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A Phosphate and Potassium-Solubilising *Aspergillus niger* Strain SA1 Isolated from Spoiled Rice for Potential Biofertilizer Application

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ABSTRACT

This study aimed to screen and isolate efficient fungal strains from spoiled rice with phosphate (P) and potassium (K) solubilisation potential. Screening was conducted using solid and liquid Pikovskaya (PVK) medium with tricalcium phosphate (TCP) as the P source and Aleksandrow (AS) medium with potassium alumino silicate as the K source. Among 12 fungal isolates, 8 exhibited positive solubilisation activity, with phosphate solubilisation index (PSI) ranging from 2.00 to 4.00 cm and potassium solubilisation index (KSI) ranging from 2.00 to 3.50 cm. Based on structural morphology under a light microscope, all of them belong to the Aspergillus species. The SA1 strain demonstrated the highest P and K solubilisation in liquid media, releasing 836.67 µg/mL P (pH 3.55, $p < 0.05$) and 78.90 µg/mL K (pH 3.57, $p < 0.05$) after 7 days of incubation at 30°C. The pH reduction was associated with organic acids secretion, primarily gluconic acid (10.09 mg/L for P; 4.58 mg/L for K), followed by citric, oxalic, and lactic acids. The fungal strain SA1 was further identified using ITS region sequencing and was confirmed to be *Aspergillus niger*. These findings highlight the potential of this fungal strain as biofertilizer to enhance plant growth and improve soil health.

INTRODUCTION

Phosphorus (P) and potassium (K) are two of the most important macronutrients for plants, after nitrogen (N) in supporting plant growth and development. Agricultural soil often lacks P and K which remain as a challenge and usually fixed by the excessive usage of chemical fertilizers. Even though these fertilizers help the farmers to increase plants yield, the overuse can lead to many environmental issues such as soil fertility degradation, eutrophication, groundwater pollution, and the accumulation of toxic residues. A critical concern is the limited bioavailability of these nutrients in soil. For example, it is estimated that about 1% of all the P in the soil is in a form that plants can easily take up. This directly causes P deficiency and reduced crop yields [1,2]. Similarly, K is also very important for activating enzymes, photosynthesis, and osmoregulation. However, deficiencies in K due to a lot of K soil is bound up in minerals, making it unavailable for plant uptake. According to findings by Flatian et al. [1] and Muthuraja & Muthukumar [3], between 90–98% of soil K stays in forms that are not accessible by the plant, while only 2–10% is easily available for plant uptake. This limited nutrient availability making farmers to rely more on chemical fertilizers application in which raises concerns for the long term environmental sustainability and soil health. To mitigate these issues, phosphate-solubilising fungi (PSF) and potassium-solubilising fungi (KSF) have been found as a promising biological resource due to its ability converting insoluble P and K, making it more available in soils. These fungi help in nutrient cycling by converting the insoluble P and K into soluble forms that plants can easily take up, thereby supporting more sustainable agricultural practices.

Although extensive research has been conducted on P and K solubilising bacteria, fungal solubilizers from food waste remain under reported, despite the industrial relevance of *Aspergillus* spp. for acid-mediated nutrient mobilisation. Filamentous fungi like *Aspergillus* and *Penicillium* have been found to be some of the best solubilizers of both P and K according to Muthuraja & Muthukumar [3] and Vassileva et al. [4]. Additionally, PSF and KSF can improve the health and quality of the soil while also practicing sustainable farming and using fewer chemical fertilizers. Therefore, this study aims to isolate and evaluate

fungal strains from spoiled rice for their potential in solubilising P and K.

MATERIALS AND METHODS

Isolation of Fungi Isolate

Spoiled rice was collected from Serumpun Café, Universiti Putra Malaysia in May 2022 and taken to the laboratory under aseptic conditions. The dilution plate method was used to get fungal isolates from the spoiled rice using. 10 g of fungal sample was mixed with 90 mL sterile distilled water inside a 250 mL Erlenmeyer flask. The solution was vigorously shaken and left to stand for 5 minutes [5]. 1 mL of homogeneous fungal solution was serially diluted from 10^{-1} to 10^{-6} using 0.85% saline solutions [6]. From each serially diluted fungal suspension, 0.1 mL were transferred and spread evenly on potato dextrose agar (PDA) which contain (per L): 15 g agar; 200 g Potato and 20 g glucose with some modifications. The PDA agar were supplemented with 25 µg L^{-1} of chloramphenicol to inhibit bacterial growth and allowed to solidify before inoculation [6]. Predominant and morphologically distinct colonies appearing on the medium were selected, purified by sub-culture and maintained on PDA. Fungal isolates were identified by observations based on their colony characteristics, spores' morphology and microscopic observations. The pure cultures were preserved on PDA slant at 4 °C for further analysis.

Characterisation of Fungal Isolates

The selected fungal isolates were cultivated on potato dextrose agar (PDA). Preliminary identification was carried out based on colony characteristics and microscopic examination of conidial structures. Detailed observations of cell morphology were examined through a compound light microscope (Olympus USA Inc., NY) with methylene blue as a staining agent to enhance contrast and clarity.

Screening of Fungi Isolates with P and K solubilising Activity

A freshly grown disc of fungi on PDA agar was inoculated on Pikovskaya agar (PVK). The PVK agar which contain (per L): 15 g agar; 10 g glucose; 5 g tri-calcium phosphate, $Ca_3(PO_4)_2$; 0.5 g ammonium sulfate, $(NH_4)_2SO_4$; 0.5 g manganese sulfate, $MnSO_4 \cdot 7H_2O$; 0.5 g ferrous sulfate, $FeSO_4 \cdot 7H_2O$; 0.5 g yeast extract; 0.2 g sodium chloride, NaCl; 0.2 g potassium chloride, KCl and 0.1 g magnesium sulfate, $MgSO_4 \cdot 7H_2O$ [7] with pH adjusted to 7.0 before autoclaving. Furthermore, 0.5% tri-calcium phosphate $Ca_3(PO_4)_2$ or known as TCP was added into PVK agar as an insoluble P source [8]. All the plates were incubated at 30°C for 3 to 7 days during which the clear halo zones formation around the fungal colonies was monitored as P solubilisation indicator [9]. The diameter and of the halo zones were measured and the phosphate solubilisation index (PSI) was calculated for each isolate. PSI was determined using the formula proposed by Edi Premono et al. [10]:

$$Solubilisation\ Index\ (SI) = \frac{(Width\ of\ fungus\ colony + halo\ zone)}{Width\ of\ fungus\ colony}$$

