphate Solubilizing Fungi (PSF) by GC-MS Analysis

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ABSTRACT

Background: Microorganisms are an important factor in solubility of nutrition minerals; phosphate solubilizing fungi (PSF) considered one of the main sources of sustainability of agriculture production systems. Some fungi are well known for their natural capability to produce various organic acids, due to the lowering pH in the soil. The study amid to isolate PSF from rhizosphere soil and some plant residue and identify its secrenning compound using GC-MS analysis.

Methods: in laboratory investigation during 2016, isolates of PSF and check their solubilizing activities using selective artificial media PVK and NBRIP in soild and broth form. The secrening of secondary organic compound in broth culture extracted and identify using GC-MS analysis.

Result: the results inventory six isolates, among these isolates *Aspergillus niger* AD-A2 and AG-B1 were Distinguished by formed halo-clear zones around their colonies as a result of their phosphate-solubilizing ability in soild PVK and NBRIP media. pH decrese of the final product of PSF isolate in broth medium. Various compounds formed by submerged fermentation of *A. niger* isolates were 1,3-dioxan-5-ol (96.47%); octanoic acid, 4-chlorobenzyl ester (0.14%); 1,3,5-cycloheptatriene (0.58%); Crotonic acid (2.64%); 2-Propanone, 1,1-diethoxy (0.18%); 2-Butanone, 4-hydroxy (13.11%); 1-Tetrazol-2-ylethanone (86.4%); Propanethioamide (0.49%); Acetic acid, ethyl ester; Acetidin (100%); Methylolacetone (100%) and *Trichoderma hamatum* (T-113) were identified: Ethane, 1,1-diethoxy (100%) and 2-Butanone, 4-hydroxy-(100%).

Key words: *Aspergillus* spp, GC-MS analysis, Organic compounds, *Trichoderma hamatum*.

INTRODUCTION

Phosphate solubilizing fungi (PSF) are considered important source for maintaining the sustainability of agriculture production systems. So, the links between PSF and ecosystem processes need to be understood. Many soil-microorganisms especially fungi have ability to change the phosphorus (P) from insoluble to available to plant by chelation, acidification, exchange reactions *etc.* (Hamdali *et al*., 2008; Javaid, 2009). Fungi are considering good acid producers, so consequently show higher phosphate solubilization activity than other microorganisms like bacteria (Xiao *et al.,* 2013).

The fungal isolates which expressed P-solubilizing ability in produced a clear zone around their colony on precipitated phosphate agar and acidity (pH decrease) in liquid medium (Whitelaw, 2000). Several of soil fungi, especially *Aspergillus, Penicillium* and *Trichoderma*, have ability to change insoluble soil phosphates to soluble forms by some mechanisms such secreting organic acids (Xiao *et al*, 2015). They also exhibit some other characteristics such as solubilize of minerals (Raaijmakers *et al*, 2009) in addition to secretion other compounds in secondary metabolites (Calvo *et al*., 2002).

Some fungi mostly from the *Aspergillus* species have a natural capability to produce some various organic acids, *e.g.* malic, itaconic, gluconic, palmitic, citric and leinoleic acid (Khan and Javaid, 2021). Some of these organic acids can be produced through large-scale bioprocesses, showing a high potential of fungi as organic acid production plateforms (Li and Punt, 2013). The Aspergilli produce high concentration of organic acids. Some strains showed the ability to produce a variety of enzymes and other secondary metabolites such fatty acids especially unsaturated ones and sterol (Al-Taie and Alwan, 2014).

Elkot and Derbalah (2011) reported that using GC-MS analysis for culture filtrates of bacte-rial and fungal revealed that it contained alkenes, alcohol, esters, ketones, aldehydes and fatty acids. Recently, it was demonstrated as a volatile organic compounds (VOCs), produced by some *Aspergillus* spp. can influence the growth of fungi (Schalchli *et al*, 2011). Moreover, PSF provides a suitable environment for the plant rhizosphere for nutrition availability, especially phosphate by reducing pH, producing hormones in addition to secretion organic acids and phosphatase. This will improved plant growth and it will be positively reflexes on plant yield (Al-Taie *et al*, 2016).

