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Isolation and characterization of rhizosphere soil associated phosphofungi from hybrid brinjal *Solanum melongena* L. var. MDU1

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The current study focuses on isolation and identification of potent filamentous fungi from the rhizosphere soils associated with high yielding hybrid brinjal (*Solanum melongena* var. MDU1) plants to solubilize tricalcium phosphate (TCP) under *in vitro* conditions using Pikovskaya (PVK) medium. A total of 68 fungal isolates were obtained from 15 rhizosphere soil samples from three *S. melongena* fields, and they demonstrated a clear halo zone of dissolved phosphate solubilization in PVK medium, indicating that these fungi had the requisite P-solubilizing ability. Among, maximum solubilization index (SI%) of TCP on PVK plates was recorded with the fungal isolate *Aspergillus* spp. (TSPS 2) with 2.3%, whereas the lowest of 0.9% was detected from the isolate *Trichoderma* spp. (TSPS 9). In addition, quantitative TCP solubilization in PVK broth inoculated with *Aspergillus* spp. (TSPS 2) revealed higher amount of P mobilization ranged between (216.43–490.56 $\mu g/mL^{-1}$) in different days of incubation. Similarly, found the greatest decline in pH from an initial pH of 7.0 to 4.5 at varied time interval. The current investigation found a variety of plant-soil-associated P-solubilizing fungi that could be used as biofertilizers.

Keywords: *Aspergillus* spp., crop plants, phosphate solubilization, rhizosphere soil

INTRODUCTION

Phosphorus (P) is naturally occurring and one of the most indispensable macronutrients next to nitrogen among 17 elements that are essential for the growth and development of plants. It is one of the major limiting factors for crop production on many tropical and subtropical soils (Yasser *et al*. 2014).

It accounts for 0.2% of plant dry weight and restricts plant growth and agricultural productivity (Pradhan and Sukla, 2006). A greater part of soil phosphorus, approximately 95–99%, is present in an insoluble form complexed with cations like iron (Fe), aluminum (Al), and calcium (Ca) that cannot be utilized by plants (Gizaw *et al*. 2017). Plant mineral nutrition is mostly determined by soil P content, which can be digested as soluble phosphate (Wang *et al*. 2018). Furthermore, P contributes significantly to photosynthesis, energy and sugar production, nucleic acid synthesis, N2 fixation in legumes, and plant disease resistance (Kanse *et al*. 2015).

Phosphorus is the kingpin of Indian agriculture, with a unique role in both traditional and alternative agriculture (Pradhan and Sukla, 2006). A large variety of microbial species have been identified in soil, particularly in the rhizosphere, where soil plays an important role in P solubilization (Yasser *et al*. 2014). Phosphate solubilizing microorganisms (PSM) use a unique process to transform these insoluble phosphates into soluble forms. That is, they carry out the acidification, chelation, exchange reaction, and gluconic acid synthesis processes (Elias *et al*. 2016; Sabbir *et al*. 2022). The organic acids and inorganic acids produced by bacteria convert tri-calcium phosphates into di- and monobasic phosphates, resulting in increased

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element availability to plants (Kanse *et al*. 2015; Elias *et al*. 2016). Different microorganisms produce different types and amounts of organic acids. The action of organic acid generated by soil microorganisms and the activity of the phosphatase enzyme is the primary mechanism of mineral phosphate solubilization. The production of these organic acids causes the microbial cell and its surroundings to become acidic. Phosphorus solubilizers not only provide phosphorus to plants, but also aid in plant growth by increasing the efficiency of accelerating the accessibility of other trace elements and synthesizing important growth-promoting substances (Mittal *et al*. 2008). They are also known to produce amino acids, vitamins, and growth-promoting substances such as indole-3-acetic acid (IAA) and gibberellic acid, all of which aid in plant growth (Nenwani *et al*. 2010). PSM is made up of bacteria, fungi, and actinomycetes that aid in the conversion of insoluble phosphate into a simple and soluble form of phosphate. Soil bacteria from the genera *Bacillus* and *Pseudomonas*, as well as fungi from the genera *Aspergillus* and *Penicillium*, are the most frequent (Diba *et al*. 2007). PSM solubilization is generally caused by the synthesis of organic acids by these organisms.

