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Molecular characterization of phosphate solubilizing fungi *Aspergillus niger* and its correlation to sustainable agriculture

Y.S. Mazrou^{1,2*}, A.H. Makhoul³, E.R. Elbealy⁴, M.A. Salem⁵, M.A. Farid⁶, M.F. Awad^{7,8}, M.M. Hassan^{7,9} and M. Ismail⁹

¹Department of Business Administration Department, Community College, King Khalid University, Abha, 62529, Saudi Arabia

²Department of Agric. Economics, Faculty of Agriculture, Tanta University, Tanta, 3111, Egypt

³Department of Botany, Faculty of Agriculture, Menoufia University, Menoufia, 32514, Egypt

⁴Department of Biology, Faculty of Science, King Khalid University, Abha, 61413, Saudi Arabia

⁵Department of Chemistry, Faculty of Science and Art, King Khalid University, Asir, 61421, Saudi Arabia

⁶Department of Biology, Faculty of Science, Taif University, Taif, 21944, Saudi Arabia

⁷Department of Genetics, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh, 33516, Egypt

⁸Department of Botany and Microbiology, Faculty of Science, Al-azhar University, Assuit, 71511, Egypt

⁹Department of Genetics, Faculty of Agriculture, Menoufia University, Menoufia, 32514, Egypt

*Corresponding Author Email : mazrou_y@yahoo.com

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Abstract

Aim: The present study aimed to investigate the phosphate solubilization potential of agriculturally important fungi, i.e., *Aspergillus* sp. isolated from the rhizosphere of healthy plants in Abha city, Saudi Arabia.

Methodology: Sixteen *Aspergillus* sp. isolated and tested for phosphate solubilization potential were identified by 5.8S-ITS region sequencing and characterized by 11 ISSR-PCR markers. Finally, the highest phosphate solubilization potential isolates were used in field experiments on cucumber and tomato plants.

Results: All *Aspergillus niger* isolates showed 96–100% similarity to *A. niger* strains available at GenBank database. Isolate ASAB-5 was most efficient at solubilizing phosphate on Pikovskaya's medium, with a solubilization index of 2.67, and 235.22 mg l⁻¹ of solubilized phosphate. ISSR-PCR markers revealed is total 142 bands in all isolates, with about 32.3% showing monomorphism and 67.6% polymorphism. Based on genetic similarity and intraspecies variability, the *Aspergillus* isolates were grouped into two different clusters with about 67.9% genetic similarity. The results of field experiments showed no significant difference between seeds treated with culture filtrate or conidial suspension of ASAB-5; however, both differed remarkably from untreated seeds.

Interpretation: The current study confirms the existence of several useful phosphate solubilizing fungi in plants, which may serve as potential biological fertilizers. They are safer than chemical fertilizers and increase the bioavailability of soil phosphates for plants.

Key words: *Aspergillus niger*, ISSR markers, Phosphate solubilization, 5.8S-ITS sequencing

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Introduction

Phosphate is one of the most important macronutrients for plant growth and development. However, in soil, it is relatively found in low concentration, ranging from 500 to 10,000 kg P in the upper parts of the soil. Phosphorus compounds commonly found in soils are mostly unavailable for plant uptake, as they are highly insoluble complex (Son *et al.*, 2006; Baig *et al.*, 2010; Chauhan *et al.*, 2017). When soluble sources of phosphorus, such as fertilizers and manures, are added to soils, they may become fixed (changed to unavailable forms) and in time form highly insoluble compounds (Khan *et al.*, 2010; Gupta and Pandey, 2019). Some farmers address this problem by using phosphate fertilizers, which become incorporated in the soil. Unfortunately, this practice is costly, environmentally troublesome, and may lead to insolubility of phosphorus (Dawwam *et al.*, 2013). Moreover, only 5% of the added phosphorus is actually available to plants (Khan *et al.*, 2010; Pande *et al.*, 2017). As a result, chemical fertilizers are not suitable for regular use in agricultural operations. Phosphate solubilizing microbes, which convert phosphate into a form suitable for plant uptake, represent a safe, as well as cheap and environmentally friendly alternative to chemical-based phosphate fertilizers (Sharma *et al.*, 2013). Some fungi, such as *Aspergillus* sp. (*A. awamori*, *A. niger*, *A. terreus*, *A. flavus*, *A. nidulans*, *A. foetidus*, and *A. wentii*) and *Penicillium* sp. (*P. digitatum*, *P. lilacinum*, *P. balaji*, and *P. funiculosum*) are known phosphate solubilizing microbes (Chuang *et al.*, 2007; Pandey *et al.*, 2008; Jain *et al.*, 2012).

