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Penicillium oxalicum Isolated from Zea mays Rhizosphere as a promising Plant Growth Promotor via Multiple Mechanisms.

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ABSTRACT

The use of synthetic fertilizers in agricultural production has harmful impacts on the ecosystem . Therefore, there is an emergent demand for using natural and sustainable practices to save the environment. Some of the soil microflora are plant-growth-promoting fungi (PGPF) which promote plant growth and are considered potential biocontrol agents against phytopathogens. In the present investigation, more than100 fungal species were isolated from the rhizosphere and rhizoplaneof Zea mays and Vicia faba from AlRaswa Ferry of Port Said governorate and characterized for their potential as an Arabidopsis thaliana plant growth promotor. Four species of four genera namely Penicillium oxalicum, Fusarium oxysporum, Aspergillus niger, and Paecilomyces lilacinus revealed to have the highest capacity to enhance A. thaliana growth. The most observed effects on A. thaliana are a significant improvement in root length development and number of leaves due to multiple mechanisms, e.g., the fungal phytohormone, indole acetic acid production, volatile components and siderophore production, and phosphate solubilization. Fungal volatiles of Penicillium oxalicum increased the shoot length of seedlings by 11.8%, the root length by 250%, the number of leaves by 60%, and the indole acetic acid production by 110% compared to the controls. Obtained results suggest Penicillium oxalicum as a natural biological plant growth inducer whether individually applied or in mixed form with the other identified fungal species which may act as a practicable substitute for harmful chemicals in the agricultural field.

Key Words:
Penicillium oxalicum , plant growth promotors, siderophore,volatile compound , phosphate solubilization.

1. INTRODUCTION

The rhizosphere and rhizoplane regions of plants are most biological interactions of microorganisms with plants occur. Plant microbiomes can chemically and physically alter the soil surrounding the roots

by changing the pH, nutrient availability, gas exchange, and other factors. It involvesa significant energy flow impacting the surrounding ecosystem. The rhizosphere microbiota improve soil fertility and encourages plant growth, which benefits agroecosystems and the agricultural sector. Included within this microbiota consortium are the filamentous fungi that have undergone extensive research for sustainable agriculture [1, 2]. Soil microorganisms transform organic matter into a variety of biological molecules and active compounds. These altered molecules can be easily assimilated by plants, thus promoting plant growth and also affecting the biodiversity of the soil microbiota. For instance, the rhizosphere mycobiota can promote the development of plants by establishing symbiotic relationships, stimulating growth hormones production, inducing phosphate solubilization, or acting as a biological control against phytopathogens and agricultural pests [3;4]. An example of such associations is the facultative biotrophic dark septate endophytic fungi that have been found to act as plant growth promoters by establishing a symbiotic relationship with host plants, facilitating the absorption of various elements such as nitrogen and phosphorus. Many fungal species, including *Cladosporium cladosporioides* [5], *Phoma* sp [6], and *Trichoderma atroviride* [7] are revealed to promote plant development by stimulating auxin hormone productions.

Fungi are a central factor of the plant microbiome that emit large amounts and different mixtures of volatiles [8, 9]. Agricultural ecosystem benefit from the roles played by microbial volatiles. They promote plant growth and offer systemic protection against phytopathogens. Additionally, they function as a signaling substance in plant-microbe interactions [10]. A variety of volatiles, including alcohols, acids, hydrocarbons, benzenoids, fatty acid derivatives, esters, organic sulfur compounds, etc., are produced by some fungi [11,12]. These volatile compounds are mainly associated with fungal metabolism.

Different fungal genera, such as *Aspergillus, Fusarium, Trichoderma* and *Penicillium* were recorded as plant growth promotors [13]. Generally, fungi could use several mechanisms to promote plant growth like the production of phytohormones [14], volatile compounds [15], siderophores [16], and solubilization of minerals [17]. In addition, fungi can indirectly promote plant growth by preventing the harmful effect of plant pathogens and diseases; for example, *P. oxalicum* protected the Pearl millet from Downy Mildew disease [18].

The goal of the current study was to investigate the potentiality of various fungi isolated from the Port Said governorate to promote plant growth and elucidate their different mechanisms. Findings are of particular importance to agricultural applications as they introduce naturally occurring fungal species able to enhance and stimulate plant.

2. MATERIALS AND METHODS

2.1. Materials:

Plant – Microbes – Culture media, Ms medium, Czapex- Dox – PDA – Pikovskaya medium-cas medium

2.2. Methods

Isolation and identification of fungi from plants and soil

Different fungal species were isolated from the rhizosphere and rhizoplane regions of *Zea mays* and *Vicia faba* from AlRaswa Ferry of Port Said governorate in the winter season. Polythene bags were used tocollect the root and soil samples. The rhizosphere soil was separated from the roots using a brush in a

petri dish. Ten gm of rhizosphere soil was diluted in 90 ml of sterile dH_2O and stirred on a magnetic stirrer for 15 minutes (1:10 dilution) Serial dilutions (i.e., 10^{-3} to 10^{-4}) were prepared, and 1 ml of each dilution was transferred into pre-labeled culture Czapex- Dox medium plates. The experiment was done for at least three biological replicates of each dilution. Roots were washed with 100 ml of sterile dH_2O three times before being transferred to a beaker containing tween 80 by using shaker to overcome surface tension between fungi and surface. Fungal identification was performed using traditional mycological methods that include conventional cultivation in Potato dextrose agar (PDA), malt extract agar and Czapex- Dox media and microscopic identification [19]. Hardly recognized fungal isolates were identified using molecular identification methods through DNA sequencing of the internal transcribed spacer (ITS) and large subunit regions of rRNA, followed by comparative sequence analysis.