The isolates that have halo zone formation around the colony, indicating P solubilising capabilities, were selected and cultured on PDA agar for further testing. All experiments were performed in triplicate. The Aleksandrow medium was used to observe K solubilisation activity in which contain (per L); 20.0 g agar; 2.0 g potassium alumino silicate, $KAlSi_3O_8$; 5.0 g glucose; 2.0 g calcium phosphate, Ca_3PO_4; 0.5 g magnesium sulfate, $MgSO_4.7H_2O$; 0.1 g calcium carbonate, $CaCO_3$; 0.005 g ferric chloride, $FeCl_3$ and pH of the solution was adjusted to 7.0 before autoclaving. The actively growing fungal discs from PDA agar was transferred to the centre of Aleksandrow agar containing 2.0 g of $KAlSi_3O_8$ as a K source and the plate was left for 3 to 7 days in incubator at 30°C [11]. Afterwards, the potassium solubilisation index (KSI) of fungal cultures was measured by halo zone formation indicating the K solubilisation activity using the SI formula. The fungal isolates with a significant halo formation were selected and preserved on PDA agar until further investigation.

Quantitative measurement of Phosphate Solubilisation Activity

The actively growing colonies on PDA were flooded with 5 mL of sterile distilled water containing 1% Tween 80. The conidia were then gently rubbed and transferred to a sterile tube [12]. The culture was adjusted to 10^7 spores/mL by serially diluting 1 mL of the homogenized spore suspension with 0.85% sterile saline to achieve the desired spore concentration [9]. Spore concentration was estimated using a hemocytometer (LO-Laboroptik Ltd., UK) by counting the number of spores. After preparing the spore suspension, the cultures were inoculated into 100 mL of PVK liquid medium supplemented with 0.5% tricalcium phosphate (TCP) as a phosphorus source (~997 µg/mL) in a 250 mL Erlenmeyer flask [13]. The medium's pH was adjusted to 7.0 before autoclaving. Non-inoculated media served as control.

All cultures were incubated in a rotary shaker (ISF-700, Lab Companion, MA, USA) at 30 °C and 150 rpm for 7 days [14]. Samples were harvested every 2 days throughout the incubation period. After transferring to centrifuge tubes, the cultures were centrifuged at 10,000 rpm for 10 minutes at room temperature. The resulting cell-free supernatant was filtered and stored for further analysis. The dissolved phosphorus concentration in the culture supernatant was estimated using the yellow phospho-molybdo-vanadate colorimetric method [15] with a UV-Vis spectrophotometer (Secomam UviLine 9400, France) at 430 nm. A standard curve was generated by plotting absorbance against the concentration of a KH_2PO_4 standard solution at 430 nm to determine the phosphorus concentration.

Quantitative measurement of Potassium Solubilisation Activity

The ability of the selected fungal isolates to solubilize and release K from potassium alumino silicate was evaluated. The cultures were inoculated into 250 mL Erlenmeyer flask containing 100 mL Aleksandrow broth with the addition of 2 g L^{-1} of insoluble K sources. The pH of the medium was adjusted to 7.0 before autoclaving. The non-inoculated media were used as control. All cultures were grown in incubator with a rotary shaker (ISF-700, Lab Companion, MA USA) at 30 °C, 150 rpm for 7 days [16]. The cultures were harvested and centrifuged to get a cell-free supernatant. The solubilised K concentration was determined using an inductively coupled plasma optical emission spectrophotometer (ICP-OES) Optima 7300DV (Perkin Elmer) at 766.49 nm [17].

Organic Acids Determination

After 7 days cultivation, the cell-free supernatant was collected from both PVK and Aleksandrow liquid media. The cultures were filtered with a 0.22 µm nylon filter (Millipore, USA). A clear supernatant was analysed for organic acids by high-performance liquid chromatography (HPLC). Analysis was performed using Rezex (Phenomenex) organic acid (ROA) column at 60 °C with a flow rate of 0.6 mL/min by UV detector via comparison with a set of standards. The cultures were analysed for the presence of citric, lactic, malic, oxalic, tartaric and gluconic acids [17,18]. The sample's peak area and retention

time were compared with those of the standards for the quantitative measurement of the organic acids.

Fungus Identification

The selected fungal isolates were cultivated on PDA. Colony characteristics and microscopic observation of conidial structures were used for preliminary identification. The cell morphology of the isolates was examined using compound light microscope (Olympus USA Inc., NY) and methylene blue as a stain. The fungal DNA was identified based on the gene sequencing of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) and was extracted by following the manufacturer's procedures using Fungal Genomic DNA Barcoding Kit. The ITS DNA sequencing and identification was analysed by Apical Scientific Sdn. Bhd., Selangor. The obtained sequences were compared with the closest strains in the GenBank database in the National Center for Biotechnology Information (NCBI) using standard nucleotide BLAST programme to determine similarity percentage. The sequence was carried out using Clustal W and the phylogenetic tree were constructed using MEGA 12.0 software [19] with the neighbor-joining method [20] subjected to 1000 bootstrap replications [21,22].

Statistical Analysis

All statistical analyses were performed using the SPSS Software Package Version 29.0 (SPSS Inc., USA). A one-way analysis of variance (ANOVA) was conducted, followed by Tukey's test to determine significant differences between treatment groups at $P < 0.05$.

RESULTS

Isolation of Fungal Isolates

In this experiment, a total of 12 fungal strains were successfully isolated from spoiled rice samples. The preliminary identification was performed through a combination of macroscopic and microscopic analyses. Firstly, fungi isolates were identified based on the observation of colony morphology including texture, pigmentation, and growth patterns on agar plates to assess morphological traits indicative of fungal classification. To support these observations, microscopic analysis was conducted to examine key structural components such as hyphae, conidiophores, and spore arrangements. The morphological and microscopic characteristics were then compared with standard taxonomic descriptions. All 12 isolates were identified as members of the *Aspergillus* genus. These findings provide an initial confirmation of fungal identity, which may be further validated using molecular-based identification techniques.