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Secretion of different organic compounds which increase "acidification" is considered as the mechanisms of inorganic phosphate solubilization especially in the alkaline soils which most of Iraqi soils are. So, the content of PSF filtrate from organic compounds under in vitro conditions needs to screening to understand their role in the ecosystem. The present study aimed to isolate PSF from rhizosphere soil of some plants grown in Wasit province and some organic wastes, also to characterise it with respect to screening the submerged fermentation of selected isolates of PSF to identify bioactive compounds in their extract using GC-MS techniques because it represent the allelopathic effect of these fungi, which lead to decrease pH and inhibited the growth of pathogenic microorganisms; that can be used in future for crop improvement and their filtrate compounds as bio-fertilizer.

MATERIALS AND METHODS

The study conducted to isolate and characterise PFS from rhizosphere soil of plants that grown in Wasit province, south central Iraq (32.5086651°N, 45.790801°E) and some organic wastes. Five (g) of soil from rhizosphere and plants residue were collected, then suspended in 100 ml of sterile distilled water and shaken on a rotary shaker for 5 min on to separate the microorganisms from the sample. All sampling procedures were repeated in triplicate.

The samples were serially diluted to 10^{-3}, 10^{-5} and 10^{-7} and spread on petri dish contain potato dextrose agar (PDA). The plates were incubated at 27°C for 5 days or until the colonies developed and then transferred to fresh medium and purified. The pure strains were maintained on PDA slants at 4°C. The isolates were identified by their colony characteristics, spores morphology and microscopic observations. The fungi frequency ratio was determined according to the following equation:

Percentage of frequency (%) =

$$\frac{\text{Number of fungle colonies}}{\text{Total number of colonies}} \times 100$$

For investigation of phosphate-solubilizing isolates activity, laboratory study conducted at mycology laboratory, plant protection department, college of Agriculture, university of Wasit in September 2016. The cultures were spot inoculated on solid phosphate solubilization media: PVK[1] (Pikovaskya, 1948) and NBRIP[2] (Mehta and Nautiyal, 2001). After 5 days of incubation at 27°C, the plates were examined for the presence of clear zones around colonies. The experiment was repeated in triplicate for each isolates. For indicating P solubilization activity, colonies forming a clear halo were counted and further used to determine the P-solubilization index. Solubilization index (SI) was measured using following formula (Edi-Premono *et al.*, 1996):

$$SI = \frac{\text{Colony diameter + Halazone diameter}}{\text{Colony diameter}}$$

Fungal culture of P solubilization activity were inoculated in to 100 ml in broth of phosphate solubilization media (PVK and NBRIP) in conical flasks 250 ml and incubated on a shaker incubator 120 rpm at 27°C for 14 days. Un-inoculated Sterile medium served as control. Initial pH and changing in pH was noted after 14 days of inoculation using pH digital meter (Nenwani *et al.*, 2010), in addition to weight of mycelia dry masses of each isolates at the end of incubation periods by drying the biomass colony in oven at 90°C till their weight are to be constant. The dry biomass was calculated in g/l of fermentation medium. The experiment was performed in triplicate for each isolates.

Secondary (Organic) compounds formed by PSF with GC-MS

For organic compounds determination, on the 14th day of incubation, 10 ml of each inoculated flasks was removed and filtered, then pooled to be extracted and also used for GC-MS analysis. The extracted and analysis of GC-MS conducted in laboratories of ecology department, ministry of science and technology, Iraq.

Sample extraction

Organic compounds in PSF culture broth, samples were extracted into 10 ml tube contain 5 ml of ethyl acetate using mechanically shaking for 10 min, then the tube was immediately centrifuged at 800 g for 5 min. The organic layer was transferred to a second tube and solvent was evaporated under reduced pressure. The residue was dried in vacuum desiccators over P_2O_5-KOH.

GC-MS analysis

GC-MS QP 2010 plus shimadzu, A fused-silica capillary column coated with Optima-5 m scroos-linked 5% phenylmethyl silicone (SE-30 m, 0.25 mm i.d., 0.32 mm film thickness used). The GC temperature program was as follows: initial temperature was 50°C, held for 2 min) increased to 200°C at a rate of 5°C/min, then to 280°C rate of 10°C/min and finally to 280°C held for 10 min. The split ratio was 1:10 injection temperature was 280°C, transfer line temperature) was 250°C and ion source temperature was 200°C). The mass spectrometer was operated at 70 eV in the electron impact mode with SCAN or selected ion monitoring, The selected ion groups for the identification of organic acid and organic compounds.

Statistical analysis

All experiments were performed in triplicate. Genstat statistical program was used to analyse the results that conducted in Completely Randomized Design-CRD in experiments, a one and two-way ANOVA were used and the averages were compared using the LSD test (p<0.01) to compare between means.

