Isolation and Molecular Profiling of Halotolerant Plant Growth Promoting Rhizosphere Fungi from Salt Affected Agroforestry Plantation

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Abstract

Soil salinity is a major abiotic stress that adversely affects plant growth, and productivity. About 20% of irrigated lands are affected by salinity worldwide. In India, there are 6.74 million hectares of salt-affected lands. Salt-tolerant Plant Growth Promoting (PGP) microorganisms can enhance the growth of plants in such salt-stressed areas. Therefore, this study aimed to investigate the diversity of beneficial fungal communities, screen for their ability to support plant growth, and evaluate the production of various essential compounds in view of plant growth in salt-stressed lands. A total of 68 fungal colonies were isolated from 5 different agroforestry plantation sites in Karur, Tamil Nadu, South India at quarterly intervals. The isolates were screened for sodium chloride (NaCl) tolerance (0%, 5%, 10%, 15%, and 20% concentration). A total of 7 isolates showed considerable salt tolerance and were tested qualitatively in-vitro, for PGP traits such as phosphate, potassium, and zinc solubilization, nitrogen fixation, hydrogen cyanide production, siderophore production, and ACC deaminase production. Finally, 5 isolates with maximum values for PGP properties under 20% NaCl concentration were tested for the quantity of Indole-3-Acetic Acid (IAA) and Exo-polysaccharide (EPS) production. All 5 isolates were identified up to the species level using 18S rRNA gene sequencing. To the best of our knowledge, this is the first report on isolating saline-tolerant PGP Fungi (PGPF) from the rhizosphere region of Casuarina equisetifolia and Eucalyptus camaldulensis in Karur, Tamil Nadu, India. In the future, the bioformulation of PGPF and its application will boost the cultivation of tree saplings in this salt affected regions.
Introduction
One of the major abiotic stresses that adversely affects plant growth, productivity, and quality, is soil salinity. About 20% of irrigated lands are negatively affected by salinity. In India, it was estimated that there are 6.74 million hectare of salt-affected lands that cause high economic losses. Among the various environmental stresses, soil salinity is one of the most devastating; it generally occurs either due to natural or anthropogenic causes and involves the accumulation of dissolved salts in the soil. In salt-affected areas, the crops suffer from stunted growth, poor biomass, and low yield, which are mainly attributed to osmotic stress, excessive absorption of sodium ions, generation of reactive oxygen species (ROS), alteration of metabolic processes, reduction of cell division and expansion, membrane disorganization, and genotoxicity. In addition, salt stress makes plants more prone to pathogenic diseases.

Due to ground water scarcity in study location (Karur district, Tamil Nadu), villages nearby to Tamil Nadu Newsprint and Papers Limited (TNPL), depends on the usage of paper mill treated effluent as water source. These leads to high level of deposition of salt in this area. Therefore, in the study area, traditional farming is impractical due to the continues use of industrial treated effluent water as irrigation source, results in reduced crop yields. The high cost for physical remediation of salt issue makes conventional agriculture as economically unviable for most farmers in the region. Here, the primary agricultural activities traditionally involved the cultivation of paddy, sugarcane, plantain, groundnut, millet, maize, and com. However, the use of industrial effluent for irrigation has resulted in the growth of only coconut and cattle grasses. Consequently, farmers with saline lands are exploring alternative income avenues through the implementation of agroforestry systems.

Several reports focus on the cultivation of trees for improving soil fertility, plant health under stressed conditions, and an alternative income source for farmers. However, an excess of salt in the soil restricts the development of new roots, which is important for the growth of transplanted seedlings and the healthy development of trees under stressed conditions. The levels of salinity differ from site to site, and the salt tolerance capacity also differs greatly among the plant species. Planting of trees on such soils without considering their level of tolerance to salinity results in heavy mortality.

A tremendous amount of fundamental research has focused exclusively on characterizing an array of salt stress-related genes in plants with the aim of improving plant salt tolerance using genetic modification approaches. However, these approaches have limited success and are highly expensive. Rhizosphere microorganisms as bio-inoculants are used as an eco-friendly and viable alternative to improve salt tolerance in plants and to ameliorate the soil salinity problems. The salt tolerance mechanisms of plant growth-promoting microorganisms isolated from saline habitat and halophilic bacteria that directly increase agricultural crop yields in salt-affected lands, are well understood. Halophilic (salt-loving) organisms have diverse physiological adaptive mechanisms to endure high salt concentrations.

Plant-associated microbial community could be the key for understanding the adaptation of plants to their habitat; they include PGPF such as Fusarium, Gliocladium, Trichoderma, Talaromyces, Phytophthora, Pencilliun, Phoma, and Rhizoctonia. These increase plant growth, by improving the innate immunity and the levels of various important secondary metabolites in plants. High soil salinity impacts the growth of several plant species, particularly that of glycophytes (salt-sensitive) compared to that of halophytes (salt-tolerant plants). Therefore, it is not economical to grow salt-sensitive plants on extremely salt-affected lands. The use of salt-tolerant PGP microorganisms enhances the growth of salt-sensitive plants in salt-stressed areas. The apparent results of these beneficial microorganisms may not be evident under all natural conditions because of their insufficient naturally-occurring population in the soil. Therefore, the external application of the most suitable beneficial microorganisms becomes imminent. The effective utilization of bio-inoculants or bio-fertilizers in tree seedlings provides economic benefits, improves the soil fertility, and maintains the natural soil ecosystem.

