# Isolation and Characterization of Phosphorus-Solubilizing *Aspergillus spp.* from Soils of Maiduguri, Borno state.

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**Abstract**

Soil samples were collected from different locations within Maiduguri metropolis for isolation and characterization of phosphorus-solubilising *Aspergillus spp.* Serial dilutions were carried out for isolation of *Aspergillus spp* on Potato Dextrose Agar and light microscope was used for morphological identification of the isolates. Isolates obtained were further inoculated on Pikovskaya medium to determine their solubilisation capacity. Molecular detection of Glucose Dehydrogenase (gdh) gene was performed to detect the gene coding region responsible for the production of gluconic acid; which is responsible for most solubilisation activity in many *Aspergillus* strains. Universal oligonucleotides ITS1 and ITS4 were used to amplify the 5.8S-ITS region of *Aspergillus spp.* isolates. The isolates were sequenced and their nucleotide sequences were deposited in GenBank with accession numbers as follows: OM463600 *Aspergillus niger* (567 bp), OM463601 *Aspergillus quadrilineatus* (538 bp), OM463602 *Aspergillus terreus* (576 bp), OM463603 *Aspergillus flavus* (563 bp) and OM463604 Aspergillus nidulans (535 bp). A Phylogenetic tree was constructed using NCBI database to evaluate specie similarities. Aspergillus terreus was recorded to be the only strain capable of displaying solubilisation ability on PVK medium and also positive for gdh gene detection; this proves its potential to be used as phosphorus-solubilising biofertilizer.

**Keywords**: Phosphorus -solubilising *Aspergillus* spp., 5.8S-ITS sequencing

## Introduction

Aspergillus is an important filamentous and ubiquitous fungi belonging to the phylum Ascomycota commonly isolated from soil and plant debris. It plays important roles in agriculture in degrading crop debris and in solubilizing soil nutrients (Choudhary *et al.*, 2016). Phosphorus-solubilising activities of these microbial populations have attracted their use as biofertilizers and as a result, they are receiving more attention by agronomists than microbiologists (Khan *et al.* 2007). Some *Aspergillus* spp. such as *Aspergillus tubingensis*, *Aspergillus fumigatus*, *Aspergillus terreus and Aspergillus awamori*, have been widely reported as phosphorus solubilizers; their phosphorus-solubilizing capability and stability has been reported to be superior to that of phosphorus-solubilizing bacteria (Sharma *et al.*, 2013; Mendes *et al.*, 2014). Phosphorus-solubilizing microorganisms are recognized as a solution to the challenges in phosphorus fertilization management due to their abilities to mobilize phosphorus from recalcitrant sources (Mendes *et al.*, 2014). Phosphorus (P) is the second most important and most limiting macro nutrient in agricultural soils required for many physiological mechanisms after nitrogen. It is one of the most essential macro-elements required by crops in large quantity relative to the supply of available P in many soils for cell division, nitrogen fixation in legumes and development of new tissue (including photosynthesis, energy and sugar production). Plant mineral nutrition mainly depends on the P content of soil and can only be assimilated as soluble P. The fate of P fertilizer when applied to the soil has been an important priority in the field of Soil Science (Riskin *et al.*, 2013).The total P present in soils constitutes about 0.5% out of which only 0.1% is available to plants (Khan *et al.*, 2010). The deficiency of P in turn severely restricts growth and yield in plants. The problems of P deficiency are generally addressed through the application of P fertilizers where maximum plant productivity is achieved, but this practice is expensive (Khan *et al.*, 2010). The repeated and uncontrolled applications of inorganic P fertilizers leads to the loss of soil fertility by disturbing microbial diversity and consequently reduces yield of crops (Gyaneshwar *et al.*, 2002; Khan *et al.*, 2010). Generally, roots absorb P in the form of orthophosphate (H2PO4- and HPO42-) through diffusion and can also absorb certain forms of organic P (Havlin *et al.*, 2006). The orthophosphates, $H_2PO^{4-}$ and $HPO_4^{2-}$, are the primary forms of P taken up by plants. The soil pH for optimum P availability is 6.5, when the soil pH is

