Egypt. J. Bot. **Vol. 61**, No. 1, pp. 167-176 (2021)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Quantitative and Qualitative Analysis of Organic Acids Produced by Phosphate Solubilizing Fungi

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THE aim of this study was to identify and quantity the organic acids produced by phosphate solubilizing fungi using high performance liquid chromatography (HPLC) apparatus. Eight phosphate solubilizing fungi (PSF) were selected from 23 fungal isolates according to their potential to solubilize tricalcium phosphate in broth Pikovskaya·s medium.These fungal isolates were collected from culture bank in Botany and Microbilolgy department, Beni-Suef University.

PSF were identified as *Aspergillus*, *penicillium* and *Trichoderma*. The PSF isolates decreased the initial pH of the media when they were incubated for seven days. The pH of the media decreased from 6.3 to 2.4 in case of *Aspergillus japonicus* 2. The decreased pH clearly indicated the production of organic acids. The highest total concentration of organic acids was secreted by *Aspergillus japonicus* 2 (2642.1mg/L). By using high performance liquid chromatography (HPLC), seven different organic acids were detected (salicylic, ascorbic, citric, formic, lactic, oxalic, and malic acids). Salicylic acid was the highest quantitatively produced organic acid by all PSF; its concentration was 1823.8 mg/ l by *Aspergillus japonicus* 2. There was a significant positive correlation between the titrable acidity and phosphate solublization ($r = 0.34$, $P \leq 0.05$) and a negative significant correlation between pH and phosphate solublization ($r = - 0.27$, $P \leq 0.05$).The immobilized phosphorus was negatively correlated with titrable acidity ($r = - 0.24$, $P \leq 0.05$).

Keywords: Acid production, HPLC, Phosphate solubilizing fungi, Phosphate immobilization.

Introduction

Phosphorus deficiency in soil is a world problem which affects crop productivity. Most of the agricultural soils contain high amount of phosphorus because of the application of synthetic fertilizers. The inorganic forms of phosphorus in the soil are not utilized by the plants. Phosphate solubilizing fungi convert the insoluble forms of phosphorus into soluble forms which are readily taken up by the plants. A large number of fungal and bacterial isolates characterized for their activity to solubilize insoluble phosphorus to soluble form. The phosphorus content and plant growth are increased when the phosphate solubilizing microorganisms (PSMs) are introduced as biofertilizer (Wang et al., 2007) and this leads to the reduction of chemical fertilizers that have a negative impact on the environment. Fungi constitute the most components of soil microbes depending on soil depth and nutrient conditions (Koorem et al., 2014). Fungi are better than bacteria in solubilizing insoluble phosphorus (Sharma et al., 2013).

Fungi are superior to all other microorganisms because their hyphae can reach far distances in soil than bacteria. Their large biomass also favors high metabolic activity (Kucey, 1983). Furthermore, bacteria lose their solubilizing activities by repeating sub- culturing (Khan et al., 2009). *Aspergillus niger* and *Penicillium* sp. are

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Received 11/7/ 2020; Accepted 11/10/ 2020
DOI: 10.21608/ejbo.2020.35150.1518
Edited by: Prof. Dr.: Khaled M. Ghanem, Faculty of Science, Alexandria University, Alexandira, Egypt.

soil fungi found to solubilize insoluble phosphorus (Yasser et al., 2014). Several mechanisms explained the phosphate solubilization by microbes (Kalayu, 2019). Rashid et al. (2004) compared the phosphate solubization by fungal isolates and bacteria strains. The results showed that fungi immobilized phosphorus better than bacteria. One of these mechanisms is the production of low molecular organic acids such as oxalic, gluconic and ketogluconic acids, which decrease pH and solubilize the insoluble phosphate. The carboxyl and hydroxyl groups of organic acids can chelate the cations attached with phosphate salt and convert the phosphate into soluble form (Khan et al., 2009). The siderophores production, phenolic compounds secretion and humic substances are considered as another mechanism by which microorganisms solubilize inorganic phosphate (Vassilev et al., 2006; Patel et al., 2008). There is a belief that the secretion of organic acid in soil refers to the existence of PSF in the soil (Fenice et al., 2000; Vassilev et al., 2001). The difference of type and amount of organic acid produced by fungi depends on fungal species (Kannahi & Maragini, 2013). Strobel (2001) concluded that the concentration of organic acids produced by microorganisms found in the rhizosphere is low and it differs from 1 to 50mM. It is important to increase our knowledge on this study because the determination of organic acids by the PSF has not been studied in details.