Fungi have a significant role in soil microorganisms. Fungi typically make up more of the soil's biomass than bacteria, depending on soil depth and nutrient levels (Gizaw *et al*. 2017). According to various scientists, about 0.1-0.5% of all fungi in the soil are P-solubilizing fungi. Because fungi in soil can travel longer distances than bacteria, they may be more important in soil P-solubilization than bacteria. P-solubilizing fungi (PSF), on average, produce more acids than bacteria and so have larger P-solubilizing activity. PSF in soils, particularly filamentous fungi such as *Aspergillus*, including *A. aculateus*, *A. awamori*, *A. niger*, *A. terreus*, and *A. tubingensis*, *Candida* sp., *Cladosporium* sp., *Cunninghamella* sp., *Curvularia lunata*, *Fusarium oxysporum*, *Humicola* sp., *Oideodendron* sp., *Phoma* sp., *Rhizoctonia solani*, *Rhizoctonia* sp., *Talaromyces funiculosus* as well as *Penicillium* (*P. canescens*, *P. citrinum*, *P. expansum*, *P. italicum*, *P. oxalicum*, *P. radicum*, *P. rugulosum*), have shown potential for solubilization of insoluble P compounds (Mittal *et al*. 2008; Pandey *et al*. 2008; Kanse *et al*. 2015; Elias *et al*. 2016; Gizaw *et al*. 2017; Sabbir *et al*. 2022). Microbial phosphate solubilization is often considered one of the parameters related to plant growth promotion. There are several reports regarding plant growth promotion due to inoculation of phosphate-solubilizing microorganisms under greenhouse as well as field conditions (Alori *et al*. 2017). Particularly, the addition of PSFs in the field has been reported to increase crop yield. Iman (2008) recorded that the PSF significantly increased the growth of soybean (*Glycine max*) plants. Li *et al*. (2021) suggested the isolation and inoculation of PSF such as *Penicillium oxalicum*, *Aspergillus brunneoviolaceus*, and *Aspergillus tubingensis* isolates, which significantly increased the growth parameters, and this could be an alternative bioformulation for the production of eggplant, *S. melongena*, under polluted soils.

Brinjal or eggplant (*Solanum melongena* L., family Solanaceae) is a popular vegetable crop grown for its edible fruit all over the world and is a good source of vitamins and minerals. Several studies have been reported, including the isolation of rhizosphere soil fungi, bacteria, endophytes in plant segments etc. (Onyia *et al*. 2015; Li *et al*. 2021). Only a few studies have been published on the isolation of phosphate solubilization fungi from brinjal or eggplant (Onyia *et al*. 2015). In recent times, dependence on inorganic chemical fertilizers for crop production and phosphorus deficiency in soil have created several undesirable effects on soil alkalinization, fertility loss, reduction in microbial density, and reduced crop yield (Kanse *et al*. 2015; Alori *et al*. 2017). Thus, one alternative strategy for reducing the use of synthetic fertilizers is to use resident microorganisms (including bacteria, fungi, and actinomycetes) that are capable of solubilizing insoluble nutrients in the soil and are more environmentally friendly and competitive than non-endophytic microbes (Wakelin *et al*. 2004). Therefore, P-solubilizing fungi are crucial for supplying P to plants and enabling the long-term application of P fertilizers. Given the foregoing, it was important to isolate and identify native soil fungal genera with high phosphate solubilization

potential so they could be used as bioinoculants in agricultural fields to encourage sustainable farming in nearby fields.

MATERIALS AND METHODS

Study site and collection of soil sample

The study was conducted in agricultural field located on Thulaiyanur village, Thirumayam, Pudukkottai district (10° 22' 23.66" N; 78º 70' 16.51" E; 754 m a.s.l.), Tamilnadu, India. Rhizosphere soil samples belonging to Brinjal (*Solanum melongena* L. var. MDU1) were collected at a depth of 10-15 cm in and around the root system of fifteen randomly selected individuals of study plants at a distance of 10 m apart during fruiting bearing stage of the plants between December-February 2023. Sampling was done three times (BSS1, BSS3 and BSS2) at month intervals and five replicate samples were obtained from each time. The collected soil samples from each sampling month approximately 250 g was pooled and placed in individual polythene bags, labeled and taken to the laboratory, and stored at 4 °C until use in subsequent experiments. One part of soil sample was air dried in shade for the analysis of soil properties.

