Neurospora sp. SR8, a novel phosphate solubiliser from rhizosphere soil of Sorghum in Kachchh, Gujarat, India

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Phosphorus (P) is abundant in soils in both inorganic and organic forms; nevertheless, it is unavailable to plants due to its fixation. Phosphate solubilising microorganisms including fungi play a pivotal role in making P available for plants by the process of solubilisation and mineralisation. Among the fungi that solubilize phosphate, the genera Aspergillus and Penicillium are the most representative although strains of Trichoderma and Rhizoctonia solani have also been reported as P solubilizers. Here, we report Neurospora discreta strain SR8 (NCCS Pune accession No. MCC1096 and NCBI accession No. KJ676544) as a P solubiliser as the first report. The strain was isolated from rhizospheric soil of Sorghum bicolor (L.) Moench. grown in semi-arid climate of a unique ecological zone of Kachchh, western India. The organism was identified on the basis of morphological characterization and by sequencing of ITS region. The strain SR8 survived the stressed environment in terms of high salinity and low precipitation rate in this area and could be a potent P solubiliser in stressed environments.

Keywords: Bioremediation, Broomcorn, Fungi, Milo, P solubilization, PSM, Salinity, Semi-arid, Soil nutrition

Phosphorus is the second important key element after nitrogen as a mineral nutrient in terms of quantitative plant requirement. Although abundant in soils, in both organic and inorganic forms, its availability is restricted as it occurs mostly in insoluble forms. To satisfy crop nutritional requirements, P is usually added to soil as chemical fertilizer, however, synthesis of chemical P fertilizer is a highly energy intensive processes, and has long-term impacts on the environment in terms of eutrophication, soil fertility depletion, carbon footprint. Moreover, plants can use only a small amount of this P since 75-90% of added P is precipitated by metal-cation complexes, and rapidly becomes fixed in soils. Such environmental concerns have led to the search for sustainable way of P nutrition of crops. In this regard, phosphate-solubilizing microorganisms (PSM) have been seen as best eco-friendly means for P nutrition of crop and overall integrated plant nutrient management.

Microorganisms are an integral component of the soil P cycle and are important for the transfer of P between different pools of soil P. PSMs, through various mechanisms of solubilization and mineralisation, convert inorganic and organic soil P respectively into the bioavailable form facilitating uptake by plant roots. This has led to increased interest in the harnessing of microorganisms to support P cycling in agroecosystems. Several strains of bacterial and fungal species have been described and investigated in detail for their phosphate-solubilizing capabilities. Typically such microorganisms have been isolated using cultural procedures with species of Pseudomonas and Bacillus bacteria and Aspergillus and Penicillium fungi being predominant.

In soil P solubilizing bacteria constitute 1-50% and fungi 0.1-0.5% of the total respective population. The P-solubilizing fungi do not lose the P dissolving activity upon repeated subculturing under laboratory conditions as occurs with the P-solubilizing bacteria. Moreover, fungi in soils are able to traverse long distances more easily than bacteria and hence, may be more important to P solubilization in soils. Generally, the P-solubilizing fungi produce more acids than bacteria and consequently exhibit

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greater P-solubilizing activity\textsuperscript{10}. Among filamentous fungi that solubilize phosphate, the genera \textit{Aspergillus} and \textit{Penicillium}\textsuperscript{11} are the most representative although strains of \textit{Trichoderma}\textsuperscript{12} and \textit{Rhizoctonia solani}\textsuperscript{13} have also been reported as P solubilizers.

Here, we report the characterisation of efficient P solubilising fungi: \textit{Neurospora} strain SR8, isolated from saline soil. The study is, to the best of our knowledge, the first report of a P solubilising \textit{Neurospora} strain. \textit{Neurospora discreta} SR8 ((NCCS Pune accession No. MCC1096 and NCBI accession. No. KJ676544)) is now stored in the Microbial Culture Collection (MCC), National Centre for Cell Science, Pune, India.

\section*{Materials and Methods}

\subsection*{Study site description}

The fungi were isolated from the rhizospheric soil sample of Sorghum (\textit{Sorghum bicolor} (L.) Moench.) grown in Kachchh, Gujarat, western India. The soil is typical camborthids and sandy loams (calcereous). This semi-arid zone of extreme part of western India is unique in various respects, due to its oppressive weather, low rainfall, aridity, hostile terrain and seismic instability. Over the years, several natural and anthropogenic factors have affected the natural resource status of Kachchh. Drought and high salinity levels have remained a typical characteristic of the region. This semi-arid zone is unique in terms of its ecology\textsuperscript{14}.