Molecular identification and phylogenetic analysis

Fungal growth were centrifuged, harvested, and used for total DNA extraction via the ABT DNA mini extraction kit (Applied Biotech Ltd, Egypt) with respect to the manufacturer's protocol. The extracted DNA was then used to identify the isolated fungi by sequencing the 5.8S rRNA region using universal primers ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATG-3') as mentioned in [20] protocol.

The amplified PCR products were submitted to Solgent Company Ltd (South Korea) for gel purification and amplicon sequencing. Geneious software (Biomatters) was used to trim and assemble the resulting sequences. The amplified region of the 5.8S rRNA was searched for using the basic local alignment search tool (BLAST) of the Gene Bank database National center for biotechnology information (NCBI). Phylogenetic analysis was developed by using Molecular Evolutionary Genetics Analysis version 11 (MEGA11). The Neighbor-Joining approach was employed to reveal the evolutionary history. Mentioned next to the branches is a percentage of the replicate trees where the relevant taxa were grouped in the bootstrap test.

Fungi- plant co-cultivation

The isolated fungi were examined for their volatiles production and their effect on plant growth in a cocultivation framework, as shown in Figure 1. The surface of the *Arabidopsis thaliana* seeds was sterilized using 70% ethanol (1 min), 5% calcium hypochlorite (5 min), and washed several times with sterilized dH_2O [21]. Four seedlings were transplanted into glass jars with solidified murashige and Skoog medium (MS medium) which consisted of macronutrients and micronutrients, vitamins, haromones, sugar and agar, after the seeds were vernalized for three days at 4°C without light in a slanted position for 3 days. Meanwhile, tested fungi were cultured for 9 days, and a point half centimeter disk of the fungal culture developed on potato dextrose broth (PDB) medium (Potatoes, implantation 200 gm and dextrose 20 gm per liter) at 20°C was seeded to a 20 ml broth medium in a beaker. Without touching the plants directly, the little beaker wasput into the jar (or MS agar). The jar (i.e., fungal culture) was positioned at a distance of about 3 cm from the plants. The *Arabidopsis thaliana* seedlings were exposed to the volatile compounds of tested fungi for 7 days at 20°C and 85 $\mu molm^{-2}s^{-1}$ of light at a 16h light/8h dark cycle in an incubato r(model:ST6P,serial number: ST06190116,country: Poland). The aforementioned conditions were performed to prevent spores production. In control experiments, MS agar was used without fungal growth. Three technical replicates were used to calculate the fresh weight, dry weight, number of leaves, and the plant shoot and root lengths [22].

Figure 1. Co-cultivation system to evaluate the effect of fungal volatiles on *Arabidopsis thaliana* growth. The *Arabidopsis thaliana* plant seedlings were grown in an MS medium tilted in a slanted position, and the volatile- producing fungi were grown in a small beaker containing a 20 ml broth medium. Both, the plant and fungi wereplaced into a jar without any physical contact between them. The beaker was placed at a 3 cm distance from the plants. The seedlings were exposed to tested fungi volatiles for 7 days at 20°C and 84 $\mu molm^{-2}s^{-1}$ of light at a 16/8 h light/dark cycle in an incubator. **(A)** represents a lateral view of the experimental setup where the treatment is on the left and the control (without fungal culture) is on the right and **(B)** is a dorsal view of the experimental design.

Quantification of indole acetic acid

Fungal growth were seeded into test tubes containing 10 ml of Potato dextrose broth(PDB) medium mixed with 2 $mg.ml^{-1}$ ofL-tryptophan in triplicate. The cultures were left to grow for two weeks at 25°C on a shaker (model:SK-0330-pro,serial number: SC184AD000415,country:USA). Cells were harvested by centrifuge at 11,000 rpm for 4-5 min using a one ml of the supernatant was added to 2 mL of Salkowski reagent, which contains 1 ml of 0.5 M FeCl3 plus 49 ml of 35% $HClO_4$ and mixed together. Themixture was then incubated in an unlighted place at 28 ± 2 °C for 30 min. Finally, the absorbance (OD)was calculated at a wavelength of 530 nm using a spectrophotometer (model:ST-UV-1901PC,serial number: ST2018022701A,country:USALAP). A standard IAA curve was prepared using different concentrations of IAA (10, 20, 30, 40, and 50 µg ml^{-1}) [23]. Concentration is calculated from this equation: adsorbance÷ slope × (total volume ÷ volume used).

Detection of phosphate solubilization

The ability of the isolated fungi to soluble tricalcium phosphate (TCP) as an insoluble inorganic phosphate source in the Pikovskaya (PVK) medium was investigated. A clear zone surrounding the mycelium is an indication of phosphate-solubilizing capacity. The PVK medium (g/L of water) was composed of 0.5 g $(NH_4)_2SO4$, 0.1g $MgSO_4{\cdot}7H_2O$, 0.02g NaCl, 0.02 g KCl, 5 g $Ca_3(PO_4)_2$, 0.003 g $MnSO_4\,H_2O$, 0.003 g $FeSO_4{\cdot}7H_2O$, 0.5 g yeast extract, 10 g glucose, 15 g agar, and 1 liter of dH_2O and was sterilized by autoclaving at 121°C for 20 min. Inoculation was done using the pour plate method

(sterilized medium was mixed with inoculated isolates in sterilized Petri dishes). The plates (three replicates) were incubated for a week at 25°C. The distance across clear zones surrounding the growth of each fungal isolate was measured at two days intervals of plating (i.e., the 1, 3, 5, and 7 days of plating) [23]. The solubilization index (SI) was recorded on each measurement day and was calculated as follows:

$$SI = \frac{\text{Diameter of solubilization zone} - \text{Diameter of the colony}}{\text{Diameter of the colony}} \times 100$$