Analysis of soil properties

Soil properties were assessed in three subsamples. The collected rhizosphere soil texture was analyzed by Bouyocos hydrometer method (Allen *et al*. 1974). Soil pH and Electrical conductivity (EC) were determined at room temperature in the aqueous solution of soil: water (1:1, v: v) using digital pH and conductivity meters (ELICO, India). Soil organic carbon (OC) was measured by a rapid titration method of Walkley and Black (1934), and total nitrogen (N), available phosphorus (P) and exchangeable potassium (K) were analyzed after extractions with ammonium acetate were determined by Jackson (1971).

Isolation and screening of phosphate solubilizing fungi (PSF)

Collected *S. melongena* rhizosphere soil samples were used for the isolation of phosphate solubilizing fungi (PSF) on Pikovskaya's (PVK) agar medium, containing the following (g/L): 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.02 g NaCl, 0.02 g KCl, 0.003 g $FeSO_4.7H_2O$, 0.003 g $MnSO_4.H_2O$, 5 g $Ca_3(PO_4)_2$, 10.0 g glucose, 0.5 g yeast extract, 15.0 g agar, and 1000 mL distilled water (Pikovskaya, 1948). The medium was autoclaved at 121ºC for 15 minutes; about 15 mL of the sterilized molten agar medium was poured into each petri dish (90 mm diameter) and supplemented with 10 . g/mL^{-1} Streptomycin sulfate to inhibit bacterial growth and allowed to solidify before inoculation.

Approximately 10 g of soil sample was transferred to an Erlenmeyer flask containing 90 mL of sterile water and shaken at 120 rpm for 10 min. Subsequently, a series of 10-fold dilutions of the suspension were prepared like aliquots of 1 mL of the supernatant from the sample was transferred to 9 mL of sterile distilled water into test tubes and serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. From the serially diluted soil suspension (10^{-4}, 10^{-5}, and 10^{-6}), 0.2 mL aliquots were transferred and spread plated on Pikovskaya's agar plates and incubated at 25 °C – 28 °C for 2–7 days. After incubation, fungal colonies showing clear halo zones around the colonies were identified as PSF. The pure cultures were preserved on Potato Dextrose Agar (PDA) slant at 4 °C for further investigation (Elias *et al*. 2016).

Identification of PSF

PDA was used to accelerate the growth rate and the production of enough conidia as reported by Diba *et al*. (2007). The characteristics of isolated PSF cultures were compared with mycological identification keys and taxonomic description (Stevens, 1974).

Determination of phosphate solubilization index

To assess the TCP solubilization efficiency, the isolated fungi were inoculated (5.0 mm mycelial disk) on PVK agar containing 5 g / L of $Ca_3(PO_4)_2$ at pH 7.0. The inoculated culture plates (in triplicate) were incubated at 28 ± 2 ºC for 7 days. Uninoculated PVK agar plate served as control. A halo/clear zone around the fungal colony

represented phosphate solubilization and was measured. For qualitative estimation of phosphate solubilization in solid medium, the solubilizing index (SI%) was calculated according to Wakelin *et al*. (2004) as follows, Solubilization index (SI%) = Colony diameter + Clearing zone / Colony diameter × 100.

The fungus with higher phosphate solubilization was selected for further studies.