\subsection*{Soil sampling and chemical parameters}

Six sorghum fields were earmarked on two different farms in the semi-arid zone of Kachchh, Western India (23°13'48.00"N, 69°42'35.06"E). Samples were collected from the rhizosphere of the crop up to the depth of 12 cm using standard soil sampling procedure. Four samples were collected per hectare and pooled to form one composite sample. Samples were analyzed in triplicates. Soil samples were divided into two parts; one part of the sample was air dried and sieved through a 2 mm sieve and analyzed for chemical characteristics, and another part was stored at 4°C for microbiological analysis. The study was carried out during the Rabi season of 2012. Soil samples were analyzed for pH and EC in a soil suspension of ratio 1:2 (w/v) with a glass electrode and digital EC meter, respectively\textsuperscript{15}. Available Phosphorus was determined following the Olsen’s method\textsuperscript{16}. The total P in soil samples was extracted by a mixture of concentrated sulphuric acid, hydrofluoric acid and hydrogen peroxide\textsuperscript{17}. Soil organic Carbon was analysed by rapid titration method\textsuperscript{18}.

\subsection*{Isolation of fungi}

The plants were uprooted at 25 DAS (days after sowing) and loosely adhering soil was removed by mechanical shaking. The soil was then suspended in sterile saline (0.85%). The serial soil dilutions of the sample ($10^3$, $10^5$ and $10^6$) were individually spread plated on Pikovskaya (PVK) agar plates containing 0.5% tricalcium phosphate (TCP) as insoluble phosphate source. After 6 days of incubation at 25-27°C, the plates were examined for the colonies developing clear halo zone around them. Such colonies were picked up, purified and maintained on PVK agar slants at 4°C.

\subsection*{Selection of efficient P solubilising fungi}

Erlenmeyer flasks (250 mL) containing 100 mL of PVK broth were inoculated in triplicate with the pure culture of fungal isolates, incubated at 27°C and 120 rpm on a rotary shaker for 11 days. The quantitative estimation of soluble P in the culture supernatant was done every 48 h by the molybdenum blue method\textsuperscript{19}. The pH was recorded using glass electrode pH meter. The amount of phosphate released into culture supernatant was criteria for choosing the most efficient PSF. The isolate showing the highest insoluble phosphate-solubilizing activity, named SR8, was selected for further study and characterized to the species level.

\subsection*{Identification of fungi}

The most effective fungus (SR-8) was identified on the basis of colony morphology and cultural characteristics. Later it was sent to Microbial culture collection (MCC) of National Centre for Cell Science, Pune, India for molecular identification.

\subsection*{Morphological and molecular characterization}

The microscopic characters of the fungus SR-8 were studied in water and lactophenol (Hi-Media, India) and stained with cotton blue (Hi-Media, India) on Nikon YS100 (Nikon, Japan). Measurements and microphotographs taken on BX53 (Olympus Corporation, Japan) fitted with ProgRes C5 camera (Jenoptik, USA). For molecular analysis, the fungus was grown on PDA for 7 days. Mycelial mat was...
scrapped from the plate and taken for DNA extraction. DNA was isolated using Mini-Prep DNA extraction kit (Fischer Scientific, USA). The purity and quantity of DNA was checked on gel and spectrophotometer (NanoDrop, USA). Internal transcribed spacer region (ITS) of rDNA was amplified using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers\(^\text{20}\) using Gene Amplifier PCR System 9700 (Perkin Elmer, USA) thermocycler. The reaction mixture (25 µL) for the PCR was: MgCl\(_2\)-1 µL, PCR Buffer-2.5 µL, dNTP-1.25 µL, Forward Primer-1 µL, Reverse Primer-1 µL, DNA template-1 µL, Taq polymerase-0.15 µL, PCR grade distilled water-17.1 µL. The ITS region was amplified using an initial denaturation of 94°C for 5 min, 35 cycles of denaturation 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 10 min and stored at 15°C. The amplified product was checked on 1% agarose gel and purified by PEG-NaCl (polyethylene glycol-NaCl) method\(^\text{21}\). Sequencing was done on ABI 3730xl Automated Sequencer using ‘ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit’ (Perkin Elmer Applied Biosystems Division, Foster City, CA).