RESULTS AND DISSCUSION

The isolation results revealed that *Aspergillus* genus was

[1] (PVK)- Pikovskaya's media
[2] (NBRIP)- (National Botanical Research Institute's phosphate growth medium

the highest frequency in the Rhizosphere soil and some organic residue by using dilution method (Table 1), sixth fungal strains were identified on the basis of colonies microscopic characters as *Aspergillus niger* , *A. terrus* and *Trichoderma hamatum* based upon their colony morphological characteristics. The *A. niger* was found in all replicates and ranged by 15.3% in the rhizosphere soil of Alfa-Alfa plant to 100% in the garlic Rhizosphere soil, followed by 60.7% in the sample containing Date palm residue, While the lowest frequency was *T. hamatum* with 22.5 % in the sample containing cucumber.

Kalaf *et al.* (2018) reported that numbers of different microorganisms were isolated from two samples of soil with and without plant residues and the *Aspergillus niger* was record about 25.5 to 41.6% respectively, While the lowest frequency was *T. harzianum* with 8.7% in the soil sample containing plant residues.

Fungal culture of phosphate solubilizing activity

In solid media

The study results showed that all fungal isolates formed a clear halo zone indicate of phosphate solubilisation activity. after 5 days of incubation a clear halo zone was formed around isolate colonies on solid PVK and NBRIP medium which supplemented with calcium phosphate, indicating fungal isolates ability of phosphate-solubilizing (Table 2). *Aspergillus niger* isolates AD-A2 and AG-B1 showed strong phosphate solubilization with average colony diameter 7.20 and 6.73 cm in PVK and NBRIP medium respectively, while other fungi was less effective solubilization by making smaller clear zones around their colonies.

The results also reflex on Solubilization Index (SI) of Phosphate, were it ranged 1.03 to 1.29. Data recorded high SI for *Aspergillus niger* isolate AD-A2 and AG-B1 with average 1.29 and 1.28 respectively than other isolates especially *Trichoderma hamatum* which showed least phosphate-solubilizing activity with SI 1.03 in both PVK and NBRIP medium respectively.

The results also showed that *Aspergillus niger* have significant ability to solubilizing phosphorus and make it available for absorbs by plants and improves plant growth more than other fungi. However, solubilization of various forms of precipitated calcium phosphate in solid agar plates has been widely used as an initial isolation criterion for Phosphorus solubilizing microorganisms (Alikhani *et al*, 2006). Whitelaw, (2000) and Al-Taie and Alwan, (2014) indicates that precipitated phosphorus agar techniques are useful for isolating and selecting microorganisms for further investigations but have limited sensitivity.

Results of the present study are supported by the observation of Al-Taie, (2014) and Saber *et al.* (2009) who studied ability of isolate *Aspergillus niger* to produce a clear halo zone around their colony in NBRIP solid media have similar results, this indicates its ability to precipitated phosphorus due to produce of organic acids.

In broth media

After fungal isolates phosphorus solubilizing ability was confirmed on solid medium, this ability was carried out in

Table 1: Fungal frequency percentage isolated from rhizosphere soil and organic residue.

Fungus \ Rhizosphere soil	Garlic	Alhagi	Alfalfa	Date palm residue	Cucumber residue	Average %
Aspergillus niger	100					100
A. niger		66.66				66.66
A. terrus			15.3			15.3
A. niger			15.3			15.3
A. niger				60.7		60.7
Trichoderma hamatum					22.5	22.5

Table 2: Ability of fungal isolates of phosphate solubilizing fungi (PSF) in two solid media after 5 days of incubation at 27°C.

Fungal isolate	Isolate code	Colony diameter with clear halo zone (cm)			Colony diameter without clear halo zone (cm)			SI		
		NBRIP	PVK	Average	NBRIP	PVK	Average	NBRIP	PVK	Average
Aspergillus niger	AG-B1	8.63	4.83	6.73	6.66	3.80	5.23	1.29	1.27	1.28
A. niger	AD-A2	8.20	6.20	7.20	6.23	4.88	5.58	1.31	1.27	1.29
A. terrus	AAT-C2	3.03	4.43	3.73	2.66	3.50	3.08	1.13	1.26	1.21
A. niger	AA-C3	6.00	6.73	6.37	5.9	5.90	5.90	1.01	1.14	1.07
A. niger	AD-11	3.40	6.12	4.81	3.07	5.08	4.08	1.10	1.20	1.17
Trichoderma hamatum	T-113	2.90	8.30	5.60	2.73	7.70	5.21	1.06	1.07	1.07
Average		5.36	6.10		4.55	5.19		1.14	1.19	
L.S.D 1% between fungal isolates			0.29			0.30			0.011	
L.S.D 1% between solid media			0.19			0.21			0.012	
L.S.D 1% for interaction between fungal isolates and solid media			0.49			0.55			0.020	