Evaluation of quantitative phosphate solubilization efficiency in liquid broth

Quantitative estimation of phosphate solubilization was carried out using Erlenmeyer flasks containing 100 mL PVK liquid broth supplemented with 0.5% tricalcium phosphate (TCP) in amounts equivalent to "P" 997 μg/mL. The initial pH of the medium was adjusted to 7.0 before sterilization. After sterilization, the medium was inoculated with the spore suspension of 10% (v/v) of *Aspergillus* spp. TSPS2 containing 10^6 spores / mL. Ten milliliters of sterile distilled water inoculated sample was treated as the control. Three replicates were maintained. Incubation was done at 28 ± 2 ºC in an incubator shaker (Sanco, India) at 120 rpm for 20 days. The amount of Pi released and pH in the broth flasks were estimated at different times (day 5, day 10, day 15, and day 20) in the presence of TCP. An aliquot of 5 mL culture supernatant was aseptically withdrawn periodically from each culture flask at 5-day interval. The cultures were filtered through Whatman number 1 filter paper and the filtrates were used for estimation of Pi released. The pH was measured with a digital pH meter (Deluxe pH meter-101). The amount of P-solubilized in culture supernatant was estimated using chlorostannous acid reduced molybdophosphoric blue colour method of Jackson (1971) and Elias *et al*. (2016) expressed as equivalent phosphate (μg/mL).

Statistical Analysis

All the experimental results related to qualitative and quantitative phosphate solubilization activity were expressed as mean ± SD.

RESULTS AND DISCUSSION

Rhizosphere soil properties

The rhizosphere soils of *S. melongena* var. MDU1 had a pH of 5.8, an EC of 0.17 dSm^{-1} and an organic C of 1.8% (Table 1). Total N, available P and exchangeable K of the soils were 95.12 kg/ha^{-1}, 7.5 kg/ha^{-1} and 196.45 kg/ha^{-1}, respectively. The studied field soils were sandy loam in nature.

Isolation and identification of PSF

In this study, a total of 68 fungal isolates were obtained from 15 rhizosphere soil samples of three adjacent brinjal (*S. melongena* var. MDU1) fields (Table 2) and these showed a clear halo zone of dissolved phosphate in solid PVK medium, which indicated that these isolates exhibited the desired P-solubilizing ability (Fig. 1). The pure cultured fungal species were identified on the basis of morphological characteristics (colony colour, reproductive structures and other phenotypic features based on identification manuals) revealed the 9 morphotypes belonging to 4 different general i.e. *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma* (Table 2). Majority of identified fungal species belonged to the Ascomycetous group (Table 2). Out of all, *Aspergillus* and *Penicillium* were found to be highest with 3 species each. Whereas, two and one species, were recovered from each of the genus- *Penicillium* and *Trichoderma*, respectively. The identified fungal morphotypes from *S. melongena* var. MDU1 rhizosphere *as* follows *Aspergillus niger* (TSPS1), *Aspergillus* spp. (TSPS 2), *Aspergillus flavus* (TSPS6), *Fusarium oxisporum* (TSPS3), *Fusarium* spp.1 (TSPS4), *Fusarium* spp.2 (TSPS5), *Penicillium citrinum* (TSPS7), *Penicillium* spp.1 (TSPS8), and *Trichoderma* spp. (TSPS9). Furthermore, fungal species *Aspergillus niger* (TSPS1) and *Fusarium oxisporum* (TSPS3) was found to all the examined brinjal fields, whereas *Aspergillus flavus* (TSPS6), *Fusarium* spp.2 (TSPS5) and *Trichoderma* spp. (TSPS9) were only recovered from any one of the fields viz, BSS1, BSS3 and BSS2, respectively (Table 2).

Similrly, Chuang *et al.* (2007), Pandey et al. (2008), Oniya *et al.* (2015), and Gizaw *et al*. (2017) isolated

Table 1: Rhizosphere soil properties of *Solanum melongena* var. MDU1 cultivated in natural fields

Soil variables	*Solanum melongena* var. MDU1
Soil type	Sandy loam
Ph	5.85±0.30
Electrical conductivity (dSm^{-1})	0.17±0.12
Organic carbon (%)	1.80±0.09
Total nitrogen (kg ha^{-1})	95.12±2.24
Available phosphorus (kg ha^{-1})	7.50±0.30
Exchangeable potassium (kg ha^{-1})	196.45±11.24

Means ± SD (n=3)