**Phylogenetic characterization**

*Nucleotide sequence accession number*

The comparative analysis of sequences was done using comprehensive database of NCBI (http://www.ncbi.nlm.nih.gov) and nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) to identify the unknown fungi based on their ITS sequence data. The sequences were edited using ChromasPro version 1.34 and SeqScanner software and contig was formed. The FASTA sequence was used and NCBI and DDBJ BLASTn search done for sequence similarity of ITS region of SR-8 strain. The sequence of strain SR-8 was generated in lab and all other sequences were downloaded from NCBI Gene Bank database. All sequences were aligned in CLUSTALW\(^\text{22}\) and gaps at both the ends were deleted. The aligned dataset was used to make phylogenetic tree. Phylogenetic analysis was accomplished using MEGA5 program\(^\text{23}\). Neighbour-joining (NJ) tree was made using Kimura-2 model in MEGA5 and bootstrap value <50% was excluded. The strain was given an accession number MCC1096.

### Results and Discussion

The rhizospheric soil of *Sorghum bicolor* contained around 14 groups of fungi when plated out on the PVK agar. Three isolates coded as SR8, SR1 and SR11 showed a marked insoluble phosphate solubilizing activity as visualized by the clarified zone developed around the colony. These three strains were isolated at 10\(^3\) dilutions. In case of isolate coded SR8 in the PVK broth, the soluble P showed a gradual increase and reached a solubilisation percentage of 9.67% at the 15\(^{th}\) day (Table 1). This isolate was the most potent isolate amongst the isolated strains and hence was chosen for further characterisation. Earlier studies by Rachana et al.\(^\text{24}\) have shown P solubilisation potential of fungi *Aspergillus awamori* S19, which was isolated from the rhizosphere soil of *Pennisetum glaucum* grown in semi-arid climatic conditions and the P solubilisation in PVK broth was as high as 1008 mg/L at the 10\(^{th}\) day. Similarly, in a study by Bhattacharya et al.\(^\text{25}\), a potent fungal strain *Emericella* (*Aspergillus*) *nidulans* isolated from vermicompost had shown Phosphate solubilisation potential.

The morphological and phylogenetic study of strain SR-8 confirms that it is a species of *Neurospora* sp. The morphological observations have shown fast growing orange colony formed by *Neurospora* sp. SR-8 forming typical brown-black *Neurospora* spores. The NCBI BLAST result of ITS region with both NCBI and DDBJ database showed similarity with *Neurospora discreta* AS-2-2 (KC215135), *N. discreta* ATCC MYA-4616 (GU327632) and *N. discreta* FGSC 6794 with 99% similarity and 100% query coverage. It also showed 98% similarity with *N. pannonica* CBS 270.91 (GQ922532; query coverage 100%), 97% similarity with *N. tetrasperma* CBS 377.74 (GQ922530; 100% query coverage),

<table>
<thead>
<tr>
<th>Soil location</th>
<th>PSM strain</th>
<th>P solubilization in PVK broth (%) at different DAI</th>
<th>pH of medium at 15 DAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(days after inoculation)</td>
<td>(Initial pH of medium was 7.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Bhuj, Kachchh, western India</td>
<td>SR8</td>
<td>3.78</td>
<td>4.56</td>
</tr>
<tr>
<td>(23º13’48.00’’N, 69º42’35.06’’E)</td>
<td>SR1</td>
<td>3.56</td>
<td>4.98</td>
</tr>
<tr>
<td></td>
<td>SR11</td>
<td>3.23</td>
<td>5.56</td>
</tr>
</tbody>
</table>

Table 1—P solubilisation potential and pH of medium at different days after inoculation for three potent strains.
97% similarity with *N. pachypodioides* CBS 164.52 (GQ922526; 100% query coverage) and 97% similarity with *N. tetrasperma* CBS 377.74 (GQ922530; 100% query coverage) (Table 2 and Fig. 1). Phylogenetic analysis accomplished by doing NJ tree (Fig. 2) shows that the present strain is positioned with *N. discreta* ATCC MYA-4616 and *N. discreta* AS-2-2 in a separate sub-clade within the clade of *N. discreta*. The alignment had total of 498 bp, out of which 241 were conserved, variable 237 and parsimony informative 53 and 180 were singletons. Hence based on the morphology, BLASTn with NCBI database and phylogenetic studies conducted, the strain SR-8 is identified as *Neurospora* sp. SR-8 (NCCS Pune acc no. MCC1096 and NCBI acc. No. KJ676544).