liquid (broth) medium. The PSF isolates ability was accompanied by a decreasing pH of the medium. The results (Table 3) showed that PSF more significant of decreasing pH as compare with control treatment in both PVK and NBRIP broth medium at 14 days of shaking incubation 120 rpm at 27°C.

Aspergillus niger isolate AD-11 was dropped pH of final product from fermentation significantly 3.52 and 4.71 followed by the isolate AD-A2 with 5.05 and 5.30 in both PVK and NBRIP broth medium compared with control treatment where it remained constant around pH 5.75 and 6.61 respectively, while *Trichoderma hamatum* which showed least decreasing of pH compared with other fungal isolates with 5.32 and 5.39 in both PVK and NBRIP medium respectively.

The study indicated that mycelia dry masses production by *Aspergillus niger* isolate correlated with acidity (reducing pH). The results (Table 3) showed that isolates AD-11 were recorded high amounts of fungal biomass 0.30 and 0.35 g and 190.8 and AD-A2 were recorded 0.28 and 0.34 g at both PVK and NBRIP broth medium respectively after 14 days of shaking incubation at 27°C. Such results revealed that the fungal strains have ability to solubilizing P and change it to available form and consume enough P for its own growth.

Yuan *et al.* (2005) reported that the significant decrease in pH broth medium as a result from organic acids production, that effect comes from consequence consumption of sugar. Barroso and Nahas (2008) revealed that rhizosphere microorganisms also associated the acid producing activity by decrease in the pH of the culture medium. This effect were explain how organic acids can release soluble phosphate by chelating the cations complex to inorganic phosphate (Whitelaw *et al.*, 1999), which sutable to plant because of P availability to plants. Also, releasing of organic acids by non-vesicular mycorrhizal fungi can sequester cations beside create a microenvironment acidifying near the roots, this effect is considered to be a major mechanism of P-solubilization, as well as Mn, Fe and Zn by plants (El-Azouni, 2008).

Due to the fact that many different mechanisms effects were involved in organic compound in the fungal filtrate (Zeng et al, 2001). The organic-chemical of PSF effects on cucumber seedling were demonstrated with *Aspergillus* sp. (14) and *Trichoderma hammatum* (Zayed and Motaal, 2005).

Table 3: pH and dry biomass of phosphate solubilizing fungi isolates in broth media after 14 days of shaking incubation 120 rpm at 27°C.

Fungal isolate	Isolate code	pH			Dry biomass (g)		
		NBRIP	PVK	Average	NBRIP	PVK	Average
Aspergillus niger	AG-B1	5.03	6.00	5.52	0.19	0.25	0.22
A. niger	AD-A2	5.05	5.30	5.18	0.28	0.34	0.31
A. terrus	AAT-C2	4.45	6.10	5.28	0.20	0.35	0.27
A. niger	AA-C3	4.43	5.30	4.87	0.26	0.33	0.29
A. niger	AD-11	3.52	4.71	4.12	0.30	0.35	0.32
Trichoderma hamatum	T-113	5.32	5.39	5.36	0.11	0.13	0.12
Control		5.75	6.61	6.18			
Average		4.79	5.63		0.22	0.29	
L.S.D 1% between fungal isolates			0.14			0.022	

Table 4: Natural products, organic compounds identified in the extract of filtrate of PSF by GC-MS analysis.

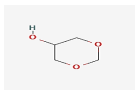
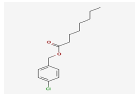
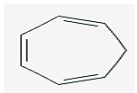
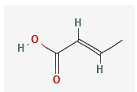
Isolate code	Name of compound	Molecular formula	Molecular weight	Retention time (min)	Area (%)	Activity	Chemical structure
AG-B1	1,3-Dioxan-5-ol	$C_4H_8O_3$	104	2.091	96.47	Antibacterial, Antimycotics, Antivirals	
	Octanoic acid, 4-chlorobenzyl ester	$C_{15}H_{21}ClO_2$	268	2.267	0.14	Antimicrobial	
	1,3,5-Cycloheptatriene	C_7H_8	92	2.413	0.58	Volatile compound-antimicrobial	
	Crotonic acid	$C_4H_6O_2$	86	2.941	2.64	Antifungal Antibiotic	

Table 4: Continue...