Identification of most common and important species of the genus *Aspergillus* remains problematic due to variability in the phenotypic characters (Nayak *et al.*, 2019). However, a validated and careful approach of phenotypic classification (taxonomy) together with phylogenetic treatment of DNA sequence data is a prerequisite for a reliable and rapid identification (Khan *et al.*, 2007; Godet and Munaut, 2010; Manjunath *et al.*, 2017). Recently, advancements in molecular techniques have created new possibilities for the selection and genetic improvement of live fungal stocks. Internal transcribed spacer (ITS) region on rDNA, in particular, is the most useful method for inter- and intraspecific sequence-based identification of *Aspergillus* genus and other fungi (Toju *et al.*, 2012; Riaz *et al.*, 2017; Hassan *et al.*, 2019). In view of the above, the present study aimed to investigate the phosphate solubilization potential of *Aspergillus* sp. isolated from the rhizosphere of healthy plants in Abha city, Saudi Arabia.

Materials and Methods

Soil samples: Twelve different tomato rhizospheric soil samples were collected from farms situated in different areas of Abha city, and surrounding locations. The collected soil samples were brought to the laboratory, dried, and stored for further analyses.

Isolation of fungi from the rhizosphere: All soil samples were subjected to serial dilution in potato dextrose agar (PDA) medium for isolation of fungi. The fungi were identified based on the

morphological, conidial and culturing characteristics, and then identified at molecular level using 5.8S-ITS region sequencing.

Screening for phosphate solubilizing fungi: To identify phosphate solubilizing fungi, the isolates were cultured separately on Pikovskaya's agar plates and incubated at $28 \pm 2^\circ\text{C}$ for 7 days. The ability of fungi to produce a halo/clear zone due to solubilization was selected as an indicator of potential phosphate solubilizers. Phosphate solubilization index (SI) was calculated by the formula (Pikovskaya, 1948) given below:

$$\text{SI} = (\text{Colony diameter} + \text{Halo diameter}) / \text{Colony diameter}$$

DNA extraction: Fungal mycelia of selected phosphate solubilizers were inoculated into Czapek Dox broth and incubated for 5 days. Genomic DNA of each selected fungus was extracted using the Norgen Plant/Fungi DNA isolation Kit (Sigma, Canada) following manufacturer's instructions.

Sequence analysis of 5.8S-ITS region: ITS1 and ITS4 primers were designed according to White *et al.* (1990). PCR products were sequenced by Macrogen International Co. Seoul, Korea. The produced sequences were aligned and nucleotide BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST/>) was selected to determine the homology between the obtained fungal sequences and related sequences in the GenBank database. MEGA software version 7.0 was used to draw a phylogenetic tree of the obtained sequences and related NCBI sequences.

Inter simple sequence repeats (ISSR)-PCR analysis: For repetitive sequence analysis, PCR of selected fungal isolates was performed according to Lakhani *et al.* (2016). Eleven ISSR primers (ISSR-Asp, ISSR-G4, ISSR-G5, ISSR-2, ISSR-8, ISSR-9, ISSR-10, ISSR-12, ISSR-16, ISSR-18, and ISSR-28) were used to amplify the genomic DNA. A simple matching coefficient was estimated by means of Jaccard's coefficient and was used to construct a similarity matrix by NTSYS-pc, ver. 2.2.

Molecular characterization of selected fungi: Total DNA extracted from the *Aspergillus* sp. isolates were used for PCR amplification of fragments in the calmodulin gene. Specifically, NIG1/NIG2 and TUB1/TUB2 primer pairs were used to discriminate between *A. niger* and *A. tubingensis*. The extracted DNA was used for detecting phosphate solubilization genes, glucose dehydrogenase (*gdh*), and its cofactor pyrroloquinoline-quinone synthase (*pqq*). Finally, total DNA of these isolates were used to assess the presence of Ota and Aopks genes and production of ochratoxin A (Ferracin *et al.*, 2012). (Primers are listed in Table 1).