less than 7.0, $H_2PO^{4-}$ is the predominate form in the soil. At high or neutral pH, P reacts with calcium to form minerals, such as apatite. Under acidic conditions, P may react with iron and aluminium to form minerals, such as strengite and varescite respectively (Brady and Weil, 2002). A variety of fungi have been isolated and characterized for their capability to solubilise inorganic and unavailable forms of P to available forms of P which are readily taken up by the plants. These phosphorus-solubilizing microorganisms (PSMs) are known to be used as fertilizers to increase P uptake and plant growth (Wang *et al*., 2007). Moreover, P solubilizing fungi have been observed to secrete more acids than bacteria and display greater P solubilizing activity (Sharma *et al*., 2013). Inoculation of P solubilizing fungi into soil has been reported to increase the level of available P in the soil by mobilizing P fixed in soil particles which results in improved plant growth (Nagaraju *et al*., 2012). These PSMs render insoluble P into soluble form through the process of acidification, chelation, and exchange reactions (Saxena *et al*., 2013). The main mechanism for the solubilization of inorganic P is the production of organic acid. In terms of P solubilization, fungi are more efficient than their bacterial counterpart on precipitated agar and in liquid medium and they have the ability to reach greater distances in soil more easily than bacterial strains (Saxena *et al*., 2013). The present study aims to isolate, characterize and screen P -solubilising Aspergillus spp. from soils of Maiduguri metropolis using morphological and molecular techniques.

## Materials and Methods

### Soil sample collection

Soil samples were collected from different locations within Maiduguri metropolis with a hand trowel at a depth of 0-20cm and put into sterile polythene bags for microbial isolation.

### Isolation and screening of fungi

All soil samples were subjected to serial dilution and spread plated on Potato Dextrose Agar (PDA) medium (treated with chloramphenicol) for isolation of fungi and further screened for solubilization using Pikovskaya (PVK) medium at the University of Maiduguri Microbiology laboratory. 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 of isolates for phosphorus-solubilization

Following the appearance of fungal colonies on PDA medium, these isolates were identified morphologically using Olympus CH-2 light microscope using X40 and X100 magnification and following the identification manual by Samson *et al*., (2004). All strains identified as Aspergillus spp. were recorded and further sub-cultured on PVK medium. The identified Aspergillus spp. isolates were screened for their phosphate solubilizing ability by point inoculation on Pikovskaya Agar (glucose 10g; $Ca_3(PO_4)_2$, 5g; $(NH_4)_2SO_4$, 0.5g; NaCl 0.2g; $MgSO_4*7H_2O$ 0.1g; KCl 0.2g; yeast extract 0.5g; $MnSO_4*H_2O$, 0.002g; and $FeSO_4*7H_2O$, 0.002g). After 7 days of incubation at 28±2°C, formation of a partial halo zone around the fungal growth on PVK agar were observed. The degree of phosphate solubilization was observed by rapid growth of isolates on the medium.

### Genetic characterisation of *Aspergillus* spp.

### DNA extraction and quantification

DNA of fungal mycelia of selected isolates were extracted using Zymo Quick-DNA Fungal/Bacterial Miniprep extraction kit at Inqaba Biotech. West Africa, Nigeria using the manufacturer's instructions. DNA were quantified using Nanodrop Spectrophotometer (NanoDrop One Microvolume UV-Vis Spectrophotometer Thermo Scientific™, USA).

### PCR amplification

The gene coding section in the 18S rRNA was amplified by PCR using specific primers for gdh gene; forward primer gdh-f [5'- ATGTATCCGGATTTAAAAGG -3'] and reverse primer gdh-r [3'- TTAACCGCGGCCTGCCTGG -5'] by Polymerase Chain Reaction (Chauhan et al., 2016) respectively. Universal oligonucleotide primers ITS1 [TCCGTAGGTGAACCTGCGG] and ITS 4 [TCCTCCGCTTATTGATATGC] were used to amplify the 5.8S-ITS region in Aspergillus spp. isolates. Mastercycler nexus gradient (Eppendorf Germany) was used for PCR amplification using One Taq Quick Load 2X Master Mix with Standard Buffer (New England Biolabs, USA), 10 µl, genomic DNA (10-30ng/µl) 1 µl, forward primer (10µM) 1 µl, reverse primer (10µM) 1 µl and Nuclease free water (Quality Biological Inc.) 7 µl using the following PCR conditions for denaturation, annealing and elongation;

| | |
|---|---|
| 50°C for 30 sec | |
| 53°C for 30 sec | 35 Cycles |
| 55°C for 30 sec | |
| 68°C for 1 min | |
| 68°C for 10 min | |
| Hold at 4°C | |

#### Agarose gel analysis

The integrity of the PCR amplicons were visualized on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with Safe View Fire Red DNA Dye and visualized using E-Box gel documentation system (Vilber Lourmat, Italy).