Li et al. (2016) identified the organic acid produced by *Aspergillus niger* and *Penicillium oxalicum* and estimated their total concentrations as 10,000 and 4000ppm at pH values of 2.4 and 3.6, respectively after five days culture. Oxalic acid dominates acidity in the medium due to its high concentration. Athakorn et al. (2014) showed that *Trichoderma* species produced citric acid in higher concentration than lactic acid. Rathi & Packialakshmi (2017) isolated *Aspergillus* sp. and *Penicillium* sp. from rhizosphere and showed that tricalcium phosphate was solubilized by production of organic acids like citric acid, gluconic acid and malic acid. In Egypt, studies on phosphate solubilizing fungi are limited and the recent researches aim to study the organic acid production by phosphate solubilizing fungi which is considered as a renewable source for organic acids. Therefore, this study aimed to quantify the organic acids produced by these phosphate solubilizing fungi using high performance liquid chromatography (HPLC) apparatus.

Materials and Methods

Fungal isolates

Aspergilli and *Penicillia* were isolated from different habitats (reclaimed land, canal bank, island, cultivated soil, orchard and saline soil) in Beni-Suef governorate, Egypt by serial dilution method on Pikovskaya agar plates.

The Pikovskaya agar medium contained g/L: Glucose, 10g; $Ca_3(PO_4)_2$, 5g; $(NH_4)_2SO_4$, 0.5g; NaCl, 0.2g; $MgSO_4.7H_2O$, 0.1g; KCl, 0.2g; yeast extract, 0.5g; $MnSO_4.7H_2O$, 0.002g; $FeSO_4.7H_2O$, 0.002 and agar 15g.

The phosphate solubilizing ability can be detected by formation of transparent zone around the fungal colony after 5 days of incubation at 28°C (Yasser et al., 2014). The purified isolates were maintained on potato dextrose agar (PDA) slants at 5°C. The identification of the isolates was carried by their colony shapes, spores morphology and microscopic observations according to Moubasher (1993). Isolates of fungal species were obtained from culture bank of Botany and Microbiology Department, Beni-Suef University.

Measurement of pH and titrable acidity

The pH of 23 filtrates of fungal isolates from rhizosphere soil was measured by pH meter then the filtrates were centrifuged at 1000rpm for 10min. Few drops of Phenolphthalein indicator were added to each five milliliter of supernatant. 0.01N NaOH was used to titrate the filtrates of each fungal culture. The titrable acidity was expressed by volume of 0.01N NaOH consumed for each 5mL of culture filtrate (Reena et al., 2013)

Quantitative estimation of phosphate solubilization.

Discs (5mm diameter) of 23 fungal isolates from four days old PDA cultures of *Aspergillus*, *Penicillium* and *Trichoderma* were inoculated to 100mL Pikovskya broth medium. Tricalcium phosphate (TCP) (0.5%) conical flasks was added to the medium**.** Each inoculation was replicated three times. The incubation temperature of cultures was at 28°C ± 2°C, for 7 days at shaking conditions (121rpm). The control treatment was done by un-inoculated flask with fungal culture but supplied with TCP. The fungal cultures were filtered by using Whatman No. 4 filter paper after seven days

of incubation. The pH of the media, titrable acidity and organic acid analysis were measured on 7th day in the culture filtrate. P was determined by Inductively Coupled Plasma Spectrometry (ICP) (Ultima 2 JY Plasma) according to the procedure of Environmental Protection Agency (Jackson, 1967).