Qualitative phosphate solubilization

The solubilization index (SI%) of the isolated PSF ranged from 1.4 to 2.3% at seven days of incubation at 28°C (Fig. 2). Among the screened fungi morphotypes, maximum solubilization of TCP on PVK plates was recorded with the fungal isolate *Aspergillus* spp. (TSPS 2) with SI% = 2.3, whereas the lowest SI% of 0.9% was detected from the isolate *Trichoderma* spp. (TSPS9). As previously stated by Elias *et al*. (2016), the differential potential of phosphate solubilization based on SI on agar plates in the current investigation may be due to the varying type, quantity, and diffusion rates of distinct organic acids released by fungal isolates. The SI of the test phosphate-solubilizing fungal strains (*Penicillium italicum* and *Aspergillus niger*) were 2.42% and 3.15%, respectively, according to Iman (2008). In contrast, Elias *et al*. (2016) showed SI ranging from 1.1% to 3.0% for various fungal strains recovered from tomatoes and other crops. Alam *et al*. (2002) found that the SI of fungal cultures isolated from the maize rhizosphere ranged from 1.53% to 1.80%. Fungal isolates with higher SI on solid agar medium did not exhibit a similar trend in liquid broth medium, which is consistent with the findings of Alam *et al*. (2002), who reported that some isolates with little clear zone on solid agar medium had higher efficiency for dissolving insoluble phosphates in liquid medium. Some fungal isolates had wider clear

Table 2: Fungal isolates obtained from different rhizosphere soil samples of brinjal plants

Fungal isolates	Strain ID	*Solanum melongena* fields			Number of isolates
		BSS1	BSS2	BSS3	
Aspergillus niger Tiegh.	TSPS1	+	+	+	16
Aspergillus flavus Link	TSPS6	+			3
Aspergillus spp.	TSPS2		+	+	12
Fusarium oxisporum E.F. Sm. & Swingle	TSPS3	+	+	+	8
Fusarium spp.1	TSPS4	+	+		7
Fusarium spp.2	TSPS5			+	7
Penicillium citrinum Thom	TSPS7		+		4
Penicillium spp.1	TSPS8		+	+	6
Trichoderma spp.	TSPS9		+		5
					68

+ - indicates presence

P-solubilizing fungi from eggplants and other crop rhizospheric soil samples, including *Aspergillus niger* and *Penicillium* spp. Furthermore, *Aspergillus* spp. (53%) was the most common P-solubilizing fungus among the identified genera. This could be attributed to *Aspergillus* sp. efficiency in different soil (Elias *et al*. 2016). The current finding is consistent with previous findings of several researchers (Chuang *et al*. 2007; Yasser *et al*. 2014; Onyia *et al*. 2015; Elias *et al*. 2016; Gizaw *et al*. 2017), who discovered that P-solubilizing fungi belonging to the genus *Aspergillus* predominated in the rhizosphere of different crop plants. Because phosphate solubilizing microorganisms are primarily dependent on carbon-rich sources from plant roots for active production of organic acids that are used for solubilizing soil-bound phosphate (Pradhan and Sukla, 2006; Yasser *et al*. 2014), the effectiveness of P-solubilizing fungi in the current study is most likely due to sufficient root exudates.