There are only a few reports on the isolation and application of halo-tolerant fungal species, especially those from salt-affected agroforestry plantations. This study aimed to investigate the diversity of beneficial
fungal communities in salt-affected areas, screen for their ability to support plant growth under salinity conditions, and evaluate the production of various compounds essential for processes critical for plant growth such as phosphate solubilization, potassium solubilization, zinc solubilization, nitrogen fixation, siderophore production, HCN production, ACC deaminase production, phyto-hormone production, and EPS production, under in vitro conditions.

Materials and Methods
Sample Collection
The research site in Karur district is located in Tamil Nadu, South India, lies between 11°08’00” North and 77°98’39” South. The study sites are in close proximity to TNPL in Karur, where treated effluent from paper mill is utilized for irrigation. The introduction of this treated effluent has resulted in the fluctuations of soil physicochemical parameters. In response to increased salinity, local farmers have transitioned to agroforestry plantations. To enhance the growth of plants cultivated in this area, we have devised a plan to isolate and bio formulate native halotolerant PGPF from the rhizosphere of tree plantations.

To implement the work, we collected rhizosphere soil samples from five distinct agroforestry plantations near TNPL Karur area. It was collected quarterly (Nov-Jan, Feb-Apr, May-Jul, and Aug-Oct) during the year 2016−2017. The selected plantations are as follows: (1) Kagithapuram Casuarina equisetifolia plantation (KACA), (2) Kagithapuram Eucalyptus camaldulensis plantation (KAEU), (3) Masagoundapudur C. equisetifolia plantation (MACA), (4) Moolimanglam C. equisetifolia plantation (MOCA), and (5) Murthypalayam E. camaldulensis plantation (MUEU). Soil samples were carefully collected in clean ziplock polybags and promptly transported to the laboratory, where they were stored at 4°C for subsequent analysis.

Physico-Chemical Analysis of Collected Soil Samples
The soil samples collected from different study locations were analysed for their physico-chemical parameters such as pH, electrical conductivity (EC), and exchangeable sodium percentage (ESP) with three replicates, at the soil and water testing laboratory, Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore by following standard procedures, as described by Dandwate.

Enumeration of the Total Fungal Count in Rhizosphere Soil
Standard procedures were employed for serial dilution and plating to quantify the population density of fungi in the soil samples collected. Ten grams of soil sample was weighed and used for serial dilution (up to 10⁶). Then 0.1 ml of aliquots were serially transferred to sterile, labelled petri plates containing potato dextrose agar (PDA) and rose bengal agar (RBA). The plates were incubated at room temperature for 48 h. Three replicates were performed to access the Colony Forming Unit (CFU) per gram of soil sample.

Effect of Different Concentrations of Sodium Chloride (NaCl) on Fungal Growth
Due to fluctuations in soil salinity throughout the study period, we outlined a design to assess the maximum salinity tolerances of fungal isolates from various study locations. Accordingly, we conducted in-vitro tests to evaluate their varying levels of salinity tolerance by incorporating different NaCl concentrations viz., 0% (control), 5%, 10%, 15%, and 20% by following the method prescribed by Ji. The fungal disc of 6 mm was placed on the center of PDA medium containing different concentration of NaCl and incubated at 28°C for 48 h with three replicates. The growth of the fungal mycelium was compared with the control plate (without NaCl), and the percentage of mycelium growth was calculated. The isolate showing the maximum salt tolerance and the fastest growth was selected for further studies.

Screening for Qualitative PGP Traits
Identification of Phosphate- (P), Potassium- (K), and Zinc (Zn)-Solubilizing Isolates
Phosphate, potassium, and zinc solubilization under different NaCl concentration (0%, 5%, 10%, 15%, and 20%) was evaluated using tri-calcium phosphate as the insoluble phosphate (Pikovaskaya medium), mica powder as the insoluble form of potassium (Alexandro agar medium), and modified Bunt and Rovira agar medium containing 0.1% of ZnCO₃ as the insoluble zinc source. Spot inoculation of the isolates was done at the center of the respective medium and the plates were incubated at 28±2°C for 10 days. The zone of clearance formed by the colonies was measured after every 24 h for up to 10 days. Each test was performed with three replicates. The solubilizing efficiency of the selected culture was calculated using the following formula as described by Sharma.
Solubilization Efficiency (SE) = (Solubilization diameter - colony diameter) / (colony diameter) x 100

Identification of Nitrogen-Fixing Isolates
Nitrogen fixation was tested by inoculating selected fungal isolates on plates of N-free agar medium incorporated with different concentration of NaCl (0% (control), 5%, 10%, 15%, and 20%) and incubating the plates for 7 days at 28°C with 3 replicates. The isolates growing after repeated inoculation in N-free medium were considered to be positive for nitrogen fixation.