#### Sequence Analysis of Aspergillus Isolates

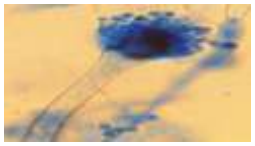
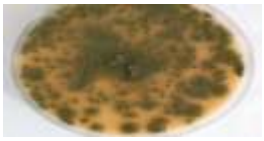
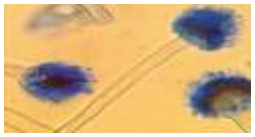
The sequences were evaluated at GenBank database using Basic Local Alignment Search Tool (BLAST) server at NCBI (https://blast.ncbi.nlm.nih.gov/blast.cgi) and the nucleotide sequences were further deposited in GenBank database.

## Results and Discussion

### Inoculation of *Aspergillus* spp. on PVK medium

Isolates grown on PDA medium were directly inoculated on PVK medium under sterilized condition. They all grew exponentially with Sample C (*Aspergillus terreus*) showing a partial zone of solubilization.

*Table 1: Growth of Aspergillus spp. isolates on Pikovskaya (PVK) medium*

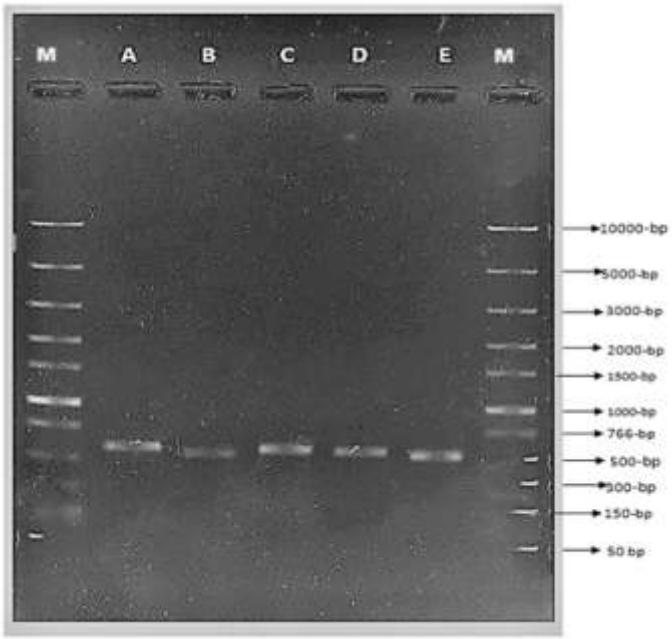
| Name ofisolates | Macroscopic obverse view | Macroscopic reverse view | Microscopic features (X40) |
|---|---|---|---|
| Sample A: *Aspergillus niger* |  |  |  |
| Sample B: *Aspergillus quadrilineatus* |  |  |  |
| Sample C: *Aspergillus terreus* |  |  |  |
| Sample D: *Aspergillus flavus* |  |  |  |
| Sample E: *Aspergillus nidulans* |  |  |  |

**Halo zone of solubilization on Pikovskaya medium (PVK)**

Pikovskaya agar medium was modified by Sundara and Sinha, 1963 for culturing phosphorus-solubilizing microorganisms. Important components of PVK medium include; glucose which serves as energy source and the different salts and yeast extract supports the growth of the organisms. A zone of clearance may be observed after spot inoculation on PVK medium, however, most strains produce no halo zone but rather causes colour change of PVK medium. According to Pande, (2017), yellowish/brownish colouration (as observed in plates A,B,D and E) of PVK medium is as a result of citric acid production while medium appearing to be cream coloured (as observed in plate C) is as a result of gluconic and formic acid production. Observations made from the Aspergillus strains inoculated on the PVK medium (in Table 1) shows a partial zone of solubilization in sample C; this observation was also reported by Rosling *et al*. (2007). According to findings by Ceci *et al*. (2008), it was confirmed that *in vitro* screening of fungi performed by measuring the solubilization halo only is inefficient in selection of phosphorus-solubilizing fungal strains. Ceci *et al*. (2008) also reported that; the halo zone produced as a result of solubilization in PVK plates represent weak indicators of the real fungal potential to make P bioavailable to plants in soil. Mehta *et al*. (2019) also reported that detection of halo zone on agar medium is not an authentic test for P-solubilization. According to Ceci *et al*. 2008, saprotrophic fungi can modulate uptake and availability of P by different mechanisms. PSMs can store P in mineral forms that precipitate in their mycelium and assimilate P in organic forms that could be released after the death of the same fungus. According to; Cerezine *et al*., 1988; Reddy *et al*., 2002; Wakelin *et al*., 2004; Maurya and Kumar, 2006; Qiu and Lian 2012 and Posada *et al*., 2013; the species A. *niger*, showed a negative correlation between the medium's pH and solubilization of P without producing halo zones.