Detection of siderophore

The isolated fungi were inoculated in CAS-agar medium plates composed of 4 solutions. The first solution (i.e., solution 1) where a 10 ml of l mmol l^{-1} $FeCl3.6\ H_2O$ was mixed with 50 ml of CAS aqueous solution (1.21 g l^{-1}) to prepare the Fe-CAS indicator. The resulting blue color was mixed gently with hexadecyltrimethylammonium bromide (1.821g l^{-1}), the yield was then autoclaved and cooled down to 40-50°C, solution 2 (32 g piperazine-N, N'-bis (2-ethane sulfonic acid) PIPES was dissolved in 0.75 liters of $ddH_2O.KH_2PO_4$, 0.3 g NaCl, 0.5g, and 1 g of NH_4Cl. The solution pH was adjusted to reach 6.8 with the help of NaOH. Water was then added to increase volume to 0.8 liters; then, the mixture was autoclaved after adding 18 g agar before being cooled down to 50°C, solution 3 (in 70 ml H_2O, added 2g glucose, 2g mannitol, 493mg $MgSO4.2H_2O$, 11 mg $CaCL2$, 1.17 mg Mn $SO4.2H_2O$, 1.14 mg H_3BO_3, 0.04 mg $CuSO4.\ 5H2O$, 1.2 mg $ZnSO4.7\ H2O$ and sodium molepedate 1 mg then autoclaved and cooled to 50°C. Solution 4 contains 10% (w\v) casamino acid. Solution 3 was mixed with solution 2 and 30 ml from solution 4 before the mixture was finally added to solution 1 [24]. After that, plates were poured and mixed with inoculum, then incubated for a week at 28°C in dark conditions (three replicates). The equation used to calculate the siderophore zone is as follows: siderophore zone= orange area - diameter of the colony.

Head space collection of volatile compounds

A culture disk (~0.5 cm) from a one-week-old *P. oxalicum* culture was seeded into 100 ml of PDB medium in sterile screw-capped glass bottles inserted into an incubation chamber of a marginally altered wind current collection system [25]. A sterile humidified tube (Charcoal-purified) was forced through a closed system. The volatile components released inside the headspace were trapped in a column. Throughout the incubation stage, the volatile compounds were collected at intervals of 24 hours for up to 9 days before being eluted with 300µl dichloromethane (Fig. 2).

Figure 2. The design of volatiles collection. Two sterile screw-capped glass bottles, each containing 100 ml of PDB medium, were prepared. (A) represents the treatment in which the 100 ml medium was inoculated with *P. oxalicum*, and (B) refers to the control conditions lacking any fungal inoculation. An air/vacuum pump was connected to each of the sterile bottle caps through a Durham tube. A Charcoal-purified, sterile humidified tube was pushed through this closed system.Volatile compounds discharged inside the headspace were trapped in a column. The volatile compounds were collected in 24 h intervals during the incubation period of up to 9 days. Volatiles were eluted from the catching material with 300µl dichloromethane.

Data analyses

The one-way analysis of variance (ANOVA) in SPSS statistics was used to determine the statisticalsignificance of differences between the means of the different groups (26).

2. RESULTS AND DISCUSSION

Identification of the obtained fungal isolates

Among fungi isolated, P1,F2, A3, and P4 were isolated from AlRaswa Ferry of Port Said Governorate, rhizosphere of *Zea mays*, rhizosphere of *Vicia faba*, rhizosphere of *Zea mays* and rhizosphere and rhizoplane soil of *Zea mays*, respectively. *Fusarium oxysporum*(F2*), Aspergillus niger* (A3*),* and *Paecilomyces lilacinus*(P4) were morphologically different and showed variation in their morphological characteristics like colony color, growth, texture,and margin when grown in Potato Dextrose Agar, malt extract agar, and czapeks medium. The shape and color of conidia and conidiophore also varied in the different isolates when observed under a microscope, which made it easy to be identified using conventional methods. On the contrary, *P. oxalicum* (P1) was identified by using molecular identification as it was difficult to be identified with the traditional methods.

Molecular identification and phylogenetic analysis

The fungal strain was identified via sequencing the 5.8S rRNA region using universal primers ITS1 and ITS4. The amplified PCR products (Fig. 3A) were sequenced and searched for using the BLAST

search tool in the NCBI. The sequence was as follows:

TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAAC
CTCCCACCCGTGTTTATCGTACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGG
GGGGCATCCGCCCCCGGGCCCGCGCCCGCCGAAGACACACAAACGAACTCTTGTCTG
AAGATTGCAGTCTGAGTACTTGACTAAATCAGTTAAAACTTTCAACAACGGATCTCTT
GGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAT
TCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCAT
GCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCTCTCGCCCC
CCGCTTCCGGGGGGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAG
CGTATGGGGCTTCGTCACCCGCTCTGTAGGCCCGGCCGGCGCCCGCCGGCGAACACC
ATCAATCTTAACCAGGTGACCTCGGATC

The BLAST search identified the strain to be from the genus *Penicillium*. Alignment and phylogenetic analysis showed a 100% identity and formed a cluster between the obtained sequence and thesequence of *Penicillium oxalicum* clone EF_475 (GenBank accession #MT529124.1) (Fig. 3B).