Identification of Siderophore-Producing Isolates
Siderophore production was assessed on solid chrome azurol S blue agar (CAS) plates with different concentrations of NaCl, as described by Hofmann. All the glassware used in the test were soaked in 2 N HCl solution for 24 h to avoid iron contamination from the glassware. Fungal disks were spot inoculated on the CAS blue agar plate and incubated at 28°C for 48 h with three replicates. The formation of a yellow-orange halo around the colony was considered as positive for siderophore production.

Identification of Hydrogen Cyanide (HCN)-Producing Isolates
HCN production was detected, as described by Joseph. All selected fungal isolates were inoculated in PDA, supplemented with glycine (4.4 g/l) and different concentration of NaCl. Whatman No. 1 filter paper was soaked with 2% sodium carbonate in 0.5% picric acid solution and placed at the top of the plate. Plates were sealed with paraffilm and incubated at 28°C for 4–7 days. Following incubation, the colour change of the filter paper was noted: yellow to brown or orange colour indicates positive result. The experiment was conducted with three replicates.

ACC Deaminase-producing Isolates
The assessment of ACC deaminase activity, followed the methodology outlined by Penrose and Glick. The colonies were screened for ACC deaminase activity on the sterile minimal Dworkin and Foster (DF) salts media supplemented with 3 mM ACC instead of (NH₄)₂SO₄ as sole nitrogen source. Fungal disks were spot inoculated on the center of the plates and were incubated at 28°C for 3 days, with daily monitoring of mycelium growth. Fungal mycelium grown on the plates were recognized as ACC deaminase producers, while those lacking observable growth were classified as non-ACC deaminase producers.

Quantitative Screening of PGP Traits in Selected Isolates
The fungal isolates showing maximum PGP properties under high NaCl concentration were shortlisted and used for testing for important quantitative PGP properties. All the experiments were conducted with three replicates.

Quantitative Estimation of IAA
We screened selected isolates for their ability to produce the plant growth hormone-IAA under in vitro conditions, as described by Turbat 24 and Bano and Musarrat. The test tubes containing PD broth supplemented with L-tryptophan (0.2 %) and different concentration of NaCl (0%, 5%, 10%, 15%, and 20%) were inoculated with selected fungal isolates and incubated for 7–8 days at room temperature. Following the incubation, the culture broth was centrifuged at 10,000 rpm for 30 min, and the pellet was discarded; 1 ml of the supernatant was transferred to a clean test tube. To this, 2 ml of freshly prepared Salkowskί's reagent was added. The tubes were incubated in the dark for 30 min and checked for the development of pink colour. Optical density at 530 nm (OD₅₃₀) was recorded, and a graph was plotted against the standard values of IAA.

EPS Production
EPS production was screened, as described by Ruhmann. Selected isolates were inoculated into 10 ml basal medium with different concentrations of NaCl (0%, 5%, 10%, 15%, and 20%) and incubated at room temperature for 5–6 days, 10 ml of culture suspension was collected and centrifuged at 3000 rpm for 15 min. Thrice the volume of chilled acetone was added to the supernatant. EPS was separated from the mixture in the form of a slimy precipitate; it was collected on a pre-dried filter paper, and the precipitates were allowed to dry overnight at 50°C. The dried filter paper was reweighed after overnight drying.

Morphological, Cultural, and Molecular Characterization of PGPF
The cultural characteristics were studied using PDA and RBA; the colonies were observed for their macroscopic appearance (colour, reverse colour,
pigmentation, growth rate, spore type, and hyphal arrangement). All selected isolates were subjected to microscopic analysis (microstructure). Fungal cultures were further identified based on 18S rRNA gene sequencing.

Bioinformatics Protocol
The 18S rRNA gene sequence analysis was performed for selected PGPF. DNA isolation was performed by using the EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd, Coimbatore, Tamil Nadu. Sanger’s di-deoxy nucleotide sequencing method was employed for the gene sequencing. Subsequently, a similarity search for the generated sequence was executed using the National Centre of Biotechnology Information (NCBI) with the help of BLAST program. The resulting sequences were deposited into the GenBank nucleotide database, and accession numbers were acquired. Following the retrieval of blast results, a phylogenetic analysis of the query sequence was conducted, which was subsequently followed by multiple sequence alignment with closely related sequences. Then the construction of the phylogenetic tree was accomplished using the Neighbour-joining (NJ) method.

Statistical Analysis
PGP traits were analysed using one-way ANOVA followed by Duncan Multiple Range Test (DMRT) using SPSS software. Statistical significance was set at P < 0.05.