Comparison of the P solubilisation in PVK broth with tricalcium phosphate (TCP) as source of insoluble P showed that strain SR8 was most efficient at 15 DAI (days after inoculation), although at 10 DAI both strain SR8 and SR1 showed similar percentage P solubilised. The decrease in pH of medium at 15 DAI was the highest in SR8 (4.5) (Table 1) which shows that this isolate secretes organic acids which lower the pH and solubilise P.

The soil properties of the rhizospheric soil show that soil are sandy loams and pH coupled with electrical conductivity (EC) of the soil is on the higher side. The soil was organically amended so that it is good in soil organic carbon (SOC). The total Phosphorus values in all the three soil locations is high but available P levels are low, indicating that the soil in the area has high total P but mostly unavailable to plants (Table 3). Biofertilizers suited to this soil environment could possibly help in solubilisation/mineralisation of this unavailable P.

**Table 2**—ITS sequences producing significant alignments (based on NCBI-BLAST search)

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query coverage</th>
<th>E value</th>
<th>Max identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC215135.1</td>
<td><em>Neurospora discreta</em> strain AS-2-2</td>
<td>941</td>
<td>941</td>
<td>99%</td>
<td>0</td>
<td>99%</td>
</tr>
<tr>
<td>GU327632.1</td>
<td><em>Neurospora discreta</em> strain ATCC MYA-4616</td>
<td>917</td>
<td>917</td>
<td>97%</td>
<td>0</td>
<td>99%</td>
</tr>
<tr>
<td>JN570255.1</td>
<td><em>Neurospora</em> sp. E10527a</td>
<td>898</td>
<td>898</td>
<td>100%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>FJ904922.1</td>
<td><em>Neurospora tetrasperma</em> strain GrS11</td>
<td>898</td>
<td>898</td>
<td>100%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>FJ360521.1</td>
<td><em>Neurospora crassa</em></td>
<td>898</td>
<td>898</td>
<td>100%</td>
<td>0</td>
<td>98%</td>
</tr>
</tbody>
</table>

**Table 3**—Soil properties of the rhizospheric soil of sorghum (*Sorghum bicolor*) from which the fungal strains were isolated.

<table>
<thead>
<tr>
<th>Soil location</th>
<th>PSM strain</th>
<th>Soil type</th>
<th>pH</th>
<th>EC (electrical conductivity in ds/m)</th>
<th>Soil organic carbon (SOC in %)</th>
<th>Total Phosphorus (Kg/ha)</th>
<th>Available Phosphorus (Kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bhuj, kachchh, western India (23°13'48.00''N, 69°42'35.06''E)</td>
<td>SR8</td>
<td>Sandy loam</td>
<td>8.04±0.69</td>
<td>0.82±0.04</td>
<td>0.92±0.57</td>
<td>94.945±8.56</td>
<td>21.685±1.23</td>
</tr>
<tr>
<td></td>
<td>SR1</td>
<td>Sandy loam</td>
<td>7.46±0.30</td>
<td>0.89±0.06</td>
<td>0.80±0.13</td>
<td>106.945±7.82</td>
<td>15.685±0.98</td>
</tr>
<tr>
<td></td>
<td>SR11</td>
<td>Sandy loam</td>
<td>8.27±0.09</td>
<td>0.79±0.45</td>
<td>0.75±0.34</td>
<td>120.945±9.84</td>
<td>10.685±0.25</td>
</tr>
</tbody>
</table>

* Values are MEAN±SEM
Conclusion

Various fungi have been reported earlier as P solubilisers. Here, we reported a strain of Neurospora discreta as a P solubiliser for the first time. As the site from where it is isolated is a stressed environment, hence it could be a potent P solubiliser for such environments. The strain was isolated from salt affected soils, which indicates its high salinity tolerance. The percentage P solubilisation potential and decrease in pH of the medium by the isolate SR8 was highest as compared to other isolates, and this property can be effectively utilised in future by biofertilizer formulation using this newly reported Neurospora sp. Supporting studies in terms of P solubilisation at different salinity levels, pH and temperature are needed to be conducted further.

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Competing Interests

The authors declare that they have no conflict of interests.

References