Universal oligonucleotides ITS1 and ITS4 were used to amplify the 5.8S-ITS region in *Aspergillus spp.* isolates. As shown on the gel image in Plate 1. The amplicons were further sequenced to determine their exact species and are found to be; *Aspergillus niger* (567-bp), , *Aspergillus quadrilineatus* (538-bp), *Aspergillus terreus* (576-bp), *Aspergillus flavus* (563-bp) and *Aspergillus nidulans* (535-bp) with their corresponding accession numbers; OM463600, OM463601, OM463602, OM463603 and OM463604 as shown in Table 2 respectively. A phylogenetic tree was constructed using the NCBI database to evaluate specie similarities as shown below.

As reported by Yong et *al*., (2018), glucose dehydrogenase encoded by "gdh" gene is the most important enzyme of inorganic phosphorus-dissolving metabolism, catalysing the oxidation of glucose to gluconic acid. The insoluble P in the sediment is converted into soluble phosphate through the activities of gdh. Among the acids produced by microorganisms, gluconic acid (GA) is one of the main and most important acid when it comes to P solubilization. phosphorus-solubilising *Aspergillus spp.* release gluconic acid from the outer surface of cytoplasmic membrane which is the site of direct oxidation pathway. Organic acid forms a complex and chelate cations linked with P which releases soluble P into the soil through ligand exchange reactions with phosphate anion (Bolan et *al*., 1996). In Plate: 2, among all the isolates tested for the presence of the gdh gene coding region using the molecular techniques, only sample C (*Aspergillus terreus*) was positive for the gdh gene with approximately 800 -bp which is also evident from the partial zone of solubilization observed on the plate inoculated by *Aspergillus terreus* strain on PVK medium as shown on the reverse view of the plate in Table 1 as a result of the gluconic acid activity of the isolate. This result is similar to the work of Son et *al*., (2006) and Chauhan et *al*., (2016).




*Gel Electrophoresis Results*

***Plate 1:*** *18S rRNA gene fragments using ITS1 and ITS4 of samples A, B, C, D and E. Band pattern of different Aspergillus isolates Lane M = marker; lane 1 = A. niger; lane 2 = A. quadrilineatus; lane 3 = A. terreus; lane 4 = A. flavus; lane 5 = A. nidulans; and lane M=marker.*

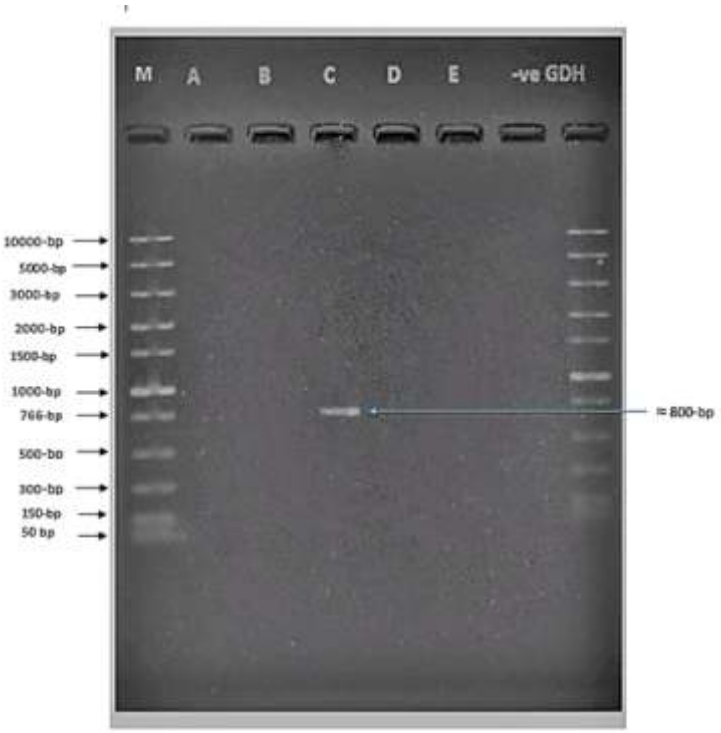



***Plate 2:*** *Band pattern of gdh gene fragment (approx.800bp) from sample C (Aspergillus terreus). Lane M = marker; lane 1 = A. niger; lane 2 = A. quadrilineatus; lane 3 = A. terreus; lane 4 = A. flavus; lane 5 = A. nidulans; and lane –ve =Negative control*