Immobilized phosphorus of fungal biomass

Twenty three isolates of phosphate solubilizing fungi were grown in PVK broth medium for seven days. After incubation, the culture filtrates were centrifuged at 2000rpm for 20min. Fungal pellets were dried in oven at 60°C for two days. Each fungal biomass was taken in Pyrex Kjeldahl's flask and 10mL mixed acid (Perchloric and nitric acid) were added to it. The flasks were heated at temperatures of 100-300°C till the solution became colorless. The cooled solution was transferred into 50mL volumetric flask. The volume was completed to 50mL by distilled water. Immobilized phosphorus and solubilized P were determined by Inductively Coupled Plasma Spectrometry (ICP) (Ultima 2 JY Plasma) according to the procedure of Environmental Protection Agency (Jackson, 1967).

Qualitative and quantitative analysis of organic acids produced by P-solubilizing fungi

The determination of organic acids was detected by inoculating the fungal culture in conical flask containing 100mL of Pikovskaya broth medium. The flasks were incubated at 30°C for seven days in a shaking incubator at 121rpm. After incubation, each fungal isolate was filtrated several times through filter papers Whatman No 4. The filtrates were sterilized by passing through syringfilter (0.45µm). HPLC (Agilent 1200) was used to determine the organic acids by using method of Van Hees et al. (1999). The column temperature of HPLC (a model Agilent 1100 DAD) was 30°C. Ammonium and methanol with the ratio of 99:1 were the mobile phase of HPLC. The pH was adjusted to 2.6 at a flow rate of 0.5mL/min by application of phosphoric acid. The UV detector set at 214nm was used to detect the organic acids. The absorbance recorded in the chromatograms relative to external standards was achieved during organic acids quantization. The typical retention times for molecules identified previously are as follows: oxalic (2.1min), citric (2.5min), malic (3.3min), lactic (2.6min), ascorbic (2.7min), formic (3.6min) and salicylic (4.5min). The acids were quantified with calibration curves constructed from the analytical standards.

Statistical analysis

Analysis of variance (ANOVA) at $P \leq 0.05$ was used to analyze the results. The correlation values were determined by simple correlation coefficient regression equation at $P \leq 0.05$ and $P \leq 0.01$ with SPSS®/Computer software package Ver.20. The results are the mean of three replicates.

Results

Effect of PSF on the medium pH

The current study revealed that the media inoculated with phosphate solubilizing fungi have reduction in pH. The results in Figs. 1, 2 and 3 showed that the pH of culture media of the 23 fungal isolates, decreased gradually during incubation period. The pH values of the liquid media inoculated with *Aspergillus japonicus* 2 decreased from 6.3 to 2.4. The inoculated medium with *Penicillium funiculosum* showed decreasing in the pH value to 5. The slight decrease in the pH values can be observed among the *Trichoderma* species. The pH of the medium inoculated with *Trichoderma harzianum* 4029 reached 6.

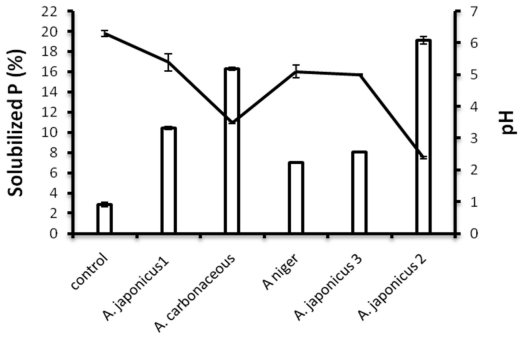


Fig. 1. The pH and the solubilization activity by different *Aspergillus* spp. [The control treatment was uninoculated medium with fungal culture]

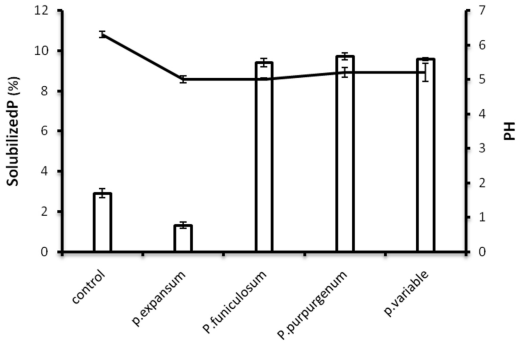


Fig. 2. The pH and the solubilization activity by different *Penicillium* spp. [The control treatment was uninoculated medium with fungal culture]