Field experiments: Seeds of cucumber (Sweet Success variety) and tomato (Super Sweet 100 variety) were sterilized for 2 min by sodium hypochlorite and rinsed several times in sterile distilled water. They were then soaked in a conidial suspension or culture filtrate of *Aspergillus* sp. ASAB-5 at 1×10^8 colony-forming units per ml. (Murali *et al.*, 2016). Treated and untreated seeds (three

replicates of ten seeds) were planted and grown in pots for 30 days to evaluate shoot and root fresh weight, dry weight and length.

Statistical analyses: Data from three replicates were analyzed by Analysis of Variance using SPSS Inc. 16.0 software (IBM, USA). Significant differences were calculated according to Duncan's Multiple Range Test (Steel and Torrie, 1980). Means within a column followed by same letter were not significantly different at $p = 0.05$ level.

Results and Discussion

In total 106 fungal isolates were collected from the rhizosphere of different plants in the Abha region of southern Saudi Arabia. Thirty-nine of them were classified as *Aspergillus* sp. Their colonies grew rapidly on PDA, and initially their white floccose mycelium spread rapidly, turning quickly into black-colored colonies that produced black spores (Godet and Munaut, 2010; Nayak et al., 2019). Four isolates of *Fusarium* sp., whose mycelia were of dirt white color, justly dense, and with yellow pigmentation at the periphery, were also found. Moreover, the mycelia grew in thick concentric rings. Some isolates displayed bright pink pigmentation at the center (Gaddeyya et al., 2012).

Fourteen of them were recognized as *Penicillium* sp., because their colonies were typically fast growing, in shades of green, occasionally white, and consisted a dense felt of conidiophores (Pandey et al., 2008). Sixteen isolates were identified as *Trichoderma* sp. when grown on PDA; they formed 1–2 concentric rings with green conidia, which were highly dense in the center and then spread to the margins (Hassan et al., 2014). Eighteen isolates were identified as *Pythium* sp.; their mycelia grew densely and presented a cotton appearance, especially on rich medium such as PDA (Gaddeyya et al., 2012). Finally, 15 isolates were identified as *Alternaria* sp.; their conidia were of muri-form shape and light brown color (Hassan et al., 2014). The isolates were serially numbered and stored prior to the phosphate solubilization experiment. All isolates were tested for phosphate solubilization using Pikovskaya's agar medium method. Fungi

that rapidly formed clear areas and were of the largest diameter indicated a superior phosphate solubilizing capacity. Among the tested fungi, 16 isolates of *Aspergillus* sp. exhibited the highest phosphate solubilization value and were selected for estimating phosphate SI. The SI of different isolates ranged from 1.79 to 2.67 after 7 days of incubation at 28–30 °C. As depicted in Table 2, ASAB-5 was the most efficient phosphate solubilizing *Aspergillus* sp. on Pikovskaya's medium with SI value of 2.67, followed by ASAB-13 with SI value of 1.96 and ASAB-11 with SI value of 1.96; whereas the lowest SI (of 1.79) was detected in ASAB-9 (Table 2). Moreover, ASAB-5 yielded the highest amount of solubilized phosphate (235 mg l⁻¹), followed by ASAB-2 (180.52 mg l⁻¹); whereas ASAB-9 was the least efficient one (171.36 mg l⁻¹). Current results are in agreement with the earlier findings (Richa et al., 2007; Khan et al., 2010; Onyia et al., 2015), where high incidence of phosphate solubilizing fungi were reported belonged to the genus *Aspergillus* and to a lesser extent to genus *Penicillium* spp. in the rhizosphere of several crop plants.

Two universal oligonucleotides (ITS1 and ITS4) were used to amplify the 5.8S-ITS region in *Aspergillus* sp. isolates. The primers successfully amplified a 600-bp fragment from the 5.8S-ITS region in all phosphate solubilizing *Aspergillus* sp. isolates. Comparing incomplete 5.8S-ITS region sequence with these in GenBank database revealed that all 16 isolates corresponded to *A. niger*. Phylogenetic analysis based on 5.8S-ITS sequencing classified the isolates into two main taxonomic lineages (Fig. 1). The first main lineage contained three sub-lineages. The first one comprised *Aspergillus* isolates ASAB-10, ASAB-11, ASAB-12, ASAB-13, and ASAB-14 with 100% similarity to *A. niger* VITAF1; as well as ASAB-7, ASAB-8 and ASAB-16 with 100% similarity to *A. niger* Asp-7136. The second sub-lineage contained *Aspergillus* isolates ASAB-2, ASAB-4 and ASAB-6 with 98% similarity to *A. niger* AHBR5. Finally, the third sub-lineage contained *Aspergillus* isolates ASAB-1, ASAB-3, ASAB-5, ASAB-9 and ASAB-15 with 96–100% similarity to *A. niger* S5. The second main lineage contained two *A. tubingensis* isolates, G20-05 and Cs72, which displayed 92% similarity to other *A. niger* isolates. ITS rDNA profiling offers a cost-effective method for