*Accession numbers were generated for all isolates as shown below;*
*Table 2: Accession numbers of isolates submitted to GenBank*

| S/N | Samples | Accession number | Name of Isolate |
|---|---|---|---|
| **1** | A | OM463600 | *Aspergillus niger* |
| **2** | B | OM463601 | *Aspergillus quadrilineatus* |
| **3** | C | OM463602 | *Aspergillus terreus* |
| **4** | D | OM463603 | *Aspergillus flavus* |
| **5** | E | OM463604 | *Aspergillus nidulans* |

*The table below shows a summary of the BLAST results for each isolate and their corresponding reference.*

***Table 3: BLAST results of ITS regions for all isolates.***

| Samples | Organisms of significant sequence alignment | Accession numbers | Percentage query coverage | Percentage identity | References |
|---|---|---|---|---|---|
| A | *Aspergillus niger*; strain ND65 | MG659659.1 | 99 | 99.29 | Nleya *et al.*, 2017 |
| B | *Aspergillus quadrilineatus;* strain CT729 | KX056236.1 | 95 | 95.0 | Irdem, 2016 |
| C | *Aspergillus terreus ;* strain DTO 403-C9 | MT316343.1 | 99 | 100 | Piazza *et al.*, 2020 |
| D | *Aspergillus flavus;* strain GFRS9 | MT447477.1 | 98 | 99.82 | Yang, 2020 |
| E | *Aspergillus nidulans;* strain DTO 402-H2 | MT316339.1 | 99 | 99.25 | Piazza et al., 2020 |

**Phylogenetic analysis using neighbour joining tree**

Phylogenetic tree and diversity of 5.8S-ITS region for all five (5) isolates of *Aspergillus* spp. were compared with reference to Aspergillus strains. It shows the evolutionary similarities and relationships among isolates. Phylogenetic tree was generated using National Centre for Biotechnology information (NCBI) for neighbour-joining (using Juke's cantor analysis) a shown below;