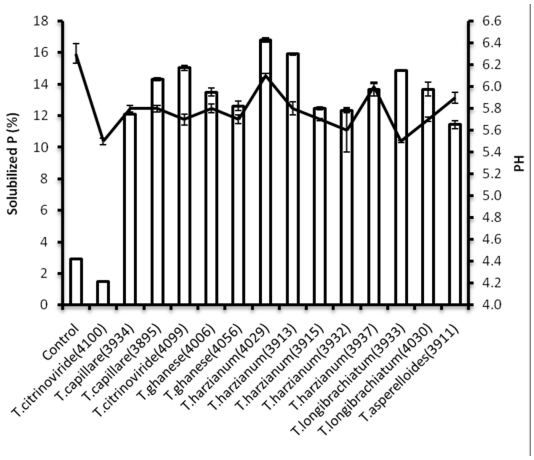


Fig. 3. The pH and the solubilization activity by different *Trichoderma* spp. [The control treatment was uninoculated medium with fungal culture]

Effect of PSF on solubilization of tricalcium phosphate

The results in Figs. 1, 2 and 3 showed that the solubilization percentage of tricalcium phosphate by PSF ranged from 1.3 % to 19.1% which clearly demarcates the variation in the efficiency of P solubilization among the isolates. *Aspergillus japonicus* showed a maximum solubilization of tricalcium phosphate (19.1%) followed by *Trichoderma harzianum* 4029 (16.8%). On the other hand, *Penicillium expansum* showed low P solubilization activity (1.3%) in liquid culture conditions. Most of the *Trichoderma* species showed P solubilization percentage in broth culture conditions ranging from 11.4 to 16.8% for *Trichoderma asperelloides* and *Trichoderma harzianum*, respectively.

Immobilized phosphorus and the dry weight of the fungal isolates.

The measured immobilized P in the fungal mass (Table 1) showed that *T. citrinoviride* (4100) recorded the highest percentage of immobilized P (0.55%) while *A. carbonaceous, P. expansum* and *T. harzianium* (4029) showed immobilized P 0.47, 0.37 and 0.47%, respectively. Regarding the dry weight of *Aspergillus* spp., *A. japonicus*1 recorded the highest dry weight value (0.87g/100mL of the medium) while *A.carbonaceous* showed the lowest dry weight (0.73g/100mL). The dry weight of *P. variable* was 0.92g/100mL. *P. purpurgenum* recorded the lowest dry weight (0.64g/100mL) among the *Penicillium* species. *T. capillare* (3934) recorded the highest dry weight (0.87g/100mL) while *T. harzianum* (3915) and (3932) showed the lowest dry weight (0.70g/100mL).

Correlation between the tested parameters of selected phosphate solubilizing fungi.

Table 2 showed a significant negative correlation (r = - 0.27, P≤ 0.05) between pH and solubilized phosphorus of the 23 isolates of PSF. Highly significant negative correlation (r = - 0.89, P≤ 0.01) was observed between pH and titrable acidity. Moreover, there was a significant positive correlation between the solubilized phosphorus and titrable acidity (r = 0.34, P≤ 0.05). The dry weight of the isolates was significantly negatively correlated with the pH values (r = - 0.24, P≤ 0.05). There was not a significant correlation between phosphorus immobilization or phosphorus solubilization and dry weights of the isolates. The titrable acidity and phosphorus immobilization showed significant negative correlation (r = -0.24, P≤ 0.05).

Detection of produced organic acids by PSF using HPLC

Seven organic acids namely ascorbic, citric, formic, oxalic, malic, lactic and salicylic acids were detected in the culture filtrates of three isolates only (*A. carbonaceous, A. japonicus* 2 and *T. harzianum* (4029). The other five isolates don't have lactic acid as shown in Table 3 and Fig. 4. It was found that salicylic acid was the most quantitatively organic acid produced by PSF. The highest concentrations of salicylic acid (1823.8ppm) and total organic acids (2642.1ppm) were recorded by *Aspergillus japonicus* 2. Generally, lactic acid was not produced by *Aspergillus niger, P. expansum, P. purpurogenum, T. citrinoviride* (4100) and *T. harzianum* (3913) but it was produced in low quantity 1.34ppm by *T. harzianum* (4029).