Table 1: PCR primers used to detect target genes (NIG, TUB, gdh, pqq, Ota, and Aopks) in phosphate solubilizing *Aspergillus* sp. isolates

Primers name	Primer sequence (5'→3')	Size (bp)	References
NIG1	TCCGTAGGTGAACCTGCGG	245	Susca et al. (2007)
NIG2	GCTGCGTTCTTCATCGATGC		
TUB1	GCATCGATGAAGAACGCAGC	505	Susca et al. (2007)
TUB2	TCCTCCGCTTATTGATATGC		
gdh-f	ATGTATCCGGATTAAAGG	798	Chauhan et al. (2017)
gdh-r	TTAACC CGGCCTGCCTGG		
pqq-f	GGCTGCTGGCCGAAGTACTT	1000	Chauhan et al. (2017)
pqq-r	GGCCGCAAGAAGCATTATTAG		
OTAF	CAATGCCGTCCAACCGTATG	776	Ferracin et al. (2012)
OTAF	CCTTCGCCTCGCCCGTAG		
Aopks-F	CAGACCATCGACACTGCATGC	530	Ferracin et al. (2012)
Aopks-R	CTGGCGTTCCAGTACCATGAG		

Table 2: Phosphate solubilization index (PSI) and amount of phosphate solubilized by the isolates of *Aspergillus* sp.

Isolates name	PSI	Solubilized P (mg l ⁻¹)
ASAB-1	1.92 ^c	175.16 ^d
ASAB-2	1.95 ^b	180.52 ^b
ASAB-3	1.91 ^c	178.91 ^b
ASAB-4	1.94 ^b	179.47 ^b
ASAB-5	2.67 ^a	235.22 ^a
ASAB-6	1.84 ^e	172.42 ^e
ASAB-7	1.83 ^e	172.61 ^e
ASAB-8	1.92 ^c	175.15 ^d
ASAB-9	1.79 ^f	171.36 ^f
ASAB-10	1.87 ^d	174.71 ^d
ASAB-11	1.96 ^b	178.11 ^b
ASAB-12	1.94 ^b	177.57 ^c
ASAB-13	1.95 ^b	178.74 ^b
ASAB-14	1.89 ^d	176.32 ^d
ASAB-15	1.85 ^e	172.94 ^e
ASAB-16	1.95 ^b	177.58 ^c

Values within the same vertical area with same letter are not significantly different at 5% probability level by Duncan's Multiple Range Test

identifying and characterizing complex microbial communities at species level, following morphological and cultural examination (Rastogi and Sani, 2011; Rao *et al.*, 2015). To confirm the identity of our isolates and discriminate between *A. niger* and *A. tubingensis*, a specific PCR assay targeting DNA sequence of calmodulin gene (Susca *et al.*, 2007) was used. The primer set N1G1/N1G2 amplified only DNA from *A. niger* strains, whereas the primer set TUB1/TUB2 amplified only DNA belonging to *A. tubingensis* (Susca *et al.*, 2007). The specific primer pair N1G1/N1G2 amplified a 265-bp fragment in all isolates, whereas primers TUB1/TUB2 failed to amplify any region, strongly suggesting that all strains belonged to *A. niger*. PCR amplification of phosphate solubilization genes, such as *gdh* and *pqq* (Son *et al.*, 2006), yielded no product for the latter but produced a 798-bp

gdh fragment with isolates ASAB-2, ASAB-4, ASAB-5, ASAB-6, ASAB-7, ASAB-10, and ASAB-13. Finally, we used primers against *Ota* and *Aopks* to ensure that ASAB-5, which were selected for field experiments, did not produce any ochratoxin and was safe to use. The *Ota* gene was amplified in most isolates except ASAB-2, ASAB-3, ASAB-4, ASAB-5, and ASAB-16. The *Aopks* gene was also found in some isolates, such as ASAB-6, ASAB-7, ASAB-11, ASAB-12 and ASAB-14. These results indicated that ASAB-5, the isolate with highest phosphate SI, did not contain the ochratoxin gene and was suitable for field experiments.