Based on colony growth patterns and microscopic observations, three distinct *Aspergillus* species were identified among the isolated fungal strains. **Figs.** 1, 2, and 3 illustrate the colony morphology on PDA plates, alongside the corresponding microscopic characteristics of these species. Strains SF1, SB3, SC2, SD4, SE1, SA1, SG5, and SH7 which designated as Group 1, all had similar morphological traits, with colonies initially started out as white and grew rapidly before turning to black due to the production of dark pigmented spores. Strains ST9, SY3, and SZ1 (Group 2) formed colonies that were yellow-green with clear white mycelial edges, while strain SS4 in Group 3 appeared as greenish-grey colonies with colourless mycelial margins (**Table 1**). These variations in colony pigmentation and structural development suggest the presence of multiple *Aspergillus* species within the isolated fungal strains, highlighting the diversity of fungal contamination in spoiled rice samples.

Table 1. Colony morphology and microscopic characteristics of fungal isolates**.**

Fungal Isolates	Colony Morphology	Microscopic Observations
Group 1 SF1, SB3, SC2, SD4, SE1, SA1, SG5	Colonies were white at first but grew rapidly producing black conidia at the centre and white mycelia towards the edges (**Fig. 1 (A)**). Reverse was dirty white colour (**Fig. 1 (B)**)	The conidia were brown, and globose. Conidia heads were biseriate, dark brown and globose. Conidiophores were smooth, hyaline and turning slightly brown towards the vesicles. (**Fig. 1 (C)**)
Group 2 ST9, SY3, SZ1	Colonies were yellow- green with white mycelia at the edges, formed sporulation rings (**Fig. 2 (A)**). Reverse was greyish ash (**Fig. 2 (B)**)	Uniseriate conidia head had radiated vesicle with phialides covering up to three quarters of the vesicle (**Fig. 2 (C)**) Biseriates conidia head had globose vesicles (**Fig. 2 (D)**)
Group 3 SS4	Colonies were greenish grey with colourless mycelia at the edges (**Fig. 3 (A)**). Reverse was greyish ash (**Fig. 3 (B)**)	Conidia heads were uniseriate, short columnar and phialides covering three quarters of the vesicles Conidiophores were smooth, colourless and expanded toward the vesicle forming flask shaped (**Fig. 3 (C)**)

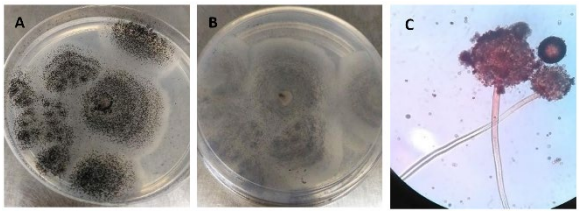

Fig. 1. Morphological and microscopic features of Group 1 fungal isolates. (A) Front view of colonies initially appearing white, later developing dense black conidia at the centre with peripheral white mycelia. (B) Reverse side of colony showing dirty white pigmentation. (C) Microscopic image showing brown, globose conidia with biseriate, dark brown, globose conidial heads; conidiophores are smooth, hyaline, and slightly pigmented toward the vesicles.

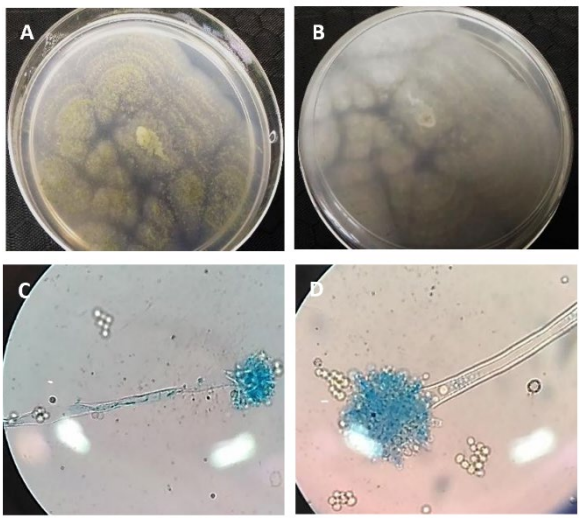

Fig. 2. Morphological and microscopic features of Group 2 fungal isolates (ST9, SY3, SZ1). (A) Colony front view showing yellow-green pigmentation with white mycelial edges and distinct sporulation rings. (B) Reverse view of colony exhibiting a greyish ash coloration. (C) Microscopic observation of uniseriate conidial heads with radiating vesicles partially covered (up to three-quarters) by phialides. (D) Biseriate conidial heads with globose vesicles.

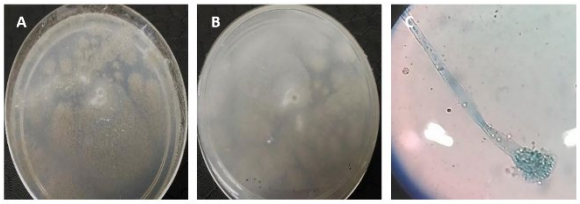

Fig. 3. Morphological and microscopic features of Group 3 fungal isolate (SS4). (A) Colony front view showing greenish-grey pigmentation with colourless mycelial edges. (B) Reverse view exhibiting a greyish ash coloration. (C) Microscopic view of uniseriate conidial heads appearing short and columnar, with phialides covering three-quarters of the vesicle; conidiophores are smooth, colourless, and flask-shaped due to expansion toward the vesicle.

Screening of Phosphate Solubilisation Activity

The screening of fungal isolates was conducted to identify efficient PSF that could serve as potential candidates for biofertilizer production. In this study, the P solubilising ability of 12 fungal isolates was tested using Pikovskaya (PVK) agar medium, which was modified with tricalcium phosphate (TCP) as an insoluble P source. After incubation for 5-7 days at 30°C, the formation of clear zones can be observed around fungal colonies indicated P solubilisation, as these zones represent the fungal isolates were able to breakdown TCP into soluble P.

A total of 8 isolates which are from group 1 demonstrated significant P solubilisation on PVK agar as shown in **Table 2**. Based on **Fig. 4**, isolate SE1 exhibited the highest PSI (3.62), followed by SA1 (3.20), which were significantly different from the other isolates ($p < 0.05$). The PSI values of these two isolates indicate strong P solubilisation potential compared to other tested isolates. Among the other isolates, SC2 displayed a moderate PSI of 2.55 cm, forming a distinct statistical group from the lower solubilising isolates, including SF1 (2.25), SB3 (2.19), and SD4 (2.31), which showed no significant differences among them ($p > 0.05$). Meanwhile, SG5 (2.14) and SH7 (2.16) had the lowest PSI values, placing them in the least effective group for P solubilisation. The statistical classification of isolates based on Tukey's HSD test further confirmed that SE1 and SA1 were significantly P solubilizers, while SG5 and SH7 were the least effective.