Discussion

The microorganism which produce organic acids or decrease the surrounding pH can be selected as Phosphate solubilizer. Moreover, organic acids have biocontrol effect by their siderophore activity (Nikolay et al., 2006; Farahat et al., 2016). The acidification is a mechanism by which PSMs can convert the insoluble forms of phosphorus to soluble forms (Sudhakara et al., 2002). The production of organic acid is the main mechanism for the solubilization of inorganic phosphate. The acidification of the microbial cells and their

surrounding is due to the production of organic acid. Li et al. (2016) found that the most efficient phosphate solubilizer that reduces the pH of the medium during growth. This agrees with the results of many researchers (Ryan et al., 2001). The reduction of pH correlates with the titrable acidity (Hwangboo et al., 2003). The observed negative correlation between the pH and soluble phosphorus indicates organic acid production by these PSF. Similar results were reported by Serrano et al. (2013). Alam et al. (2002) concluded that citric and oxalic acids were the most common organic acids produced during phosphate solubilization and our findings differ from these results. *Aspergillus niger* produced citric acid at a concentration of 99.5 ppm in comparison with other isolates which agrees with Arcand & Schneider (2006) who reported that *Aspergillus niger* can produce citric acid. Accordingly, *Aspergillus niger* is a natural source in the industrial production of citric acid and has been reported as the most effective fungus for phosphate solubilization. Lactic acid was not produced by *A. niger*. This disagrees with Padmavathi (2015) who reported that lactic acid was detected during P solubilization by *A. niger*.

The acidification of growth media by organic acid production can be explained due to utilization of glucose as carbon source during the growth, similar results were extrapolated from previous studies (Rodriguez et al., 2004; Trivedi & Sa, 2008). The secreted organic acids by fungi dissolve the mineral phosphate as a result of anion exchange of $PO4^{-3}$ by the acid anion (Gyaneswar et al., 2002).

TABLE 1. Alternation of titrable acidity and immobilized phosphorus in biomass of phosphate solubilizing fungi at 28°C for 7 days

Fungal isolates	Dry weight (g/100mL)	Immobilized phosphorus (%)	Titrable acidity M mole/L
Control	-	-	1.20 ± 0.1
*A. japonicus*1	0.87 ± 0.01	0.17 ± 0.01	4.60 ± 0.66
A. carbonaceous	0.73 ± 0.03	0.47± 0.03	21.60 ± 1.17
A. niger	0.86 ± 0.02	0.17 ± 0.01	9.50 ± 0.76
A. japonicus 3	0.80 ± 0.00	0.18 ± 0.00	18.50 ± 0.0
A.japonicus 2	0.86 ± 0. 03	0.15 ± 0.00	29.30 ± 0.66
P. expansum	0.75 ± 0 .00	0.37 ± 0.02	2.60 ± 0.057
P. funiculosum	0.73 ± 0.00	0.28 ± 0.03	13.16 ± 0.60
P. purpurgenum	0.64 ± .0.00	0.35 ± 0.01	15.80 ± 0.16
P. variable	0.92 ± 0.01	0.23 ± 0.02	14.16 ± 0.4
T. citrinoviride (4100)	0.75 ± 0.06	0.55 ± 0.06	5.16 ± 0.16
T. capillare (3934)	0.87 ± 0.13	0.39 ± 0.0	3.00 ± 0.00
T. capillare (3895)	0.83 ± 0.15	0.39 ± 0.02	3.20 ± 0.2
T. citrionviride (4099)	0.70 ± 0.01	0.46 ± 0.03	2.80 ± 0.16
T. ghanese (4006)	0.77± 0.03	0.45 ± 0.04	3.50 ± 0. 28
T. ghanese (4056)	0.81 ± 0.02	0.38 ± 0.02	2.60 ± 0.16
T. harzianum (4029)	0.72 ± 0.01	0.47 ± 0.00	4.0 0 ± 0. 57
T. harzianum (3913)	0.80 ± 0.12	0.16 ± 0.02	3 .00 ± 0.00
T. harzianum (3915)	0.70 ± 0.00	0.35 ± 0.03	2.43 ± 0.06
T. harzianum (3932)	0.70 ± 0.01	0.42 ± 0.05	2.90 ± 0.06
T. harzianum (3937)	0.71 ± 0.01	0.44 ± 0.04	3.00 ± 0.00
T. longibrachiatum (3933)	0.80 ± 0.00	0.47 ± 0.05	3.40 ± 0.20
T. longibrachiatum (4030)	0.79 ± 0.00	0.45 ± 0.03	4.30 ± 0.10
T. asperelloides (3911)	0.85 ± 0.01	0.18 ± 0.01	3.20 ± 0.12

The data are means of three replicates (± standard error).