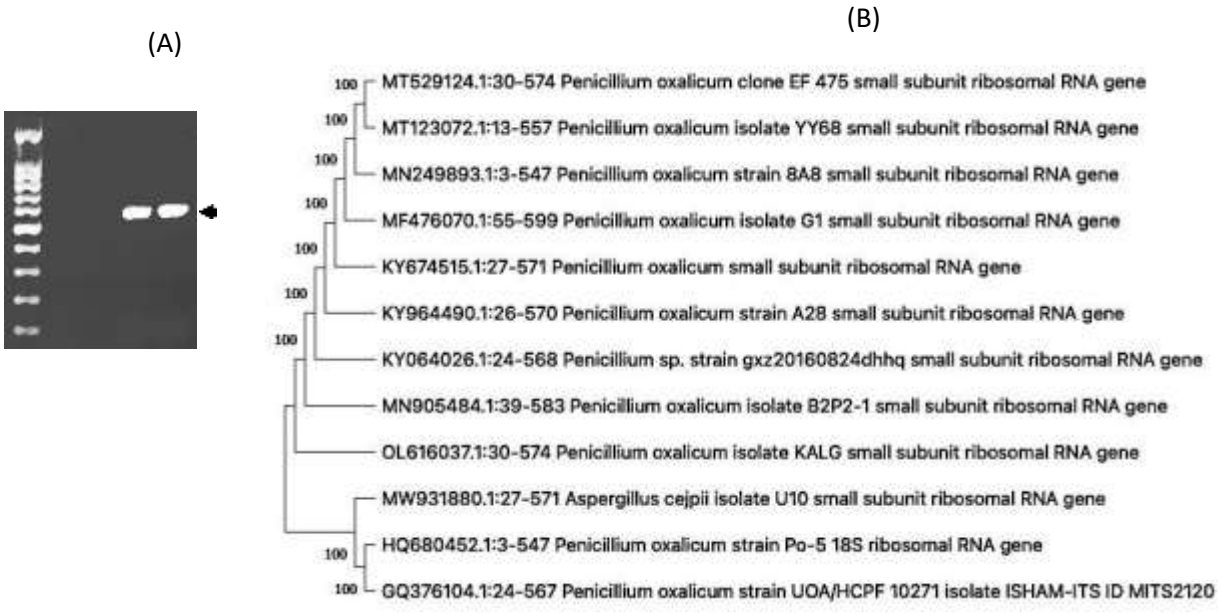


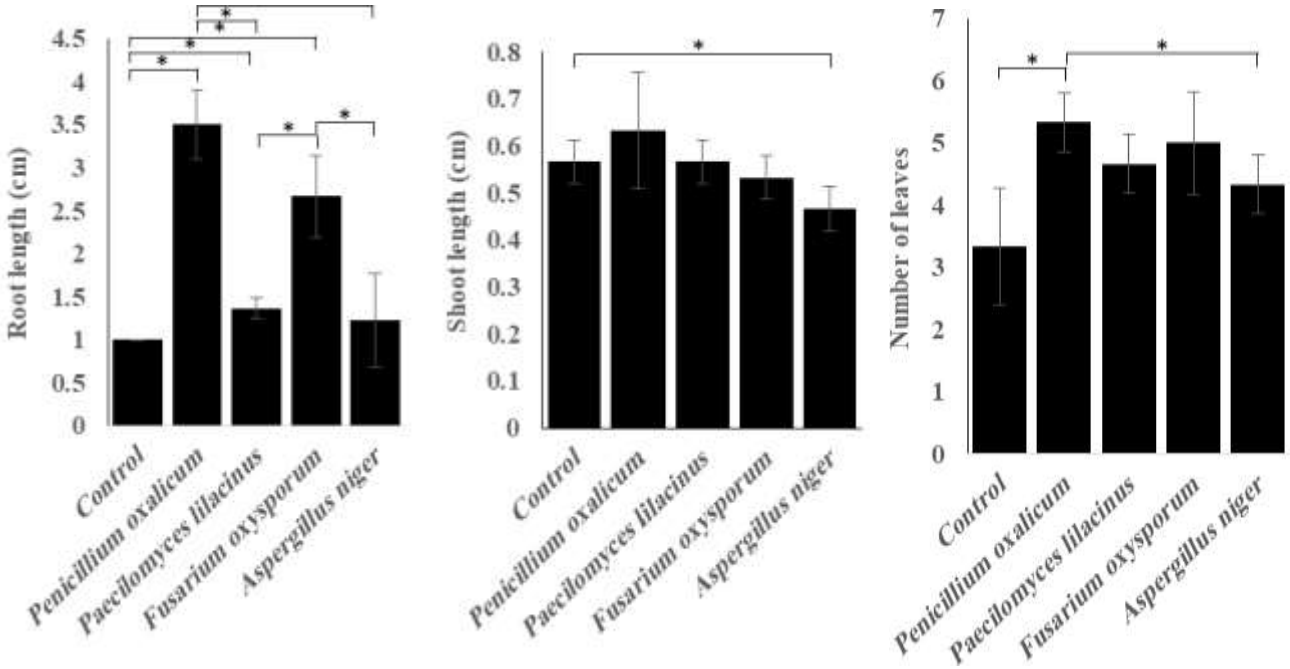
Figure 3. Phylogenetic tree of the molecularly identified fungal species. (A) A gel image shows the amplified band from the unknown fungus using the universal primers ITS1 and ITS4. **(B)** Phylogenetic tree based on the fungal sequence. The tree was developed by using MEGA11 with 1000 times bootstrap replication and a substitution model p-distance (complete deletion of gaps/missing data treatments). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The tree is drawn to scale.

The impact of fungal volatile compounds on the development of *A. thaliana*

After nine days of cocultivation and concurrent exposure of the *A. thaliana* seedlings to emitted volatiles from the different fungi, the growth and development of *A. thaliana* were evaluated. *P.*

oxalicum, F. oxysporum, A. niger, and *paecilomyces lilacinus* were with the most significant effect on the *A. thaliana* growth, where *P. oxalicum* showed the highest effect among all on the length of root and shoot regions,and the number of leaves (Fig. 4A and B). Inoculation with *Penicillium oxalicum* increased the seedlings' shoot length by 11.8%, the root length by 250%, and the number of leaves by 60% compared to the controls. Thereby, *P. oxalicum* volatiles received further investigations. Seedlings exposed to the *P. oxalicum* volatiles showed a vigorous appearance with no shrink or other side effects, unlike *A. niger* which showed some wilt on leaves (Fig. 4B). This result shows that these four fungal strains promoted the *A. thaliana* plant growth compared to control.