Results
Physico Chemical Properties of the Rhizosphere Soil Collected from Different Study Sites
Data on the physico-chemical properties of rhizosphere soil samples collected from different study locations. All the parameters varied during the quarterly intervals in the study period. Very high salinity was observed during the Feb-Apr season in all the study sites. The pH was alkaline (8.3) in the MOCA rhizosphere soil during Feb-Apr, compared to that in the other sites, which had a neutral pH. The pH ranged from 7.3–7.7 during Nov-Jan; 7.8–8.3, during Feb-Apr; 7.0–7.6, during May-Jul, and 7.0–7.7, during Aug-Oct. The soil samples collected from five different study locations showed distinct variations in EC throughout the study seasons. The EC values were the highest in the MUEU rhizosphere soil (17.5 dS/m) during Feb-Apr. The EC ranged from 1.4–4.7 dS/m during Nov-Jan; 4.4–7.5 dS/m, during Feb-Apr; 1.2–12.4 dS/m, during May-Jul; and 1.0–8.4 dS/m, during Aug-Oct interval. An EC >4 ds/m indicated that the soil was affected by salinity. ESP was below 15% at all the study sites (3.1–11.1%) (Table1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Seasons</th>
<th>Study locations</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td>KACA  KAEU MACA MOCA MUEU</td>
</tr>
<tr>
<td>Nov-Jan</td>
<td>7.7 7.4 7.7 7.3 7.4</td>
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</tr>
<tr>
<td>Feb-Apr</td>
<td>7.9 8 7.8 8.3 8</td>
<td></td>
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<tr>
<td>May-Jul</td>
<td>7.1 7 7.6 7.1 7.6</td>
<td></td>
</tr>
<tr>
<td>Aug-Oct</td>
<td>7.7 7.5 7.5 7.3 7</td>
<td></td>
</tr>
<tr>
<td>EC (dS/m)</td>
<td></td>
<td>KACA  KAEU MACA MOCA MUEU</td>
</tr>
<tr>
<td>Nov-Jan</td>
<td>4.7 4 1.4 2.2 2.6</td>
<td></td>
</tr>
<tr>
<td>Feb-Apr</td>
<td>13.2 4.4 11.9 16.5 17.5</td>
<td></td>
</tr>
<tr>
<td>May-Jul</td>
<td>7.1 1.25 11.6 6.1 12.4</td>
<td></td>
</tr>
<tr>
<td>Aug-Oct</td>
<td>6.2 1.05 8.4 5.1 8.1</td>
<td></td>
</tr>
<tr>
<td>ESP (%)</td>
<td></td>
<td>KACA  KAEU MACA MOCA MUEU</td>
</tr>
<tr>
<td>Nov-Jan</td>
<td>10.5 8.4 3.1 4.6 4.2</td>
<td></td>
</tr>
<tr>
<td>Feb-Apr</td>
<td>9.1 8.1 8 7.9 8.5</td>
<td></td>
</tr>
<tr>
<td>May-Jul</td>
<td>7.2 7.8 9.1 10.2 8.6</td>
<td></td>
</tr>
<tr>
<td>Aug-Oct</td>
<td>9.1 8.2 9.5 9.3 11.1</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean of three replicates
KACA- Kagithapuram C. equisetifolia rhizosphere soil, KAEU- Kagithapuram E. camaldulensis rhizosphere soil, MACA- Masagoundanpudur C. equisetifolia rhizosphere soil, MOCA- Moolimangalam C. equisetifolia rhizosphere soil, MUEU- Murthypalayam E. camaldulensis rhizosphere soil, EC-Electrical conductivity, ESP-Exchangeable sodium percentage

Population Density of Fungi

To understand the existence of microbial species in natural ecosystems, their isolation and characterization is important. The population density of fungi varied in all seasons at the different study locations. In PDA, the maximum population was detected from the MACA rhizosphere soil (30.2 x 10³ cfu/g) during the Nov-Jan season, followed by that in the soil from KAEU (22.0 x 10³ cfu/g) during the Aug-Oct season; a very low population was detected in the KACA rhizosphere soil (4.7 x 10³ cfu/g) during the Feb-Apr season. In RBA, the maximum population was detected from the MUEU rhizosphere soil (19.5 x 10³ cfu/g) during May-Jul, followed by that in the KACA rhizosphere soil (16.2 x 10³ cfu/g) during Nov-Jan; very less population was observed in the KACA rhizosphere soil (2.7 x 10³ cfu/g) during May-Jul season. Therefore, Nov-Jan and May-Jun were optimum for fungal growth. PDA was more suitable for fungal growth than RBA (Table 2).