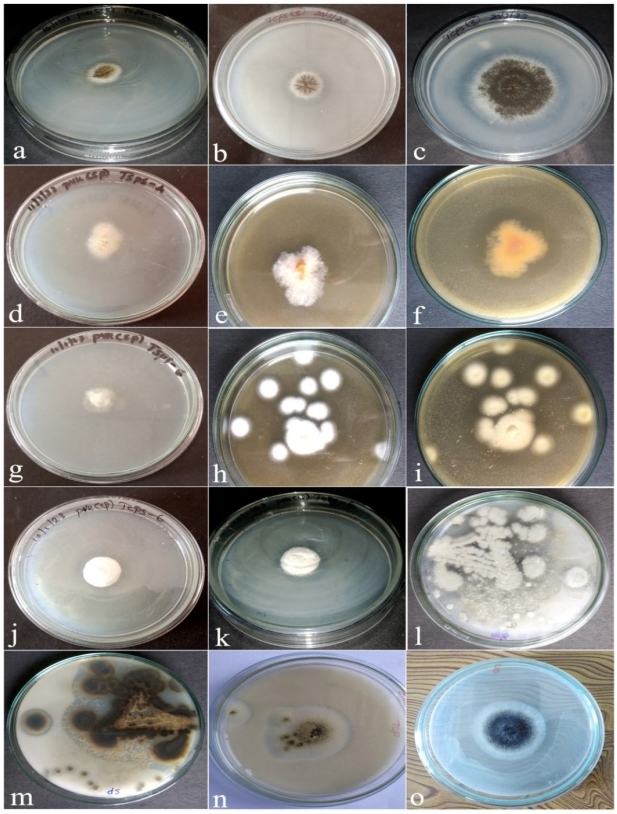

Fig. 1: Identification and TCP solubilization activity of soil fungi associated with brinjal rhizosphere. a,b,c) *Aspergillus niger*; d,e,f) *Fusarium oxysporum*; g,h,i) *Fusarium* spp.; j,k) *Fusarium* spp.; l,m) *Penicillium citrinum*; n) *Aspergillus* spp.; o) *Trichoderma* spp.

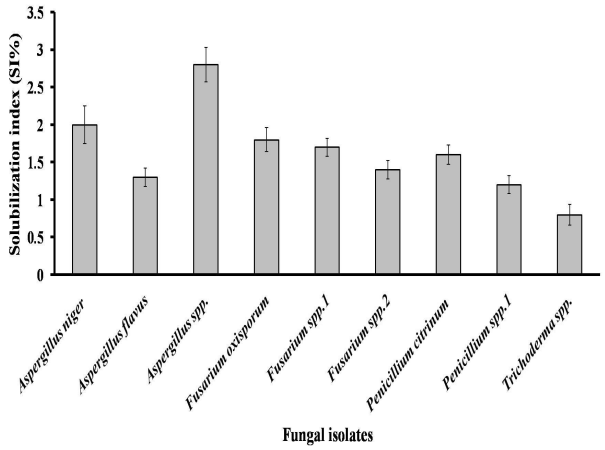

Fig. 2: TCP solubilization index (SI%) of isolated fungi associated with brinjal soils

zones on agar but poor phosphate solubility in liquid medium. This demonstrates that more SI generation on solid media does not always imply higher solubilization efficiency in liquid medium. As a result, as Elias *et al*. (2016) point out, the plate technique is insufficient for screening the best P solubilizers and detecting all phosphate solubilizers.

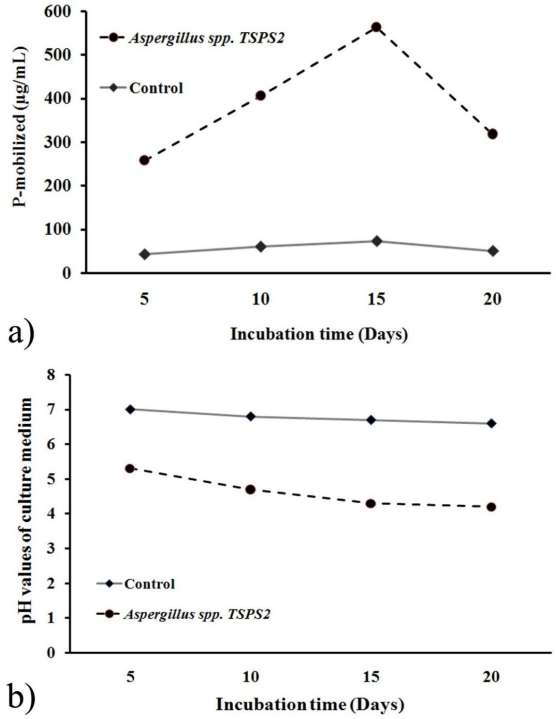

Fig.3 : Solubilized P concentrations (a) and pH values (b) of TCP containing PVK broth inoculated with PSF fungus *Aspergillus* spp. (TSPS 2) after 5, 10, 15, and 20 days of incubation time.