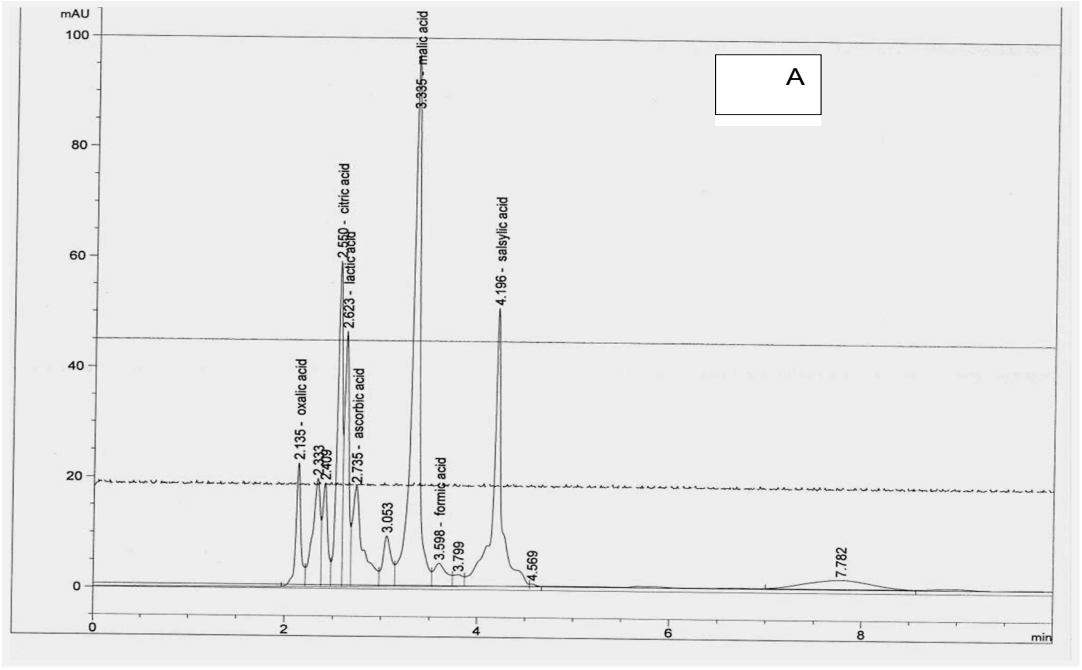
TABLE 2. Correlation coefficient among different parameters of selected phosphate solubilizing fungi

	pH	Phosphorus solubilization (%)	Titrable acidity M mole/L	Dry weight (g)	Phosphorus immobilization (%)
pH			-0.89**	-0.24*	0.11
Phosphorus solubilization (%)	-0.27*			0.12	0.14
Titrable acidity (M mole/L)		0.34*		0.21	-0.24*
Dry weight(g)			0.2		
Phosphorus immobilization (%)				0.07	

*: Significant, **: Highly significant

TABLE 3. Organic acid concentration (mg/L) by eight PSF isolates as detected by HPLC

Fungal isolates	Total organic acid (ppm)	Ascorbic acid	Citric acid	Formic acid	Lactic acid	Oxalic acid	Salicylic acid	Malic acid
A. carbonaceous	1672.5	11.28	58.97	134.28	6.03	31.22	1170.45	260.50
A. japonicus 2	2642.1	27.99	44.34	141.34	12.21	90.83	1823.86	502.33
A. niger	1153.4	9.26	99.49	77.74	-	15.5	909.09	42.95
P. expansum	771.0	9.46	99.28	91.87	_	27.38	465.91	77.50
P. purpurogenum	886.6	9.87	90.37	95.41	_	23.2	607.95	59.76
T. citrinoviride (4100)	1813	1.41	9.55	10.60	_	26.7	147.73	9.34
T. harzianum (4029)	276.9	1.61	3.82	0.00	1.34	97.9	164.77	7.47
T. harzianum (3913)	1136.3	9.87	86.97	77.74	_	17.9	886.36	49.49