Table 2: Population density of fungal colonies isolated from rhizosphere soils

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Location</th>
<th>Quarters of sample collection</th>
<th>Fungi (CFU 10³ g⁻¹)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PDA</td>
</tr>
<tr>
<td>1</td>
<td>KACA</td>
<td>Nov-Jan</td>
<td>19.75b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.750a</td>
</tr>
<tr>
<td>2</td>
<td>KAEU</td>
<td>Feb-Apr</td>
<td>2.75a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>May-Jul</td>
<td>8.000a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aug-Oct</td>
<td>16.75b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nov-Jan</td>
<td>20.75b</td>
</tr>
<tr>
<td>3</td>
<td>MACA</td>
<td>Feb-Apr</td>
<td>8.250ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>May-Jul</td>
<td>12.25a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aug-Oct</td>
<td>22.00b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nov-Jan</td>
<td>30.25c</td>
</tr>
<tr>
<td>4</td>
<td>MOCA</td>
<td>Feb-Apr</td>
<td>16.50c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>May-Jul</td>
<td>19.75b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aug-Oct</td>
<td>15.75a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nov-Jan</td>
<td>19.20b</td>
</tr>
<tr>
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<td>MUEU</td>
<td>Feb-Apr</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>May-Jul</td>
<td>11.50a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aug-Oct</td>
<td>15.00ab</td>
</tr>
</tbody>
</table>

Means, in a column, followed by common letter (s) are not significantly different at p<0.05 level according to DMRT.

Foot Note

A total of 68 fungal colonies with different morphology were selected from various study sites, and the pure cultures were stored for further use.

**Screening for the NaCl Tolerance of the Isolated Rhizosphere Fungal Colonies**

Seventeen fungal isolates from the KACA plantation showed good mycelium growth in control (without NaCl) and in the 5% NaCl supplemented medium; 16 isolates, at 10%; 5 isolates, at 15%; and one isolate, at 20% salt concentration. In the KAEU plantation, 17 fungal isolates showed good growth in the control and in the 5% NaCl concentration; 11 isolates, at 10%; 3 isolates, at 15%, and only one isolate, at 20% NaCl concentration. Among the species from the MACA plantation, 11 fungal isolates were screened for NaCl tolerance. All isolates showed maximum mycelium growth in the control, and at 5% and 10% salt concentration; three isolates, at 15% and two isolates, at 20% NaCl concentration. In case of MOCA plantation, 12 fungal isolates showed maximum mycelium growth in the control, and at 5% and 10% salt concentration. Among the 12 isolates, 6 were tolerant to 15% NaCl and one isolate was tolerant to 20% NaCl. In the samples from MUEU plantation, 10 fungal isolates displayed maximum growth in the control and at 5% salt concentration, 7 isolates, at 10%; 5 isolates, at 15%; and 2 isolates, at 20% NaCl concentration. Number of fungal isolates showing NaCl tolerance is presented in Figure 1.

![Salt tolerant fungal isolates](image)

**Foot Note**

C-medium without NaCl (0%), 5%-NaCl, 10%-NaCl, 15%-NaCl, 20%-NaCl

KACA- Kagithapuram *C. equisetifolia* rhizosphere soil, KAEU- Kagithapuram *E. camaldulensis* rhizosphere soil, MACA- Masagoundanpudur *C. equisetifolia* rhizosphere soil, MOCA- Moolimangalam *C. equisetifolia* rhizosphere soil, MUEU- Murthypalayam *E. camaldulensis* rhizosphere soil

**Plant Growth-Promoting Traits**

The data on qualitative P solubilization are presented in Figure 2. Among the 7 fungi, F2 and F62 showed maximum P solubilization, F2 had the highest value at 27.00 SE (Solubilization Efficiency) followed by F62 (12.00 SE). Two isolates (F2 and F62) show P solubilization in 20% NaCl amended Pikovskaya agar medium.

The data on qualitative K solubilization are presented in Figure 3. The isolates, F2 and F36 displayed K solubilization at 20% salt concentration; F36 exhibited 40.00 SE followed by F2 with 12.00 SE. The isolates F2 and F36 show maximum K solubilization at 20% NaCl incorporated medium.
Fig. 2: Phosphate solubilization efficiency (SE) of fungal isolates

Fig. 3: Potassium solubilization efficiency (SE) of fungal isolates

Fig. 4: Zinc solubilization efficiency (SE) of fungal isolates
The data on qualitative Zn solubilization are presented in Figure 4. Among the tested rhizosphere fungal isolates, none of the isolates showed Zn solubilization at 20% NaCl concentration.

The isolate, F45 showed 15.00 SE in up to 15% NaCl; F25 shows 10.00 SE in 10% NaCl amended medium.

The isolate, F45 showed 15.00 SE in up to 15% NaCl; F25 shows 10.00 SE in 10% NaCl amended medium.

Quantitative Production of IAA and EPS
F36 exhibited the maximum IAA production (50.6 μg/ml) at 20% NaCl concentration, followed by F2 (26.0 μg/ml), while F45 showed the least production (12.1 μg/ml) at maximum NaCl concentration; when the salt concentration was increased, the fungal isolates, F53 and F62 did not produce any IAA. EPS production was highest in F36 (3.54 g/ml) followed by F45 (2.1 g/ml) and F62 (1.56 g/ml) at 20% NaCl concentration. It was found that the increasing salt concentration affect the production of EPS in F53 isolate. The IAA and EPS production efficacy of selected isolates under different salt concentration is presented in Figures 5 and 6.

Primarily, fungal colony morphology (macroscopic appearances) including mycelial colour, nature of pigments produced, growth rate, and microscopic characteristics including spore type and hyphal structure were evaluated (Table 4).