Table 2. Qualitative screening of phosphate solubilisation.

Fungal Isolate	P Solubilisation
SF1	+
SB3	+
SC2	++
SD4	+
SE1	++
SA1	++
SG5	+
SH7	+
ST9	-
SY3	-
SZ1	-
SS4	-

Note: P solubilisation activity based on the halo zone formation. No activity of halo zone denoted as -; halo zone < 2.5 cm denoted as +; and halo zone > 2.5 cm denoted as ++.

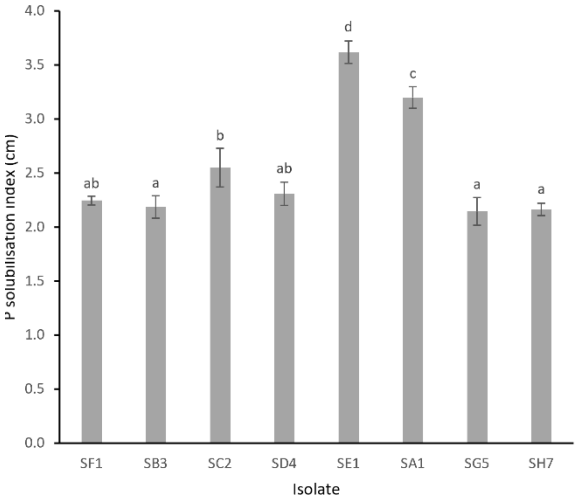

Fig. 4. P solubilisation index of 8 fungal isolates. Data represent mean values ± SD presented by the error bar (n = 3). The different letters between samples showed significant differences (one-way ANOVA + Tukey test at $P < 0.05$).

Screening of Potassium Solubilisation Activity

The selected 8 isolates with P solubilising ability were further screened for their K solubilising ability on Aleksandrow agar supplemented with an insoluble K source (potassium alumino silicate). Among the 8 isolates, SA1 exhibited the highest KSI (3.20), making it the most efficient K solubilising strain in this study. This was followed by SF1 (2.59), which also displayed a significantly higher KSI than most other isolates but remained distinct from SA1 (**Fig. 5**). The lowest KSI values were recorded for SB3 (2.18), SD4 (2.27), and SG5 (2.28), which were not significantly different from each other ($p > 0.05$) and formed the least effective K solubilising group.

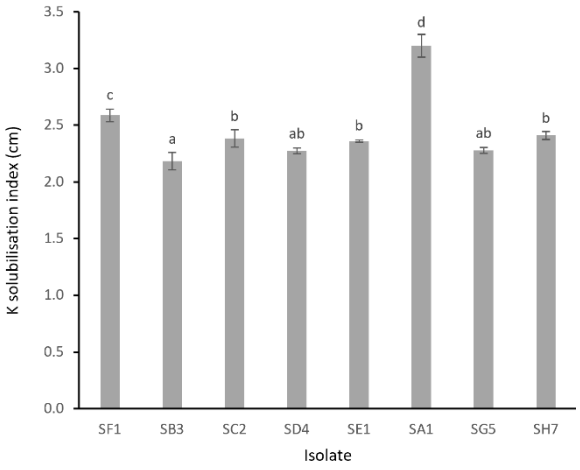

Fig. 5. K solubilisation index of 8 fungal isolates. Data represent mean values ± SD presented by the error bar (n = 3). The different letters between samples showed significant differences (one-way ANOVA + Tukey test at $P < 0.05$).

Quantitative Measurement of Phosphate and Potassium Solubilisation

Phosphate Solubilising Activity

All fungal isolates were evaluated for their phosphate (P) solubilisation potential in Pikovskaya (PVK) broth, with measurements taken at intervals on Days 1, 3, 5, and 7. The P concentration increased significantly over time, reaching its peak between Days 3 and 5, as shown in **Fig. 6**. The results indicate a steady rise in P solubilisation from Day 1 to Day 5, followed by a slight decline on Day 7. Among the tested isolates, SA1 exhibited the highest solubilisation before experiencing a minor decrease on Day 7. Similarly, SE1 demonstrated high solubilisation on Day 3, followed by a slight decline on Day 5 and a further decrease on Day 7. Overall, as depicted in **Fig. 6**, P solubilisation was significantly higher than in the control, showing an initial sharp increase before declining.

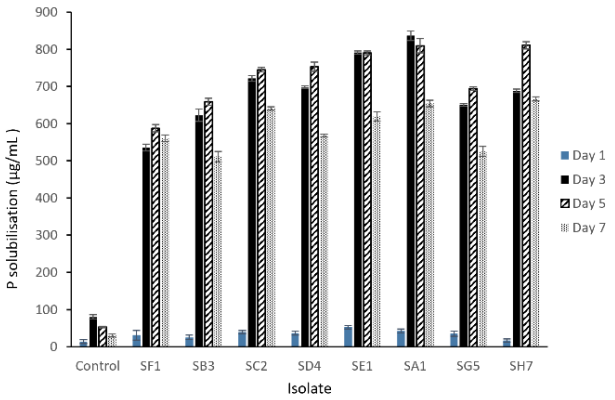

Fig. 6 P solubilisation activity of the isolates during fermentation on PVK liquid medium. Data represent mean values ± SD presented by the error bar (n = 3).

A significant decrease in pH was observed across all fungal isolates, as shown in **Fig. 7**, correlating with increased P solubilisation. The lowest pH values were recorded for SA1, which reached its peak P solubilisation on Day 3. Similarly, SE1, SC2, and SH7 exhibited strong acidification, with pH dropping to 3.55–3.70 on Day 3 and 3.73–3.99 on Day 5, further supporting their high P solubilisation levels. In contrast, the control group maintained a relatively stable pH range of 7.29–7.62, indicating that the pH decline resulted from microbial metabolic activity. On Day 3 as shown in **Fig. 8**, the P solubilisation analysis in PVK broth revealed significant differences among the fungal isolates in terms of P release and pH reduction. The Tukey HSD post-hoc test categorized the isolates into distinct statistical subsets, with SA1 exhibiting the highest solubilisation, followed by SE1, SC2, and SH7, while the control group showed the lowest solubilisation.