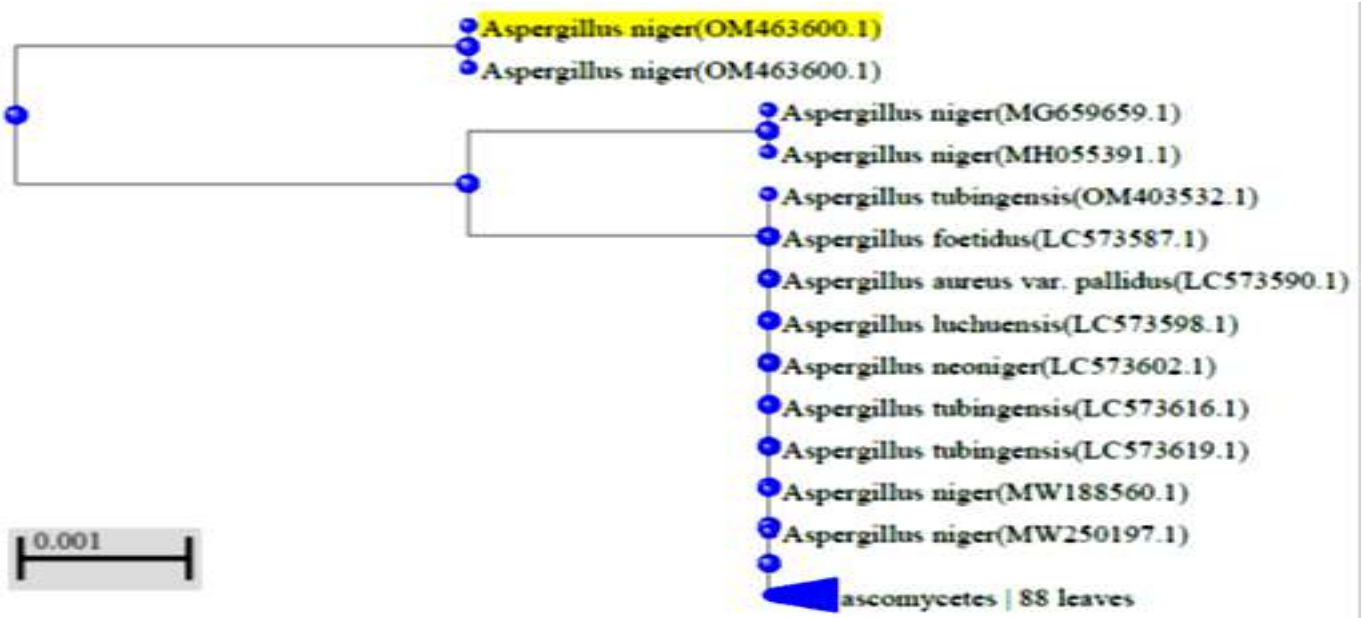



***Figure 1:*** *Neighbor-joining phylogenetic relationship of Aspergillus niger with closely related isolates (from GenBank) using ITS sequences*

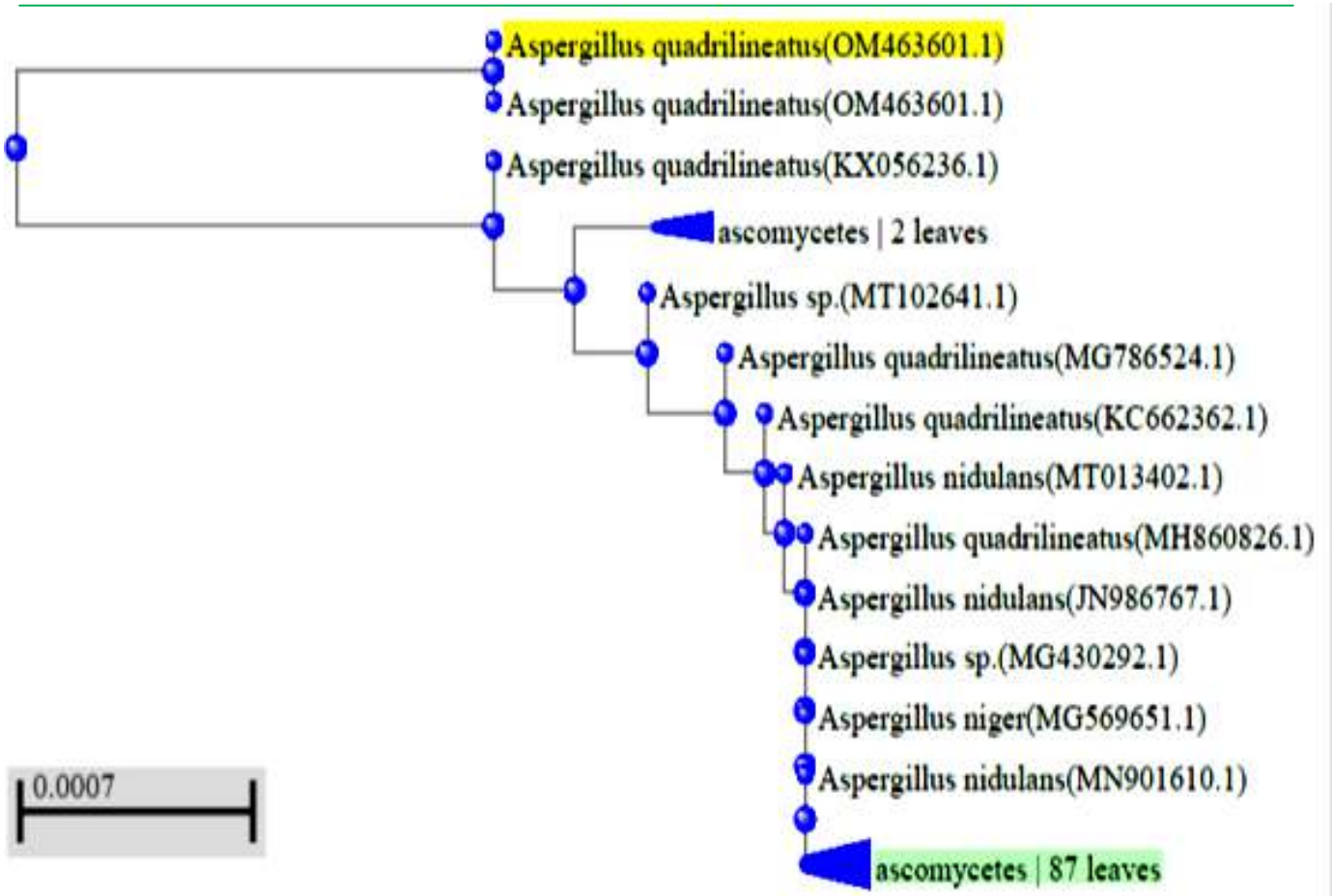



***Figure 2:*** *Neighbor-joining phylogenetic relationship of Aspergillus quadrilineatus with closely related isolates (from GenBank) using ITS sequences*