Molecular characterization of selected PGP microbes
The PGP fungal isolates showing maximum salt tolerance and in vitro plant growth-promoting activity (F2, F36, F45, F53, and F62) were selected for molecular identification. The 18S rRNA genes were successfully amplified using PCR. The BLAST comparison searches with NCBI nucleotide database revealed 99% similarity of the isolate F2 with *Pencillium citrinum* (Genbank accession number: MF170859), F36 with *Trichoderma viride* F1 (Genbank accession number: MF170862), F45 with *Trichoderma viride* F2 (Genbank accession number: MF170858), F53 with *Periconia variicolor*
(Genbank accession number: MF170861), and F62 with *Acremonium borodinense* (also known as *Bulbithecium borodinense*) (Genbank accession number: MF170860) (Table 5).

Table 4: Morphological and cultural characteristics of fungi

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Macroscopic appearance</th>
<th>Microscopic appearance</th>
</tr>
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<tbody>
<tr>
<td>F2</td>
<td>Grey green colour</td>
<td>Conidia-globose to subglobose</td>
</tr>
<tr>
<td></td>
<td>velvety colonies</td>
<td>Absent</td>
</tr>
<tr>
<td>F36</td>
<td>Blue green colour</td>
<td>Conidia-globose</td>
</tr>
<tr>
<td></td>
<td>, concentric rings</td>
<td>Absent</td>
</tr>
<tr>
<td>F45</td>
<td>Light green colour</td>
<td>Conidia-globose</td>
</tr>
<tr>
<td></td>
<td>powdery colonies</td>
<td>Absent</td>
</tr>
<tr>
<td>F53</td>
<td>White puffy colonies</td>
<td>Conidia-Verrucose</td>
</tr>
<tr>
<td>F62</td>
<td>White cottony colonies</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absent</td>
</tr>
</tbody>
</table>

Fig. 5: Quantitative estimation of IAA production

Fig. 6: Quantitative estimation of EPS production
Table 5: List of salt-tolerant fungi identified using 18s rRNA sequencing

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Isolate code</th>
<th>Organism isolation site and sample</th>
<th>Organism identified as</th>
<th>Genbank accession number</th>
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<tr>
<td>1</td>
<td>F2</td>
<td>Kagithapuram C. equisetifolia rhizosphere soil</td>
<td><em>Penicillium citrinum</em></td>
<td>MF170859</td>
</tr>
<tr>
<td>2</td>
<td>F36</td>
<td>Kagithapuram <em>Eucalyptus</em> rhizosphere soil</td>
<td><em>Trichoderma viride</em> F1</td>
<td>MF170862</td>
</tr>
<tr>
<td>3</td>
<td>F45</td>
<td>Masagoundanpodur <em>Casurina equisetifolia</em> rhizosphere soil</td>
<td><em>Trichoderma viride</em> F2</td>
<td>MF170858</td>
</tr>
<tr>
<td>4</td>
<td>F53</td>
<td>Moolimangalam <em>Casurina equisetifolia</em> rhizosphere soil</td>
<td><em>Periconia variicolor</em></td>
<td>MF170861</td>
</tr>
<tr>
<td>5</td>
<td>F62</td>
<td>Murthypalayam <em>Eucalyptus</em> rhizosphere soil</td>
<td><em>Acremonium borodinense</em> (Bulbithecium borodinense)</td>
<td>MF170860</td>
</tr>
</tbody>
</table>

The phylogenetic tree of the PGP fungal isolates was constructed using neighbor-joining bootstrap analysis (Figure 7a, 7b, 7c, 7d, 7e). The identified colonies are shown in Figure 8.
Discussion

Physico-Chemical Parameters of Soil Samples

The physico-chemical properties of five different soil samples, collected from selected agroforestry
plantation sites at quarterly intervals were evaluated. The pH of soil samples was neutral throughout the study time. The soil nutrient parameters were not constant across the different study locations during the study period; this could be attributed to differences in irrigation water, application of chemical fertilizers, temperature, and rainfall. Similar results were reported earlier for tropical soils. Rousk reported variations in soil pH, due to different biotic and abiotic factors during their study period.

The soil with EC >4 dS/m, ESP >15%, and pH <8.5 is considered as saline or salt affected soil (U.S salinity laboratory staff, 1954). In this study, EC > 4 dS/m was observed in most of the soil samples at the various study sites. Among the four seasons, the soil samples collected during summer (Feb-May) showed the highest level of EC. This may be due to less rainfall, saline water irrigation, especially the use of industrial treated effluents, use of chemical fertilizer, and high temperature. These promote the accumulation of soluble salts and their retention near the soil surface, resulting in higher EC. Similar results were reported by Iwai increased level of EC from 1.80 to 28.60 dS/m during the dry season. Sharma reported that the higher evaporation during dry seasons results in the accumulation of soluble salts at the upper surface of the soil owing to capillary rise. Kawser reported the variation in the salinity of soil samples due to climate change throughout the year; low salinity was observed during rainy season and high salinity was observed during the dry winter and summer seasons, in the upper soil horizons; these results are similar to that obtained in this study.