(A)



(B)

Figure 4. Impact of fungal volatile compounds on the growth of *A. thaliana.*

The *A. thaliana* seedlings and fungal strains were co-cultivated for 9 days at 20°C and 84 µmol $m^{-2}s^{-1}$ of light at a 16/8 h light/dark cycle. Root length, shoot length, and the number of leaves were measured and bar graphed as shown in **(A),** and the *A. thaliana* plant morphology of seedlings exposed to volatiles versus control is shown in **(B)**. Data were compared using one- way ANOVA for at least three biological test replicates. Star "*" represents significance, and Error bars represent the standard deviation of at least 3 biological replicates.

Analysis of head space volatile compounds of *P. oxalicum*

As the volatile compounds obtained by *P. oxalicum* appeared to cause the maximum significant effects on the *A. thaliana* morphology, analyzed volatile components using gas chromatography (GC) (Table1). Intriguingly, some compounds released by *P. oxalicum* were not previously recorded to have any biological activities. These compounds include Phenol, 2-Methoxy-4-(2-propenyl)- and Ristrimethylsilyl ether derivative of 1,25-dihydroxyvitamin D2.

Table 1. Gas chromatography analysis of the *P. oxalcium* volatile compounds

Compounds	RT (Retention time)	Quantity(%)	Biological activities
Ristrimethylsilyl ether derivative of 1,25-dihydroxyvitamin D2	5.76	.30	Plant growth promotion [27]
9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methy l]ethyl ester, (z,z,z	7.92	.35	Antifungal activity[28] anticancer, anti-inflammatory[29]
3-Oxo-20-methyl-11-à-hydroxyconanine-1,4-diene	10.53	.36	Anticancer and antifungalactivity [30]
Phenol, 2-methoxy-4-(2-propenyl)-	12.27	.79	Plant growth promotion [31],and Flavoring agent, anti-infective agent, and antioxidant.
1,8-Di(4-nitrophenylmethyl)-3,6-diazahomoadamantan-9-one	15.23	.58	Plant growth promotion [32]
Phenol, bis(1,1-dimethylethyl)	15.49	1.24	Antifungal and plant growth promotion (Effects of phenol 2,4-bis(1,1-dimethylethyl) on seedling growth of erticillium dahliae, no date
2-Myristynoyl pantetheine	19.02		Antifungal activity [32]
Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl	21.77	.32	Antioxidant[31] and antifungal activity
Methyl-9,9,10,10-d4-octadecanoate	22.72	.44	Cell membrane stability [33]
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	23.63	.37	Antioxidant activity and antifungal activity [34]
Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester	23.81	.57	Hypocholesterolemic, antiarthritic, nematicide, 5- alpha reductase inhibitor, antiacne and Hepatoprotective

Rhodopin	33.76	.56	Plant growth promotion [27] and Pesticide and heavy metal behavior (Morphology, no date)
9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy] propyl ester, (z, z, z)	25.90	1.96	Plant growth promotion[27]anti-inflammatory, anticancer, antimicrobial, antioxidantand hyperchloesteralemi
2-Hydroxy-3-[(9e)-9-octadecenoyloxy] propyl (9e)-9-octadecenoate	26.29		Plant growth promotion [27]
9-Octadecenoic acid (z)-	26.53	.39	Antifungal [35], and Stroke, Acute neurologic disorder, Respiratory failure, Anemiatreatment.[36]
2-Acetyl-3-(2-cinnamido) eth yl-7-methoxyindole	27.01	.57	Antioxidant [33]
3-(Tetradecanoyloxy)-2- [(Tr Imethylsilyl) Oxy] Propyl Myristate	27.43	.41	No activity recorded till now
4-Hexyl-1-(7-methoxycarbonylheptyl) bicyclo[4.4.0]deca-2,5,7-triene	27.51	.31	Plant growth promotion [27]
Hexadecanoic acid, 2,3-dihydroxypropyl ester	28.75	.73	No activity recorded till now
Hahnfett	29.36	.80	Antifungal activity
O-Tetrakis(trimethylsilyl)3,5-dihydroxy-2-(3-hydroxy-1octenyl)-cyclopenta-neheptanoate	34.55	.76	Plant growth promotion[27]
5,8,11,14-Eicosatetraynoic acid, tbdms derivative	29.47	.81	Plant growth promotion [27]
Prost-13-en-1-oic acid, 9,11,15 tris[(trimethylsilyl) oxy]-, trimethylsilyl ester, (9á,11à,13e,15s, acetic acid.	34.55	3,98	No activity recorded till now
Cholesterol, tms derivative	30.03		No activity recorded till now
Di(Isityl)di[phosphino] silane	31.41	1.01	Antibacterial activity
7-(4-Hydroxy-5-methoxy-1,5-dimet hylhexyl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-t etradecahydrocyclopenta[a]phenanth ryl ester	31.49		No activity recorded till now
Campesterol, tms derivative	34.65	3.9	Antibacterial
5ah-3a,12-Methano-1h-cyclopropa [5',6']cyclodeca[1',2':1,5]cyclopenta[1 ,2-d][1,3]dioxol-13-one, 1a,2,3,9,12,12a-hexahydro-9-hydrox y-10-(hydroxymethyl)-1,1,3,5,7,7-hex amethyl-, [1ar-(1aà,3à,3aà,5aà,8ar*,9á,12à,1 2aà)]	32.46		Antifungal activity
Psi.,. psi. -carotene, 1,2-dihydro-1-hydroxy	33.76	3.31	No activity recorded till now
À-D-GLucopyranoside, methyl 2-(acetylamino)-2-deoxy-3- o-(trimethylsilyl)-, cyclic methylboronate	34.01		Plant growth promotion[27]