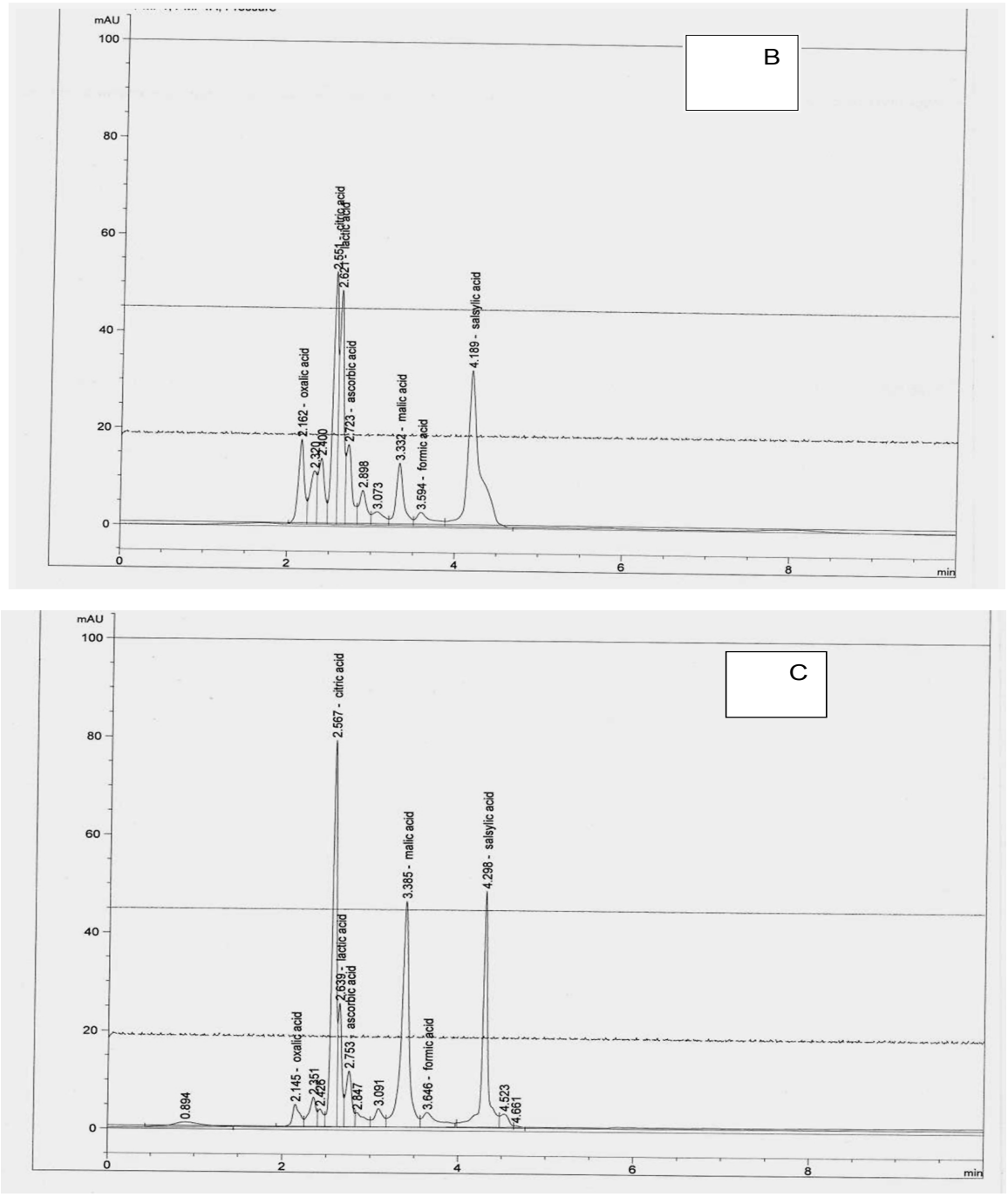


Fig. 4. Chromatogram of organic acids detected in the culture filtrate of (A) ***Aspergillus japonicus*** **2, (B)** ***Penicillium purpurgenum*** **and (C)** ***Trichoderma citrinoviride*** **4100 after 7 days of incubation in PVK broth [The observed peaks detected in culture medium were of oxalic, citric, malic, formic, lactic, ascorbic and salicylic acids]**