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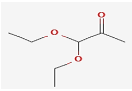
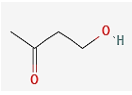
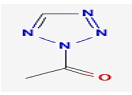
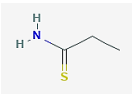
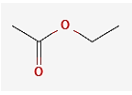
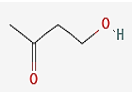
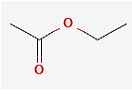
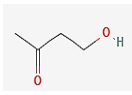
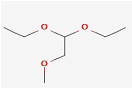
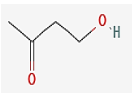
	2-Propanone, 1,1-diethoxy	$C_7H_{14}O_3$	146	3.583	0.18	Antibacterial	
AD-A2	2-Butanone, 4-hydroxy-	$C_4H_8O_2$	88	2.024	13.11	Antibacterial compound	
	1-Tetrazol-2 -ylethanone	$C_3H_4N_4O$	112	2.14	86.4	Anti fungal activity - secondary photoreactions	
	Propanethioamide	C_3H_7NS	89	2.197	0.49	Bioremediation, Antibacterial, Antimycotics, Antivirals	
AAT-C2	Acetic acid, ethyl ester; Acetidin	$C_4H_8O_2$	88	2.145	100	Volatile acidity - Fungal inhibitors	
	2-Butanone, 4-hydroxy-	$C_4H_8O_2$	88	2.024	13.11	Antibacterial compound	
AA-C3	Acetic acid, ethyl ester; Acetidin	$C_4H_8O_2$	88	2.128	100	Volatile Acidity-fungal inhibitors	
AD-11	2-Butanone, 4-hydroxy-	$C_4H_8O_2$	88	2.05	100	Fungal inhibitors	
T-113	Ethane, 1,1-diethoxy	$C_6H_{14}O_2$	118	2.016	100	Antifungal, antibacterial activity	
	2-Butanone, 4-hydroxy-	$C_4H_8O_2$	88	2.024	13.11	Antibacterial compound Fungal inhibitors	

Identification of organic Allelochemical compounds formed by PSF by GC-MS

It was observed that, ethyl acetate extract of PSF culture filtrate that analysis by GC-MS, resulted different compounds, in this study, Eleven major compounds were detected in the crude extract of PSF culture filtrate of *Aspergillus sp.* isolates and *Trichoderma hamatum* (T-113) as PSF (Table 4).

Many compounds are formed by submerged fermentation of *Aspergillus* isolates, were identified in as following: 1,3-Dioxan-5-ol (96.47%); Octanoic acid, 4-chlorobenzyl ester (0.14%); 1,3,5-Cycloheptatriene (0.58%); Crotonic acid (2.64%); 2-Propanone, 1,1-diethoxy (0.18%); 2-Butanone, 4-hydroxy (13.11%); 1-Tetrazol-2-ylethanone (86.4%); Propanethioamide (0.49%); Acetic acid, ethyl ester; Acetidin (100%); Methylolacetone (100%) and *Trichoderma hamatum* (T-113) were identified: Ethane, 1,1-diethoxy (100%) and 2-Butanone, 4-hydroxy-(100%). Most of these compounds have not been reported earlier, this screening of LF of PSF is recorded for the first time in Iraq.

Used organic solvents are reported to be more efficacious in extraction of antimicrobial compounds (Parekh *et al*, 2005). The organic solvent ethyl acetate was thought to enhance the solubility of the compound. In addition to, that ethyl acetate extracts have higher concentration of active components and thus have greater potency against the pathogen and soluble phosphorous (Bibi *et al.*, 2011).

CONCLUSION

The secondary compounds of PSF play an important role in Phosphate Solubilizing by decrease pH of rhizosphere and inhibited the growth of pathogenic microorganisms. We report the isolation of PSF from *A. niger* have been governing organic compounds producing which carried out to identify by GC-MS analysis for the first time in Iraq. More trails need to be conducted to determine which of compounds will be affected by *A. niger* isolation. Details about the mode of action of organic compounds will also be required.

Conflict of interest: None.

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