SA1 recorded the highest P solubilisation on Day 3 (836.67 µg/mL), significantly outperforming all other isolates. SE1 followed closely with 791.67 µg/mL, demonstrating strong solubilisation capability. SC2 (721.67 µg/mL) and SH7 (688.33 µg/mL) showed similar solubilisation levels, forming a separate statistical subset. Meanwhile, SD4 (697.50 µg/mL) and SG5 (650.42 µg/mL) exhibited moderate solubilisation, with SG5 showing slightly lower efficiency. The lowest P solubilisation values were recorded for SB3 (622.50 µg/mL) and SF1 (535.83 µg/mL), which were significantly lower than the highest-performing isolates but still notably higher than the control group.

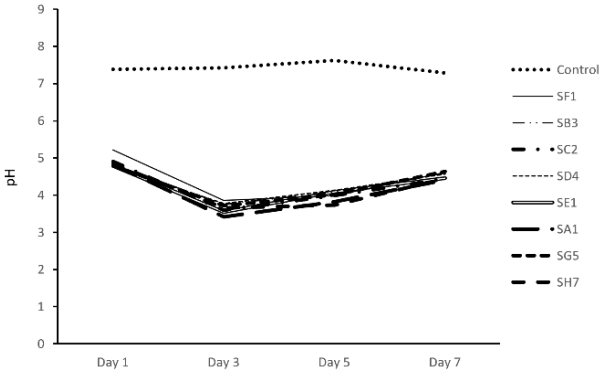

Fig. 7. pH value of PVK liquid medium during fermentation of the fungal isolates. Data represent mean values ± SD presented by the error bar (n = 3).

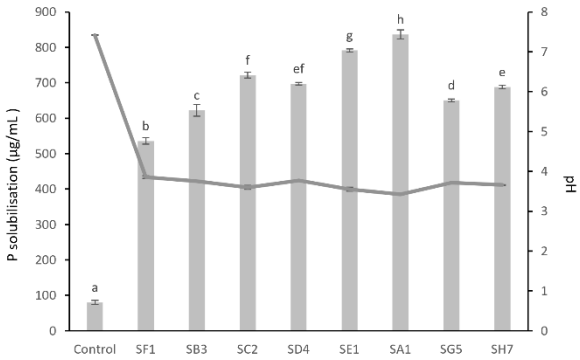

Fig. 8. P solubilisation activity of the isolates during fermentation on PVK liquid medium on Day 3. Data represent mean values ± SD presented by the error bar (n = 3). The different letters between samples showed significant differences (one-way ANOVA + Tukey test at $P < 0.05$).

Potassium Solubilising Activity (KSI)

Among the tested isolates, SA1 exhibited the highest K solubilisation (78.90 µg/mL), significantly surpassing all others ($p < 0.05$), establishing it as the most efficient K-solubilising isolate in this study. The second-highest solubilisation was observed in SF1 (51.15 µg/mL) and SE1 (51.20 µg/mL), both of which showed significantly higher K solubilisation than the control group (14.77 µg/mL), reinforcing their potential for enhancing K bioavailability (**Fig. 9**). Moderate solubilisation levels were recorded in SD4 (50.63 µg/mL), SB3 (50.11 µg/mL), SH7 (50.36 µg/mL), and SC2 (49.40 µg/mL), with no significant differences among them ($p > 0.05$). However, all exhibited significantly greater solubilisation than the control. The lowest solubilisation among the isolates was observed in SG5 (48.76 µg/mL), which was significantly lower than SA1 but still significantly higher than the control.

Following inoculation of the KSF isolates into the K-solubilising fermentation medium, pH was monitored at different time intervals (Days 1, 3, 5, and 7). As shown in **Fig. 10**, pH decreased progressively over seven days of incubation. The solubilisation of K alumino-silicate by the KSF strain in the liquid medium was accompanied by a significant reduction in the pH of the culture supernatant, dropping from an initial pH of 6.90 after 168 hours of incubation. The pH measurements in Aleksandrow broth provide key insights into microbial processes involved in nutrient mobilization.

The results indicate that SA1 exhibited the lowest pH (3.43), followed by SE1 (3.56) and SD4 (3.59), whereas the control remained stable at 6.90. The significant pH reduction in broth cultures containing fungal isolates suggests active acid production, which directly contributes to K solubilisation from its insoluble forms.

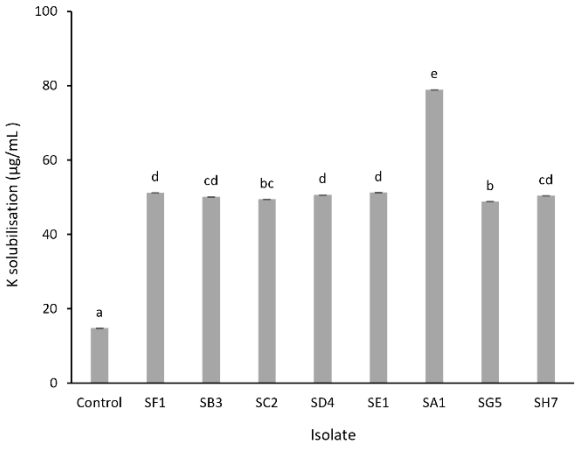

Fig. 9 K solubilisation activity of the isolates during fermentation on AS liquid medium at day 7. Data represent mean values ± SD presented by the error bar (n = 3). The different letters between samples showed significant differences (one-way ANOVA + Tukey test at $P < 0.05$).

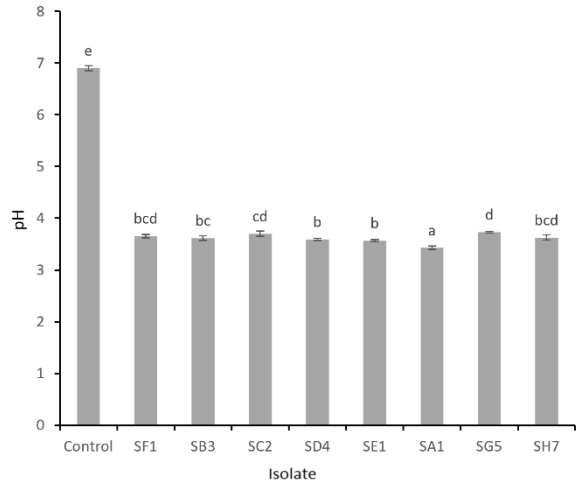

Fig. 10 pH value of the AS medium with the fungal isolates grown at day 7. Data represent mean values ± SD presented by the error bar (n = 3). The different letters between samples showed significant differences (one-way ANOVA + Tukey test at $P < 0.05$).