Diversity Status of Microorganisms in Salt-Affected Soil

Several PGP microbes are used as bio-fertilizers worldwide, contributing to increasing soil fertility and plant growth and yield. Primary metabolites such as amino acids, carbohydrates, organic acids, glucosinolates, and vitamins, as well as secondary metabolites such as alkaloids, flavonoids, phenolics, sulfur-containing compounds, and terpenoids, are secreted from the roots of crop plants, and they help the rhizosphere microflora to develop, communicate, and interact with one another. The beneficial microbial communities are better attracted to and increased by the exudates, which also destroy pathogenic organisms by preventing their growth and invasion. It was found that, numerous soil
factors impact the microbial community and its functions, thereby influencing nutrient availability and plant growth.

In this study, 68 rhizosphere fungal isolates were obtained from different rhizosphere regions. The population density of fungi varied among and within the different locations and periods. This could be attributed to the varying parameters such as physico-chemical properties of the soil, environmental conditions, salinity, and temperature. Msimbira and Smith\textsuperscript{35} reported that the biological activity and composition of soil microbes are influenced by various factors including physico-chemical properties of soil, temperature, and vegetation. Ponmurugan and Gopi\textsuperscript{36} described that the variation in population density of different beneficial microbes could be attributed to several reasons such as soil nutrients, pH levels, moisture content, organic matter, and certain soil enzyme activities. Similar to the results of this study, Grishkan\textsuperscript{37} stated that the hypersaline desert soils have a less richness of fungi. Similarly, Ibekwe\textsuperscript{38} suggested that changes in microbial density could be the first indicator of stress in saline or salt-affected soils. Zhang\textsuperscript{39} showed that salinity has a negative impact on microbial abundance, diversity, composition, and functions.

In this study, we aimed to determine the salt tolerance ability of soil fungi under \textit{in vitro} conditions. Among the 68 soil fungi studied, only seven fungal isolates showed tolerance to 20\% NaCl; a steady state of growth was observed in the maximum isolates up to 10\% salt concentration. There was a drastic reduction of number of colonies observed when the salt concentration was increased above 10\%. The reduction in population density with increasing concentration of NaCl could be attributed to hyper-osmolarity, which results in reduction of microbial cytoplasmic water activities. Dave and Desai\textsuperscript{40} isolated bacteria and fungi at different NaCl concentrations from marine salterns, Bhavnagar, Gujarat, India, and they reported similar results; the total microbial count and overall diversity of beneficial microbes decreased with increasing NaCl concentration. Oren,\textsuperscript{41} documented that halophiles can be categorized into three groups based on their salt tolerance levels: slight halophiles, moderate halophiles, and extreme halophiles. Slight halophiles exhibit optimal growth within a 0.2–0.5 M (1–3\%) NaCl concentration. Moderate halophiles thrive in environments with a 0.5–2.5 M (3–15\%) NaCl concentration, while extreme halophiles can endure and grow in conditions with a higher salt concentration, ranging from 2.5–5.2 M (15–30\%) NaCl. In the present investigation, out of the 68 isolates examined, only 7 (10.29 \% isolates) demonstrated a notable salt tolerance of up to 20\% NaCl concentration. Interestingly, among these, 5 isolates exhibited multiple PGP activities in conditions of elevated salt concentration. Effectively utilizing this extreme halotolerant PGP isolates in saline-affected soil can significantly enhance plant growth and productivity.

Mayak\textsuperscript{42} suggested that the selection of beneficial microbial isolates from naturally-stressed environment or rhizosphere is a possible measure for improving crop health because it can be used to control diseases and promote plant growth. Choudhary\textsuperscript{43} suggested that stress can be detrimental for sensitive microorganisms; it reduces the activity of surviving cells, owing to the metabolic load imposed by stress tolerance mechanisms. Arit\textsuperscript{44} reported that the adverse effects of high salt concentrations in soil leads to dispersion and flocculation of soil particles; in addition, it affects organic matter solubility and its availability. This affects the microbial community structure. In a previous study,\textsuperscript{45} we isolated 16 salt-tolerant bacterial isolates from the rhizosphere soil of \textit{Casuarina equisetifolia} and the isolates showed a maximum salt tolerance of up to 12\% NaCl.