The PCR-based ISSR markers method using arbitrary oligonucleotide primers is commonly employed for genetic analysis and as a screening genotyping method. ISSR-PCR markers have been validated as genetic markers in bacteria and fungi fingerprinting (Hassan *et al.*, 2014). ISSR markers can produce numerous fingerprint patterns with infinite number of primers. Here, 11 different ISSR primers were tested in duplicate on DNA samples of *Aspergillus* sp. isolates and all the amplified products were reproducible. Products' size ranged from about 190 bp to 2000 bp. Specifically, GTG5 primer produced in total 18 bands in all *Aspergillus* isolates, ranging from 250 bp to 1950 bp. Six shared bands were detected in all *Aspergillus* isolates, exhibiting about 37.5% monomorphism; whereas another 12 fragments showed 66.7% polymorphism (Table 3; Fig. 2). The ISSR-28 primer produced in total 12 fragments with about 50% polymorphism and 50% monomorphism among sixteen *Aspergillus* isolates. The molecular size amplified products ranged from 350 bp to 1900 bp.

According to genetic similarity and intraspecies differentiation, the *Aspergillus* isolates were grouped into two different clusters with about 67.9% genetic similarity. Based on these data, sixteen isolates were separated into two main clusters. Cluster 1 was divided into two sub-clusters; the first sub-cluster included *Aspergillus* isolates no. 1, 3, 5, 9 and 15, which displayed 56% similarity whereas the second sub-cluster included isolate no. 8. Cluster 2 was also divided into two sub-

Table 3: Polymorphic bands produced by each of the 11 ISSR-PCR primers and percentage of polymorphism in sixteen isolates of *Aspergillus* sp.

Primers	Total bands	No. of monomorphic bands	No. polymorphic bands	% monomorphic bands	% Polymorphic
ISSR-Asp	16	6	10	37.5	62.5
ISSR-G4	14	5	9	35.7	64.3
ISSR-GTG5	18	6	12	33.3	66.7
ISSR-2	9	3	6	33.3	66.7
ISSR-8	15	5	10	33.3	66.7
ISSR-9	16	4	12	25.0	75.0
ISSR-10	11	3	8	27.3	72.7
ISSR-12	11	2	9	18.2	81.8
ISSR-16	6	2	4	33.3	66.7
ISSR-18	14	4	10	28.6	71.4
ISSR-28	12	6	6	50.0	50.0
Total	142	46	96	32.3	67.6

Table 4: Effect of seed treatment with *Aspergillus* sp. ASAB-5 on cucumber plant

Treatment	Length (cm)		Fresh weight (gm)		Dry weight (gm)	
	Shoot	Root	Shoot	Root	Shoot	Root
Culture filtrate	12.0 ^a	15.2 ^a	2.92 ^a	1.33 ^b	0.36 ^a	0.09 ^a
Conidial suspension	11.9 ^a	15.0 ^a	2.85 ^b	1.46 ^a	0.36 ^a	0.10 ^a
Control	8.30 ^b	10.7 ^b	1.58 ^c	0.39 ^c	0.19 ^b	0.05 ^b

Values within same vertical area with same letter are not significantly different at 5% probability level by Duncan's Multiple Range Test

Table 5: Effect of seed treatment with *Aspergillus* sp. ASAB-5 on tomato plant

Treatment	Shoot and Root length (cm)		Fresh weight (gm)		Dry weight (gm)	
	Shoot length	Root length	Shoot weight	Root weight	Shoot weight	Root weight
Culture filtrate	12.7 ^b	8.2 ^b	1.52 ^a	0.60 ^a	0.13 ^a	0.07 ^a
Conidial suspension	13.0 ^a	9.0 ^a	1.36 ^b	0.41 ^b	0.17 ^a	0.06 ^a
Control	8.50 ^c	6.2 ^c	0.57 ^c	0.14 ^c	0.04 ^b	0.02 ^b