Organic Acids Production

Fig. 11 shows that organic acid production was the most dominant, with concentrations of 10.09 mg/mL in P-solubilising cultures and 4.58 mg/mL in K-solubilising cultures. Other organic acids, including citric, oxalic, and lactic acids, were detected in varying concentrations, while malic and tartaric acids were below 0.1 mg/mL and thus undetectable. The highest phosphate solubilisation was recorded in SA1 (836.67 µg/mL), followed by SE1 (791.67 µg/mL) and SC2 (721.67 µg/mL), all of which exhibited the lowest pH values (3.42, 3.55, and 3.60, respectively). The significant negative correlation between phosphate solubilisation and pH (r = -0.951, p = 0.000) supports the hypothesis that acidification plays a key role in phosphorus release from insoluble sources.

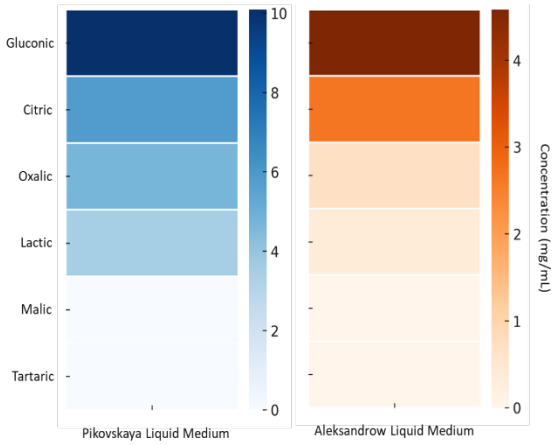

Fig. 11 Organic acids released in liquid media after 7 days of incubation.

Molecular Identification of Strain SA1

The fungal was identified as *Aspergillus* species based on its morphological characteristics. Based on microscopic observation, fungal isolate strain SA1 had rounded shape with smooth surface. SA1 also grown rapidly on the PDA agar after 7 days. It grew as black-brown colonies with white edges as shown in **Fig. 12**. This strain was selected for identification because of its ability with the highest P and K concentration after being tested for quantification analysis. This shows that SA1 have a high solubilisation of insoluble P and K in the liquid medium. Based on the observations, this fungal strain belongs to the species *Aspergillus niger* in family of Aspergillaceae. It shows smooth, colourless conidiophores and spores with radial conidial heads as shown in **Fig. 13**.

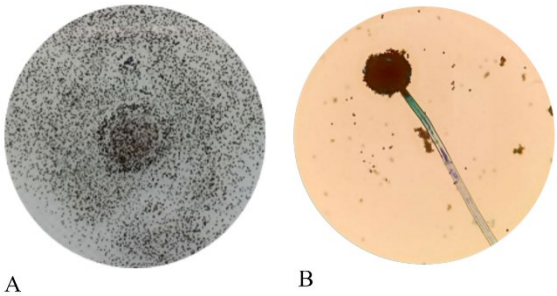

Fig. 12 Morphology of *Aspergillus niger* strain SA1. (A) SA1 growth on PDA agar; (B) SA1 under microscopic observation at 40x magnification.

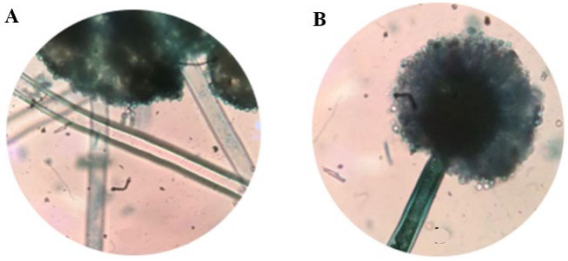

Fig. 13 Morphology of *Aspergillus niger* SA1 under microscopic observation at 100x magnification. (A) Conidium stipe/conidiophore; (B) Conidial structure displaying vesicle and sterigmata.

Molecular techniques were implemented to identify strain SA1 and it was later confirmed to be *A. niger.* This involved PCR amplification of the sample DNA from the SA1 to the ITS region using specific primers, one of the most commonly used genetic markers for fungal identification [23]. The obtained sequences were analysed using the NCBI BLASTN tool, considering percentage similarity, E-value, and query coverage. BLAST analysis indicated that strain SA1 showed high sequence similarity to *A. niger* (**Fig. 14**). The sequence was deposited in the GenBank database and the unique accession numbers were retrieved (OK176530). Phylogenetic analysis was carried out using the neighbor-joining method [20] in MEGA 12 [19], resulting in an optimal tree with a total branch length of 0.324. Bootstrap support values derived from 1000 replicates were displayed at each branch node, reflecting the reliability of clustering among taxa. Evolutionary distances were calculated using the Maximum Composite Likelihood method [24], involving 17 nucleotide sequences. Pairwise deletion was applied to ambiguous positions, comprising a final dataset of 606 positions.

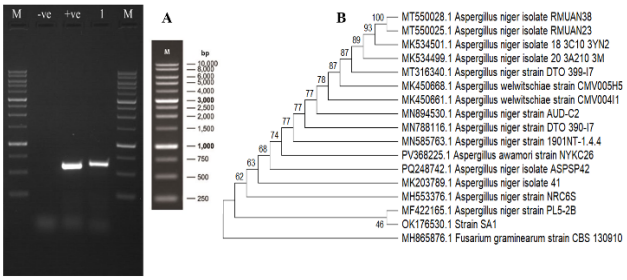

Fig. 14 Phylogenetic of *Aspergillus niger* strain SA1 via ITS DNA sequencing. (A) shows a single band in 1.5% electrophoresis gel (B) Phylogenetic tree construction of *Aspergillus niger* strain SAI using neighbour-joining method.

DISCUSSION

Most studies on phosphate-solubilising fungi (PSF) have focused on their isolation from cropping soils, such as teff [25], haricot bean [6], agricultural lands [26], and Himalayan soils [27]. In this study, a high incidence of *Aspergillus* species was recorded in spoiled rice, which aligns with previous reports implicating *Aspergillus* in food spoilage, particularly in grains [28]. *A. niger* is frequently associated with food product spoilage, especially in warmer environments, where it readily contaminates both field-grown and stored foods. *A. flavus, A. niger,* and *A. fumigatus* are known to inhabit parboiled rice [29]. These fungi are also abundant in indoor environments, where airborne spores can contaminate food production areas. Additionally, a study by Ewoh et al. [30] isolated *A. niger* from propanil-contaminated rice farms, further highlighting its prevalence in rice cultivation environments. This finding supports earlier reports identifying *A. niger* as a common contaminant in mold-damaged rice.