***Figure 3:*** *Neighbor-joining phylogenetic relationship of Aspergillus terreus with closely related isolates (from GenBank) using ITS sequences*

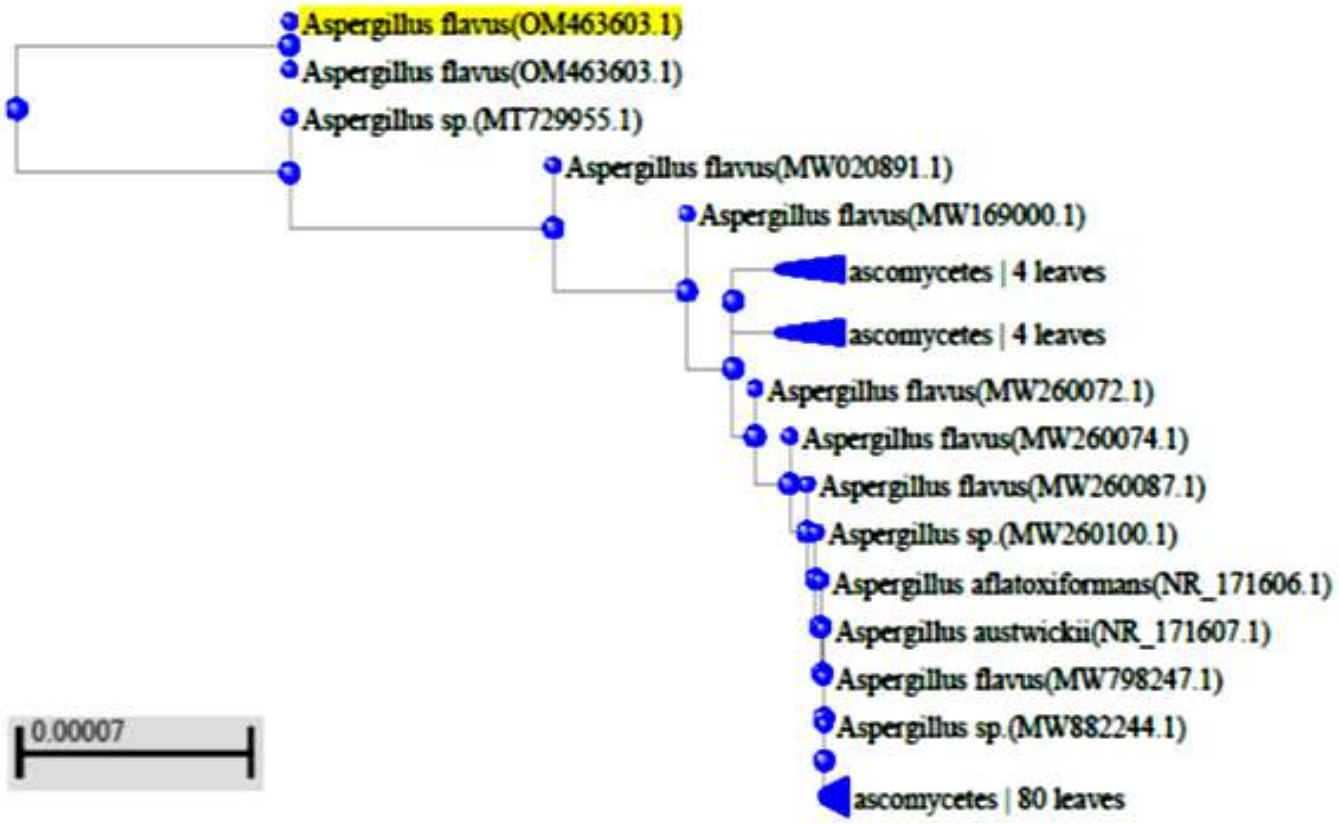



***Figure 4:*** *Neighbor-joining phylogenetic relationship of Aspergillus flavus; with closely related isolates (from GenBank) using ITS sequences*