Determination of phosphate solubilization by the isolated fungal strains

Phosphorus solubilizing fungi may promote plant growth by solubilization of phosphate to becomeavailable to plants, stimulating nitrogen fixation, synthesizing phytohormones, and enhancing iron availability. The ability of the isolated fungi to solubilize TCP was therefore tested. Obtained results showed that *A. niger*, *P. oxalicum* ,and *paecilomyces lilacinus* had the highest ability among others to solubilize the TCP, as indicated by the presence of clear zones and phosphorus solubilization index (Fig. 5). No control zone was recorded for *F. oxysporum*. This result indicates that *A. niger, P. oxalicum*, and *Paecilomyces lilacinus* may promote plant growth by enhancing phosphorus solubilization.

(A)

(B)

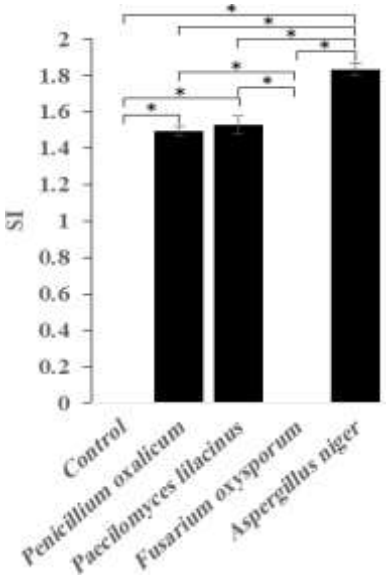
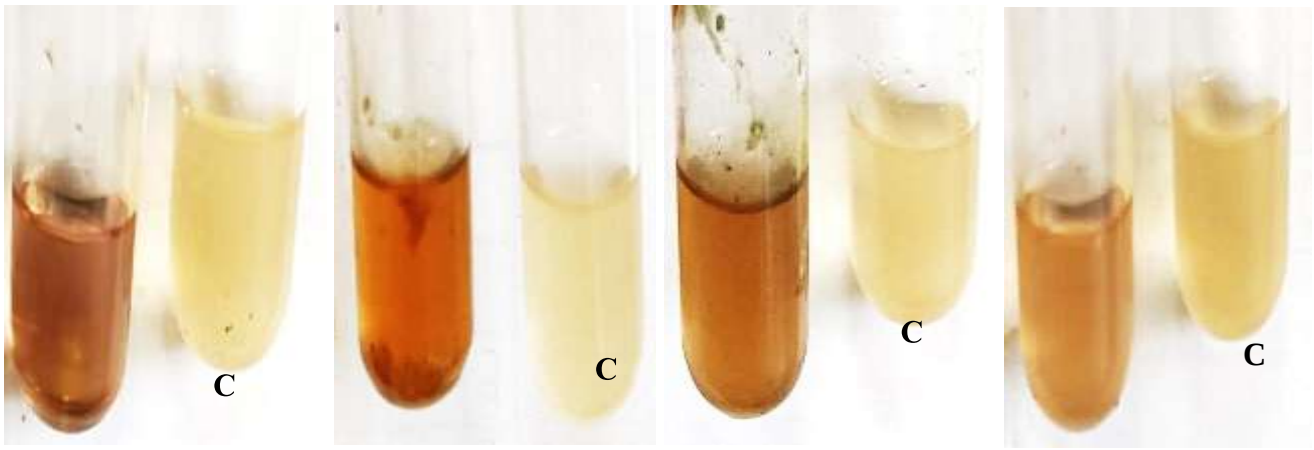


Figure 5. **Phosphate solubilization by tested fungi.** The four fungal strains were examined for their abilities to solubilize tricalcium phosphate (TCP) as insoluble inorganic phosphate sources in PVK medium. The sterilized medium was poured into sterilized Petri plates and mixed with the inoculated fungal strains. The plates were then incubated for 7 days at 25°C (Three replicates) .A clear halo zone around the mycelium is an indication of phosphate-solubilizing capacity. The distance across clear zones around the colony of each strain was measured after the 1st, 3rd, 5th, and 7th day of plating [23] . **(A)** Results showed clear zones produced by *A. niger, P. oxalicum, and paecilomyces lilacinus.* No control zones were recorded with*F. oxysporum*. **(B)** A bar graph showing the phosphate solubilization index indicating the concentration produced by *A. niger*, *P. oxalicum*, *Paecilomyces lilacinus*, and *F. oxysporum* compared to control. Data were compared using one-way ANOVA for at least three biological test replicates.Star "*" represents significance, and Error bars represent the standard deviation of at least 3 biological replicates.

Determination of IAA produced by the fungal strains

The IAA auxin stimulates the overproduction of root hairs and regulates plant growth, cell division and elongation, and responses to abiotic stresses such as light and biotic stress like the phytopathogens. IAA also stimulates the release of saccharides which attracts the plant-associated beneficial microbes .All fungal strains were tested for their IAA production. The presence of IAA was detected by measuring pink to red color development due to the Indole-Salkowski reagent reaction(Fig. 6A). Obtained results revealed that *A. niger, P. oxalicum*, and *F. oxysporum* produced the highestconcentrations of IAA among others; 0.9 μg ml^{-1}, 0.73 μg ml^{-1}, and 0.66 μg ml^{-1}, respectively (Fig. 6B). This result indicates that *A. niger, P. oxalicum*, and *F. oxysporum* may promote plant growth through enhancing IAA production.