There has been growing interest in isolating *A. niger* from food waste due to its ability to produce variety of enzymes and organic acids, which are useful in industrial and agricultural contexts. Utilizing food waste as a substrate for cultivation of *A. niger* offers a sustainable approach in managing waste while recovering valuable resources. *A. niger* can transform food waste into commercially valuable goods, including enzymes, citric acid, and other bioactive components through fermentation [31]. This strategy mitigates the environmental consequences of food waste while promoting the circular economy by converting waste into a valuable resource. Furthermore, isolation of *A. niger* from food waste has demonstrated significant potential for biofertilizer development. Previous research has demonstrated enhanced P solubilisation in composts derived from agricultural residues inoculated with *A. niger*, which also boosts soil fertility and promotes plant growth [32]. *A. niger* is recognised for its robust P-solubilizing capability and is also characterised as an efficient K solubilizer making it a promising candidate for dual-function biofertilization [2,18]. These capabilities highlight the importance of isolating *A. niger* from food waste sources to promote more sustainable agriculture methods.

The PSI values recorded in this study correspond closely with previous data on PSF. For example, *Aspergillus* sp. have been shown to have PSI values of 2.58 ± 0.04 in which indicate a strong solubilisation capabilities [33]. Similarly, *A. niger* strains obtained from Arctic regions showing a similar effectiveness with isolates SE1 and SA1, with one strain reported to release as much as 285 µg/mL of soluble P [34]. These parallels indicate a promising P solubilising capacity of SE1 and SA1 underscoring their potential for further advancement as biofertilizer agents. The variations in PSI values among different isolates are probably affected by the types and quantities of organic acids produced, together with enzymatic activity and the metabolic pathways engaged in P solubilisation. Several studies have shown *A. niger* as an effective producer of citric and oxalic acids, which significantly contribute to the breakdown of insoluble P compounds via acidification and chelation mechanisms [2].

The presence of distinct P solubilising groups among the fungal isolates indicates inherent genetic and physiological variations that may influence their efficacy in different environmental conditions. Supporting this, [35] [6] shown that the solubilisation index (SI) of PSF varied from 1.10 to 3.05 for *Aspergillus* and *Penicillium* species isolated from various plant rhizospheres when incubated at temperatures between 25°C to 28°C. Similarly, [36] also reported that SI values of 2.42 for *Penicillium italicum* and 3.15 for *A. niger*. Many studies revealed that the differences in P solubilisation frequently because of the differences in the types, amounts, and organic acids secretion by the fungal isolates. According to [37] [37], the production of organic acids is crucial in determining the solubilisation efficiency of fungal isolates. In this study, the higher P solubilisation observed in strains SA1, SE1, SC2, and SH7 may be linked to their ability to efficiently metabolise carbon sources and excreting organic acids that facilitate the dissolution of insoluble P compounds [38]. Various fungal strains exhibit different abilities to synthesise specific acids such as gluconic, citric, oxalic, and lactic acids, which are recognised for their capability to chelate cations such as Ca^{2+}, Fe^{3+}, and Al^{3+}, thereby enhancing the availability of P in the medium [39]. Overall, the results show that SE1 and SA1 are the most efficient P solubilizers, as they have higher PSI values than other isolates. Their strong solubilisation capacity underscores their potential as a biofertilizer that could help in P availability in agricultural soils.

The Pearson correlation analysis between P solubilisation and pH levels on Day 3 showed a negative correlation ($r = -0.951$, $p = 0.000$), in which explaining the increasing of P solubilisation correlated with decreasing in pH. This finding supports the hypothesis that acidification is a vital way for P release from insoluble sources, possibly due to the production of organic acids by fungal isolates [40]. The decline in pH mostly because of the excretion of low molecular weight organic acids, which release protons (H^+) into the medium, consequently making the environment more acidic and helps the degradation of P complexes [4]. The ability of SA1 and SE1 in lowering the pH shows their ability in organic acid synthesis. These observations align with the findings of Kaur & Reddy [41], which

showed that PSF capable of producing a lot of gluconic and oxalic acids demonstrate enhanced acidification and thus, increased solubilisation efficiency. The KSI values in this study are consistent with other research on fungal K solubilisation. Sheng & He [42] discovered that *A. niger* strains isolated from K-rich soils had KSI values between 2.10 to 3.30, which closely match with those documented for isolates SA1, SF1, and SC2 in this study. Similarly, Verma et al. [43] documented comparable values ranging from 2.20 to 3.50 for *A. niger* strains isolated from rhizospheric soils, further reinforcing the robust K-solubilising capacity of isolate SA1. These parallels underscore the capacity of isolate SA1 as a highly efficient solubilising strain and support its potential as a biofertilizer agent.

The high K solubilisation efficiency observed in certain fungal strains is mostly due to their capacity to excrete organic acids particularly citric, oxalic, and gluconic acids which are crucial for the decomposition of insoluble K-bearing minerals into forms that are accessible to plant [44]. The solubilisation process is affected by various factors, including the type, concentration, and diffusion rate of these acids, which can differ markedly among fungal strains [45]. The high KSI values observed for isolates SA1 and SF1 indicate that this fungus may possess the ability to generate greater volume or more effective combinations of potassium-chelating organic acids, thereby improving their solubilisation efficiency. In contrast, strains like SB3 and SG5, which have lower KSI values, may release fewer acids or have a limited ability to diffuse acids, which resulting lesser K release. Considering that K is important for some of the plant physiological activities such as enzyme activation, photosynthesis, and osmoregulation, the K availability is vital for optimal plant growth and productivity [46]. The strong solubilisation showed by SA1 and SF1 highlights their potential application in agriculture, especially in K-deficient soils. Moreover, the existence of distinct acidic halos surrounding fungal colonies on agar plates provide visual confirmation of active organic acid generation, hence reinforcing their function in K solubilisation [47].