Quantitative phosphate solubilization

The amount of phosphates solubilized by fungal isolate viz. *Aspergillus* spp. (TSPS 2) was showed to be significantly higher over uninoculated control (Fig. 3a). The minimum P-solubilized from TCP containing broth was on day 5, afterwards the solubilized P increased up to day 15 of incubation. The mobilized phosphate values in the broth ranged between (216.43–490.56 µg/mL) during 20 days of incubation time. The highest amount of solubilized phosphate (490.56 µg/mL) was recorded from *Aspergillus* spp. (TSPS 2) inoculated culture filtrates during 15 days of incubation time (Fig. 3a). In further incubation (at day 20), decline in the mobilized phosphate was recorded in the test fungal isolates that reached up to 268.16 µg/mL. In the case of pH, *Aspergillus* spp. (TSPS 2) in TCP amended broth showed a decrease in the pH over control during the 20 days of incubation (Fig. 3b). The pH values decreased to variable levels in the TCP broth during the initial days and later became increased or remained at the same

level (Fig. 3b). The highest drop in pH was recorded in the isolates *Aspergillus* spp. (TSPS 2) from initial pH of 7.0 to 4.5, after 15 days of incubation time.

Periodic estimates of P in broth media demonstrated the isolates ability to release P from insoluble phosphate sources in the current study. The fungal isolates gradually solubilized the insoluble phosphate sources, such as TCP, in the middle of the incubation period. These findings are consistent with those of Nenwani *et al*. (2010), who discovered a progressive rise in mobilized P by fungal isolate F1 in liquid cultures. At the end of the incubation period, phosphate solubilization decreased, which is consistent with the findings of Pandey *et al*. (2008).

According to Kim *et al*. (2005), this might be attributable to the availability of soluble phosphate, which inhibited further TCP solubilization, or to the depletion of carbon sources, which reduced both the generation of organic acids and microbial activity. Another possible explanation for the decrease in mobilized P is the creation of an organo-P compound generated by released organic metabolites, which reduces the quantity of accessible P (Wakelin *et al*. 2004; Pradhan and Sukla, 2006; Gizaw *et al*. 2017). During this time, fungal cells use mobilized P for growth and development, according to Elias *et al*. (2016). Furthermore, the test fungus isolated the most phosphate from TCP on day 15 and then gradually decreased. These findings are consistent with those of Pandey *et al*. (2008), who discovered that maximum phosphate solubilization occurred on day 15 of TCP incubation under controlled circumstances. When the medium was supplemented with TCP, the isolate *Aspergillus* spp. (TSPS 2) demonstrated the highest mobilization P values (490.56 μg/mL), which might be attributed to its inherent self-solubility. This shows that these fungal isolates have the capacity to solubilize insoluble phosphates, which opens up a new channel for the creation of fungal biofertilizers after the requisite qualifying studies. Similarly, Pandey *et al*. (2008) found 320 μg/mL mobilized phosphate (*P. oxalicum*) in TCP after 15 days of incubation. Acidification by organic acid has been shown to be the primary method by which microorganisms solubilize inorganic P (Pradhan and Sukla, 2006; Pandey *et al*. 2008).

The pH values of all fungal isolates decreased in the culture media in distinct ways. Several researchers have speculated that this is due to the production of various organic acids from the available nutrient (glucose) (Wakelin *et al*. 2004; Pradhan and Sukla, 2006; Iman, 2008; Nenwani *et al*. 2010; Yasser *et al*. 2014). A number of research findings have frequently documented a pH decline in cultures (Pandey *et al*. 2008; Elias *et al*. 2016). pH values declined to different levels depending on culture type and then became almost constant or increased as immobilized phosphate was reduced (Onyia *et al*. 2015; Gizaw *et al*. 2017). This discovery could be attributable to the low glucose concentration in PVK broth, which is required for organic acid synthesis. In this regard, the current findings are consistent with the findings of Elias *et al*. (2016), who found an increase in pH and a decrease in solubilized phosphate towards the conclusion of the incubation time.

CONCLUSION

A variety of fungal species with potential phosphate solubilizing activity can be found in the rhizosphere soil of brinjal (*S. melongena*). The current study discovered several plant-associated P-solubilizing fungi that could be employed as biofertilizers.

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