Values within the same vertical area with the same letter are not significantly different at 5% probability level by Duncan's Multiple Range Test

clusters; the first comprised isolates no. 2, 4, and 6 with 30% similarity, and the second isolates no. 7, 10, 11, 12, 13, and 14 with 14% similarity (Fig. 3). All treated cucumber and tomato plants were harvested after 30 days, for studying growth parameters. Cucumber plants treated with culture filtrate of *Aspergillus* sp. ASAB-5 exhibited increase in all growth parameters as compared to control plants. The percent increase was 44.5%, 85% and 89.5% for shoot length, shoot fresh weight and shoot dry weight,

respectively (Table 4). Similarity, cucumber plants treated with conidial suspension of *Aspergillus* sp. ASAB-5 showed increase in shoot parameters. The percentage increase was 43.4%, 80%, and 89.5% for shoot length, shoot fresh weight and shoot dry weight, respectively. Similar effect was also observed on the roots when treated with culture filtrate and conidial suspension of *Aspergillus* sp. ASAB-5. Using culture filtrate, the percentage increase in roots parameters was 42%, 2.4% and 80% for root

Fig. 1: Phylogenetic tree and diversity of 5.8S-ITS region in sixteen isolates of *Aspergillus* sp. compared with reference *Aspergillus* strains. Phylogenetic tree was generated using parsimony neighbor-joining and maximum likelihood analysis.

Fig. 2: ISSR-PCR profile of sixteen isolates of *Aspergillus* sp. generated with the respective ISSR primers: ISSR-Asp, GTG5, ISSR-8, ISSR-9, ISSR-18, and ISSR-28. The first lane in each panel corresponds to 100-bp molecular weight markers.

Fig. 3: Dendrogram analysis of sixteen isolates of *Aspergillus* sp. based on 11 ISSR-PCR primers.

length, root fresh weight and root dry weight. Meanwhile, using *Aspergillus* sp. ASAB-5 conidial suspension, the percent increase in root parameters was 40%, 2.74% and 100% for root length, root fresh weight and, root dry weight, respectively. Similar effect was also observed on tomato plants treated with culture filtrate and conidial suspension of *Aspergillus* sp. ASAB-5. Tomato plants treated with culture filtrate showed 49%, 1.7% and 2.25% increase in shoot length, shoot fresh weight and shoot dry weight. Tomato plants treated with conidial suspension also showed 53%, 1.4% and 2% increment in shoot length, shoot fresh weight and shoot dry weight, respectively. Similar effect was also observed on tomato roots when treated with culture filtrate and conidial suspension. Using culture filtrate, tomato roots showed 32%, 3.3% and 2.5% increment in root length, root fresh weight and root dry weight, respectively. Also using conidial suspension the increase in roots parameters was 45%, 2% and 2%. These results corroborates with the study of Fuad et al. (2019), Phosphate-solubilizing bacteria and fungi are present in the soil. Si addition increased phosphate solubilization of fungi by 50%. Statistical analysis revealed a significant difference ($p < 0.05$) between treated and untreated seeds, but no remarkable variance ($p < 0.05$) was observed between the seeds exposed to culture filtrate or conidial suspension of *Aspergillus* sp. ASAB-5. Thus, these experiments confirmed a significant enhancement in shoot fresh weight, dry weight and length in cucumber and tomato plants treated with phosphate solubilizing *Aspergillus* sp. ASAB-5.

A significant increase in tomato shoot fresh weight, dry weight and length is in line with previous reports Sudisha et al. (2013), who reported improved germination and seedling vigor in tomato seeds soaked with phosphate solubilizing fungi, such as *Trichoderma*, *Penicillium*, or *Aspergillus*. Similarly, *A. niger* and *Penicillium italicum* have been described to solubilize tricalcium phosphate and improve growth of soybean plants exposed to tricalcium phosphate-containing soil (Iman and Azouni, 2008; Eramma et al., 2020)). Tomato seeds treated with *Trichoderma harzianum* (Fahmi et al., 2012) as well as pearl millet treated with *Penicillium chrysogenum* and *Penicillium oxalicum* (Murali et al., 2016) isolated from soil rhizosphere have shown improved seed germination and seedling vigor over control plants.

The present study compared the phosphate solubilizing potential of *Aspergillus niger* strains isolated from different farm locations in Abha city, Saudi Arabia. Phosphate solubilizing efficiency was highest in isolate ASAB-5 and was confirmed by the presence of phosphate solubilization genes. Importantly, this isolate significantly improved growth parameters of cucumber and tomato plants under field trials, suggesting the potential of such fungi for boosting crop yields.

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