The results show that the fungal strains SA1 and SF1 were the best in K solubilisation compared to other fungal strains. Their strong ability to release K shows that they could be great biofertilizer alternatives for making K more available in soils. The pH reduction during the process is only due to microbial activity, but also to a fundamental biochemical mechanism that underlying the solubilisation process. This acidification is important for the decomposition of minerals like feldspar and mica, which necessitate low pH conditions to release K^+ ions into the surrounding environment [42]. Conversely, the control setup without fungal inoculation had a higher pH (6.90) and showed minimal K solubilisation, with a release of only 14.77 µg/mL. Thus, confirming that microbial activity is required to facilitate solubilisation. This further supports the hypothesis that fungal-mediated acidification is a key driver of K mobilization from insoluble sources.

The pH reduction can be attributed to various organic acids secreted by the KSF isolates. Moreover, the organic acid formation appeared to be the critical process for K solubilisation, as indicated by the acidity of the culture supernatant. The production of organic acids into the environment by microbes acidifies the niche, leading to protonation and acidification of the mineral K, resulting in the release of K ions from the mineral [44]. However, acidification does not seem to be the primary process of solubilisation, as the ability to lower pH does not always correspond to the ability to dissolve mineral K [48].

Apart from organic acids secretion, fungi also actively pump H^+ ions into the extracellular medium through proton pumps to maintain their internal pH balance. During nutrient metabolism, fungal cells exchange cations (e.g., K^+, Na^+, Ca^{2+}, Mg^{2+}) with H^+ ions, leading to further acidification of the environment. This exchange plays a crucial role in K^+ solubilisation, as it destabilizes K minerals and enhances the dissolution process [34]. Fungal isolates exhibiting the greatest pH reduction are often the most metabolically active in terms of nutrient cycling. SA1, which had the lowest pH (3.43), also exhibited the highest K solubilisation (78.90 µg/mL), indicating that it is an efficient acid-producing strain. Conversely, isolates with higher pH values (e.g., SC2 = 3.70, SG5 = 3.73) showed moderate K solubilisation, suggesting that their acid production was relatively weaker, leading to less efficient mineral dissolution.

Gluconic acid, which was found in the highest concentration (10.09 mg/mL), is a well-documented P solubilising compound that acts through chelation of Ca^{2+} ions in tricalcium phosphate ($Ca_3(PO_4)_2$), leading to the release of soluble phosphate (HPO_4^{2-}) [38]. While, citric acid, which was detected at 5.85 mg/mL, further enhances P solubilisation by chelating Fe^{3+} and Al^{3+}, which commonly form insoluble P complexes in soil [39]. Meanwhile, oxalic acid (4.74 mM) contributes to P release by dissolving P bound metal oxides, further enhancing P bioavailability [40]. The reaction for P solubilisation via acidification can be represented as:

$$Ca_3(PO_4)_2 + 2H^+ \rightarrow 3Ca^{2+} + 2HPO_4^{2-}$$

The strong negative correlation between pH reduction and P solubilisation confirms that acid-producing fungal isolates have a greater capacity for nutrient mobilization, making SA1, SE1, and SC2 the most effective P solubilising candidates. A similar trend was observed in K solubilisation, where SA1 exhibited the highest K release (78.90 µg/mL) at the lowest pH (3.43), followed by SE1 (51.20 µg/mL, pH 3.57) and SD4 (50.63 µg/mL, pH 3.59). The production of gluconic acid (4.58 mg/mL) and citric acid (2.65 mg/mL) was closely associated with higher K solubilisation, while oxalic acid (0.71 mg/mL) and lactic acid (0.38 mg/mL) were detected in lower concentrations. The solubilisation of K occurs primarily through the breakdown of potassium-bearing minerals such as feldspar and mica via acid leaching [49]. Organic acids such as gluconic and citric acid dissolve K-containing silicate minerals by displacing K^+ ions through proton exchange and chelation mechanisms [38]. The reaction for potassium release from feldspar follows this process:

$$KAlSi_3O_8 + H^+ \rightarrow K^+ + Al^{3+} + SiO_2 + H_2O$$

Gluconic acid plays a major role in both P and K solubilisation, but its concentration was significantly higher in P solubilisation (10.09 mg/mL) compared to K solubilisation (4.58 mg/mL). This suggests that P solubilisation requires stronger chelating acids to break down P bound minerals, whereas K solubilisation mainly involves proton exchange mechanisms [4]. While citric acid was also found in higher concentrations for P solubilisation (5.85 mg/mL) compared to K solubilisation (2.65 mg/mL), reinforcing its role in chelating Fe^{3+} and Al^{3+} to free P from soil complexes. However, it still plays a big role in K solubilisation by dissolving feldspar minerals [50]. Oxalic acid (4.74 mg/mL) was more relevant for P solubilisation, as it effectively dissolves iron- and aluminum-bound P, but its contribution to K solubilisation (0.71 mg/mL) was minimal, indicating that oxalic acid is not as effective in K release. Moreover, the absence of malic and tartaric acids in both P and K solubilisation suggests that these acids are not actively

involved in nutrient solubilisation processes. Instead, they are more commonly associated with metal detoxification mechanisms [39]. Results showed that strain SA1 is closely related to other *Aspergillus niger* isolates, particularly strains MF422165, MH553376, and MK203789, as identified by their GenBank accession numbers. These strains were previously classified as *A. niger*. This similarity and in addition to the molecular data that confirmed strain SA1 belongs to the same gene as *A. niger*. To support this identification, a phylogenetic analysis was done based on alignments of multiple sequences and using the neighbor-joining method, the common methods that are applied for the construction of phylogenetic trees [51].

The numbers given next to the branches represent the percentage of replicate trees in which the related taxa were grouped in the bootstrap test with 1000 replicates. The final dataset and all evolutionary analyses were performed using MEGA 12 [19]. As can be seen from the phylogenetic tree in **Fig. 14 (C)**, strain SA1 (OK176530) clustered in close relationship with other *A. niger* strains, placing it within the *A. niger* clade. The inclusion of *Fusarium graminearum* (MH865876) as an outgroup provided an additional validation step, thus also further supporting a distinct phylogenetic position of strain SA1 within the *A. niger* lineage [52]. The findings of this study confirm that the fungal strain SA1 is identified as *Aspergillus niger* which exhibits phosphate (P) and potassium (K) solubilising activity. Further studies are needed to evaluate its in planta efficacy under field conditions and to assess its effectiveness in enhancing plant growth.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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