Salicylic acid (SA), was the most quantitatively produced organic acid for all PSF isolates which has an important role in defense mechanisms against biotic and a biotic stress (Szalai et al., 2000). Tari et al. (2002) proved that application of SA enhanced the drought and salt stress resistance of plants. Arfan et al. (2007) reported that application of SA can remove the adverse effects of abiotic stress due to its important role in nutrient uptake. It was concluded from our results that all selected fungal isolates produce organic acids by utilizing the carbon source from broth medium.

Conclusion

Aspergillus japonicus, *Penicillium purpurogenum* and *Trichoderma harzianum* were the most fungal species that solubilized insoluble phosphate during phosphate solubilization. Seven different organic acids were detected (salicylic, ascorbic, citric, formic, lactic, oxalic and malic acid) during *in vitro* phosphate solubilization. The concentration of salicylic acid was higher than other detected organic acids.

Conflict of interests: The authors declare no competing financial interests.

Authors' contributions: All authors participated with different forms to complete the research. All authors discussed the results and commented on the manuscript. Pro/ Yasser, MM conceived and planned the experiment. Dr/ Marzouk, MA and Dr/ Mousa, AM supervised on the experimental work and helped in the data interpretation. Mrs/ Nasr, SH performed the experiments, analyzed the data and wrote the manuscript. The tabulation and graphics of the data were carried out by Mrs/ Nasr, SH.

Ethical approval: Not applicable.

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تحليل كمي وكيفي للأحماض العضوية المنتجة بواسطة الفطريات المذيبه للفوسفات

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تهدف هذه الدراسة إلى تعريف الأحماض العضوية المنتجة بواسطة الفطريات وتقديرها كمياً وكيفياً باستخدام جهاز تحليل كروماتوجرافي عالي الكفاءة (HPLC) .

وقد تم انتقاء (8) فطريات مذيبة للفوسفات طبقا للقدرة على إذابة ثلاثي كالسيوم الفوسفات في مرق بيئة (Pikovskaya) من (23) عزلة من الفطريات، و تم تعريف الفطريات المذيبة للفوسفات وهي تنتمي إلى جنس اسبرجلس، بنسيليوم، تريكودرما.

وقد اتضح من الدراسة أن عزلات الفطريات أظهرت انخفاض في الأس الهيديوروجيني الابتدائي من (6.3) إلى (2.4) عندما تم تحضينها لمدة (7) أيام. وانخفاض الأس الهيدروجيني له دلالة على انتاج الأحماض العضوية، وقد اتضح أن هناك تركيز عالي للأحماض العضوية ليصل إلى (2642.5) مليجرام / ملي، وتم انتاجه بواسطة فطر اسبريجلس جابونيكس2، وباستخدام جهاز HPLC تم تقدير كيفي للأحماض العضوية وتم فصل (7) أحماض عضوية وهي (حمض الساليسيليك، حمض الاسكوربك، حمض الستريك، حمض اللاكتيك، حمض الماليك، حمض الأوكزاليك، حمض الفورميك) وكان تركيز حمض السلسيليك من أعلى التركيزات بين الأحماض العضوية ليصل تركيزه (1832.8) ملي جرام / ملي، والذى تم انتاجه بواسطة فطر اسبريجلس جابونيكس2، وقد أوضحت الدراسة علاقة إيجابية ذات دلالة إحصائية بين إذابة الفوسفات ومعايره الحامضيه (titrabile acidity) وعلاقة عكسية ذات دلالة إحصائية بين الأس الهيدروجيني وعملية الإذابة كما أن هناك علاقة عكسية بين الاذابه والفسفور المثبت داخل الفطر (immobilized phosphorus).