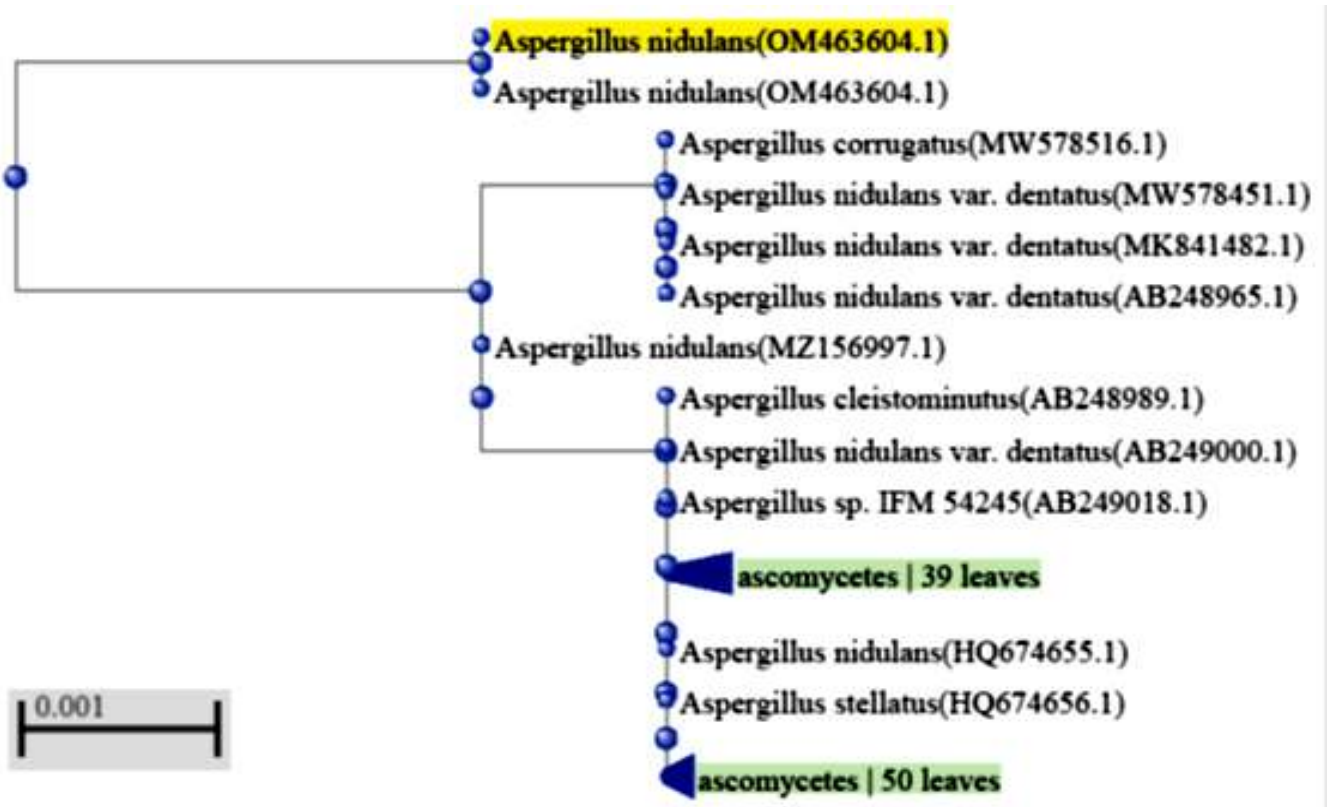



***Figure 5:*** *Neighbor-joining phylogenetic relationship of Aspergillus nidulans with closely related isolates (from GenBank) using ITS sequences*

## Conclusion and Recommendation

In conclusion, all isolates grown on PVK medium grew exponentially, among all the isolates tested on PVK medium, Aspergillus terreus was recorded to be the only strain capable of displaying zone of solubilization compared to the other isolates having reddish brown coloration. Aspergillus terreus was found to be positive for gdh gene detection. The isolates were sequenced using ITS1 and ITS4 and further deposited in GenBank. Extensive and consistent research efforts should be explored such as identifying and characterizing more phosphorus-solubilizing fungi for their ultimate application under field conditions. Genetic manipulation of such important genes contained in these strains should be explored through development of transgenic plants. The adoption of such methods will help improve utilization of P in soil by plants which can be achieved through identification of our indigenous microbial species, testing their potential and documenting these species for further research and development. By doing so, environmentally-friendly biofertilizers with lasting solution against fixation of P in the soil can be developed thereby reducing excessive use of chemical fertilizers in soil, minimizing cost and maximizing profit to farmers.

Declaration of interests

The authors declare that they have no conflict of interest.

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