(A)



A.niger ***F. oxysporum*** ***P. oxalicum*** ***Paecilomyceslilacinus***

(B)

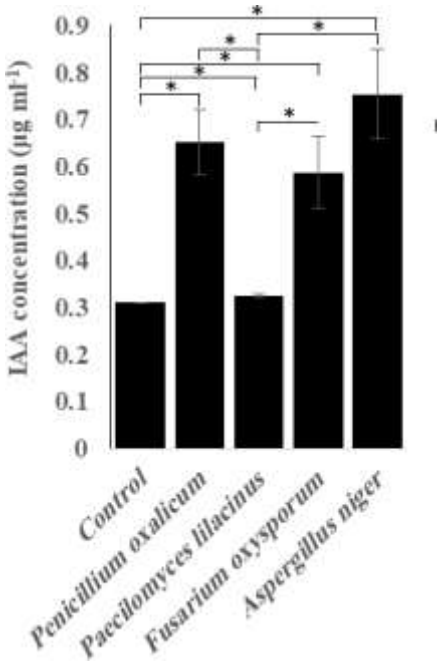
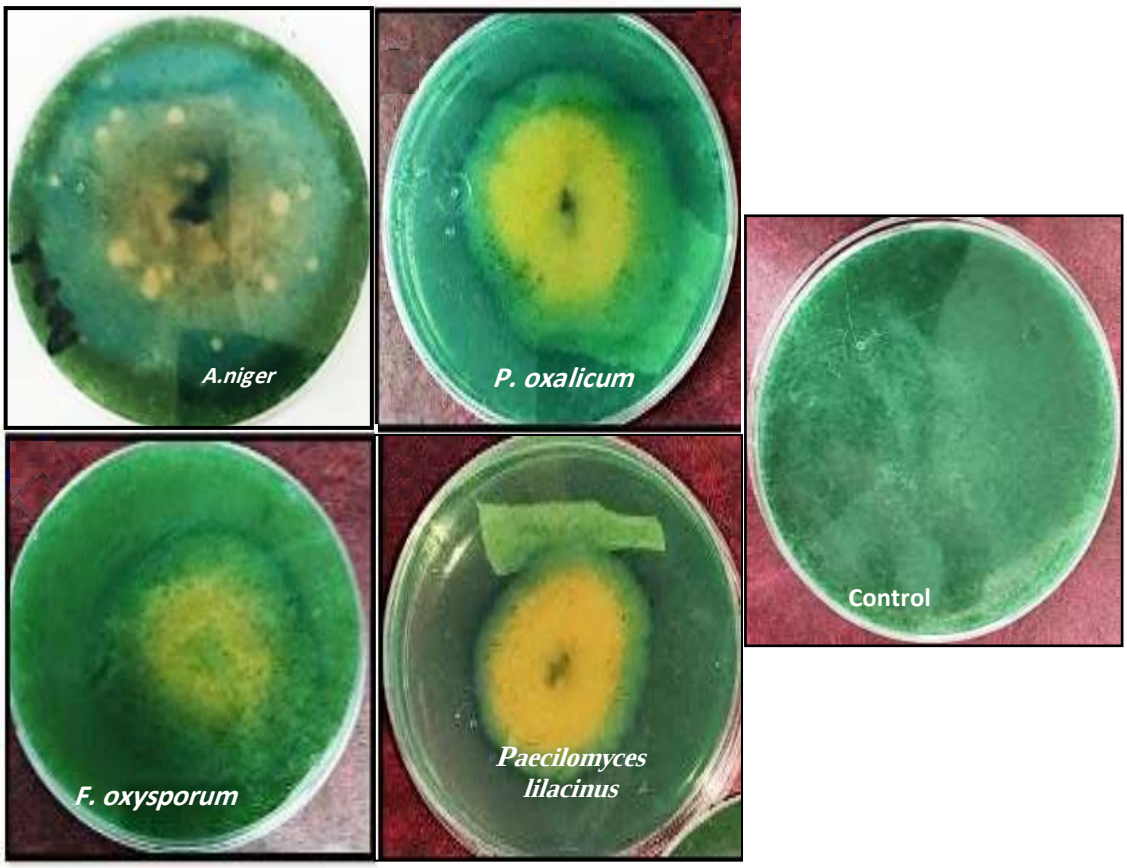


Figure 6. The concentration of IAA produced by the tested fungal strains. (A) Pink to red color formed as a result of IAA production. The presence of IAA was detected by measuring pink to red color development due to the Indole-Salkowski reagent reaction. **(B)** Bar graph representing the IAA concentrations produced by the different fungal strains. Data were compared using one-way ANOVA for at least three biological test replicates. Star "*" represents significance, and Error bars represent the standard deviation of at least 3 biological replicates

Analysis of siderophore production by the fungal strains

Siderophores are organic molecules produced by microbes, and they have a high affinity for ferric iron, making them accessible to plants (37). Color change (orange zone) on CAS agar plates was used to detect siderophore production (Fig. 7A). The fungal strains were tested for siderophore production. Obtained results revealed that the four tested fungi were apple to produce siderophores. *Paecilomyces lilacinus* and *A. niger* showed the highest ability to produce siderophores, but *F. oxysporum* and *P. oxalicum* had lower capabilities (Fig. 7B). This result indicates that the fungal strains may promote plant growth by enhancing siderophores production and iron availability

(**A)**



(B)

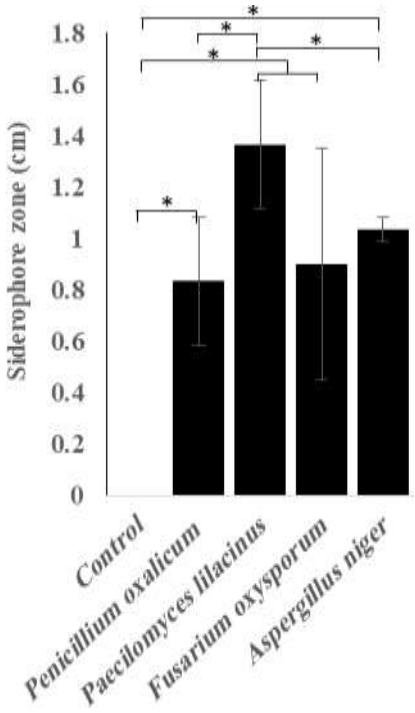


Figure 7. Siderophore production by fungi. (A) Siderophore detection using the CAS agar plates. The orange zone on CAS agar plates was used to detect the siderophore production of each of the tested fungi. The fungal strains were inoculated in the CAS-agar plates and incubated for 7 days at 28°C in dark conditions (three replicates).Bar graph showing the size of the siderophore zone produced by the different fungi. Data were compared using one-way ANOVA for at least three biological test replicates. Star "*" represents significance, and Error bars represent the standard deviation of at least 3 biological replicates.

4-Discussion

Plant growth-promoting fungi **(**PGPF) are eco-friendly alternative to harmful chemical fertilizers.

A. niger, F. oxysporum, P. oxalicum, and Paecilomyces lilacinus are well known as effective PGPF [38.39.40,41].Siderophore production by some PGPFs is one mechanism for increasing the accessibility of iron to plants[42]. Several species were previously recorded to produce siderophores in Egypt, such as A. niger [43] , Fusarium solani [44] Trichoderma harzianum [45],and Trichoderma sp [46]. In this study, A. niger and Paecilomyces lilacinus, that were isolated from rhizosphere regions of Zea mays and rhizosphere and rhizoplane regions of Zea mays, respectively, showed the highest siderophore activity. This observation agrees with [47], who found that maximum siderophore production (~ 80%) was observed with A. niger but did not agree with the case of Paecilomyces lilacinus [48]. Interestingly,P. oxalicum has not been previously recorded as a siderophore producer. Only irradiated P. oxalicum is listed to produce a siderophore [49].

Hormone production from PGPF is another mechanism to enhance plant growth. Examples of species previously recorded as hormone producers are F. oxysporum, which produces IAA, P. commune produces GA[50] Piriformospora indica produces Ethylene [51] and Glomus intraradices as ABC producers or inducer [52], A. niger, F. oxysporum, and paecilomyces lilacinus are known as producers of the indole-3-acetic acid (IAA) phytohormone, and IAA production has been suggested to promote plant root growth[53,54]. Obtained results of IAA production indicated that most of the tested fungal strains showed productivity of IAA in a cultivation medium supplemented with DL-tryptophan. The highest IAA production was detected in A. niger (0.75 μg ml^{-1}), followed by P. oxalicum (0.65 μg ml^{-1}), whereas the lowest concentration was noticed by Paecilomyces lilacinus (0.32 μg ml^{-1}). Intriguingly, P. oxalicum has not previously been recorded as an IAA producer. Solubilization of minerals is a third mechanism adopted by PGPF to increase plant growth via increased availability of some insoluble minerals. Phosphate solubilizing fungi in Egypt were studied in several regions [55] while in Port Said; there are no reports until now. A. niger, F. oxysporum, P. oxalicum, and Paecilomyces lilacinus are known as phosphate solubilizers, and its production had been suggested to produce soluble P, making it accessible to plants, improving the growth and yield of wide types of crops [56].This study revealed that the highest phosphate solubilization was produced by A. niger (SI=1.8), followed by Paecilomyces lilacinus (SI= 1.5) and P. oxalicum (SI= 1.49). The latter result agrees with [57]. Finally, the F. oxysporum strain showed no phosphate solubilization activity.

Some PGPFs are capable of producing various plant volatiles [58,59] These volatile compounds act as airborne communication signals between plants and their mycobiome with several benefits to plant health, such as growth promotion [22], inducing plant resistance [60] and tolerance. *Arabidopsis*–fungi cocultivation results showed that *P. oxalicum* affected the plant seedlings' root and shoot lengths and number of leaves compared with control. *P. oxalicum* showed a significant difference in growth parameters measured in this study and affected root hairs compared to the other four fungal strains. Interestingly, *P. oxalicum* has not been recorded as a volatile compounds producer. Chromatographic analysis of the *P. oxalicum* volatile profile introduced volatile compounds such as 2-Myristynoyl pantetheine and Hahnfett, known to have antifungal activity, Phenol, 2-Methoxy- 4-(2-propenyl) and 1,8-Di(4-nitrophenylmethyl)-3,6- diazahomoadamantan-9-one that is known to have a plant growth promotion activity.Collectively, obtained data suggested that the *P. oxalicum* isolated from the rhizosphere regions of *Zea mays* could be a promising naturally occurring plant-growth-promoting fungus. *P. oxalicum* could enhance the development of plants through various strategies such as IAA production, phosphate solubilization, siderophore production, and volatile compounds production. It will be applied as biological growth inducer.

5-Conclusion

Chemical fertilizers and fungicides are harmful to the environment. Therefore, eco-friendly biofertilizers are of growing interest for sustainable agricultural systems. Fungi isolated from Port Said are naturally occurring species that could be evaluated to act as biofertilizers to promote plant growth and health. In this study, evaluation over three hundred rhizosphere and rhizoplane fungal strains native to Port Said city in Egypt. This study concluded that *Penicillium oxalicum* could use strategies such as siderophore, phytohormones, and volatiles production to promote plant development. This study evidenced the immense ability of plant growth promoting fungi such as *Penicillium oxalicum* in the enhancement of plant development in Egypt,which may act as a viable substitute for harmful chemicals.

Declaration of competing interest

The authors declare that there is no conflict of interest

6-References

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