**Screening for PGP Traits**

PGP microbes under salt stress exhibit positive effects on plants; they positively influence the germination percentage, drought tolerance, shoot and root weight, plant growth, and yield. Abbas\textsuperscript{46} described that the plants grown in salt-affected soil harbor diverse group of microbes in their rhizosphere, which have the potential to cope with salinity. These halotolerant microbes assist plants in withstanding the increased concentration of salt through the production of different organic and inorganic compounds such as IAA, ACC deaminase, and volatile compounds.
In this study, nitrogen fixation, and the production of siderophore, ammonia, ACC deminase, IAA, and EPS were analyzed to prove the PGP ability of selected fungal isolates. Plant growth is improved by the mineralization of nutrients, mediated by PGPF, in the soil. It reduces the breakdown of the substrates into soluble molecules that can be absorbed by the plants. An essential microelement that supports plant growth is phosphours (P). In this study, P solubilization was evaluated using Pikovskaya agar medium, in which a fungal isolate (F2) showed 27.00 SE (Solubilization efficacy) at 20% NaCl concentration. There was a wide variation in the solubilization of P under different salt concentrations. Srividya reported the effect of salinity on the phosphate solubilization by A. niger F7 isolated from agricultural soils, and the isolate showed different levels of P solubilization under different saline conditions; it could tolerate a maximum salinity of 2 % NaCl, this level of tolerance is low when compared to that exhibited in this study. Malgioglio reported that the complex organic phosphorous was solubilized and mineralized into different small molecules by PGPF, through the stimulation of enzymes like phosphatases, phytase, organic acids, and inorganic acids.

K solubilization was carried out in Aleksandrov agar medium. Among the different fungal isolates, F36 showed the maximum K solubilization efficacy (40.00) at 20% NaCl concentration, when compared with the others. Similarly, Bhattacharya isolated K solubilizing microbes from saline soil. Parmar and Sindhu reported that the soil beneficial microorganisms such as bacteria, fungi, and actinomycetes solubilize the insoluble potassium to soluble forms through different mechanisms such as acidolysis, chelation, exchange reactions, and the production of inorganic and organic acids and polysaccharides. Decomposition of organic matter in the soils leads to the production of formic acid, citric acid, and oxalic acid. These organic acids increase the solubilization of K compounds by providing protons and calcium ion complex.

Zinc is a micronutrient essential for plants in very low amounts. In this study, Zn solubilization was carried out by using Bunt and Rovira agar medium; no fungal isolates exhibited Zn solubilization at 5, 10, 15 and 20% NaCl concentration. Tavallali reported that the deficiency of Zinc leads to susceptibility to heat, light, and fungal infections; in addition, it affects grain yield, pollen formation, root development, and water uptake in agricultural crops. Saravanan reported that the production of siderophore leads to Zn solubilization. Zn solubilizing microorganisms solubilize zinc through various mechanisms. These microbes produce anions by lowering the pH and chelate zinc for solubilization. Among the different PGPF, Trichoderma sp. promotes the absorption of minerals and nutrients, mainly, Fe, K, N, and P.

HCN production enhances the availability of P and other elements for plant growth. Therefore, HCN production by the microbes at high salt concentration is considered as one of the significant plant growth promoting traits. We determined the HCN production by different fungal isolates. The HCN production facilitates antibacterial and antifungal activity on plant pathogens. Anand reported that HCN could be toxic for plant pathogens. Rijavec highlighted that HCN is a bio-control agent against fungal pathogens. It has the ability to form complexes with transitional metals in the mineral substrate, the ecological function of HCN is based on its interference with the release of elements from the mineral substrate. Recent studies on mineral weathering in natural environments indicated that HCN-producing bacteria (HPB) promoted the mobilization of elements from rocks forming the minerals. HCN production is reported in various PGPFs such as Aspergillus niger, Penicillium sp., and Trichoderma sp.

In this study, the amount of siderophore production gradually increased with increase in salt concentration up to a certain level. All isolates showed siderophore production, which was a significant observation. Iron is the fourth major requirement for plants as well as microbes but is unavailable for direct assimilation. Ferric ion is pre-dominant in nature, and it is sparingly soluble (10−18 M), this makes its concentration too low to support plant growth. Therefore, plants and microbes sequester biologically available iron through secretion of siderophores in iron-stressed conditions. Crowley reported siderophore production in two PGPF, P. chrysogenum and R. arrhizus, this upgraded Fe content and promoted growth in cucumber, maize, and tomato plants, and augmented the chlorophyll content through an
increase in Fe-EDTA availability. The production of siderophores by a number of PGPF, including Absidia spp., Aspergillus spp., Thermoascus aurantiacus, Aspergillus flavus, A. niger, A. tamarii, A. nidulans, Fusarium sp., Paecilomyces variotii, Cunninghamella sp., and Penicillium are extensively reported.

All selected PGP isolates were positive for ACC deaminase activity. Synthesis of ACC deaminase under different salinity ranges, 5 to 20% NaCl, indicates its role in plant growth by reducing ethylene stress. Plant growth promotion by selected PGP microbes, irrespective of salinity can be correlated with the higher production of the ACC deaminase enzyme. Similar results were reported by Zhang67 and Viterbo68 in Trichoderma spp., which are known for their potential to hydrolyse ACC into ammonia for plant growth. ACC deaminase is found in Trichoderma sp. The inoculation of plants with PGP microbes that express ACC deaminase activity facilitate extensive root growth in seedlings due to the reduction in ethylene stress69; this imparts resistance to crop plants against various stresses including salinity.

All PGPF were screened for IAA production under in vitro conditions. Among 5 isolates, F36 and F2 exhibited the highest IAA production at a 20% NaCl concentration, surpassing the others. In contrast, the remaining isolates demonstrated a notable decline in IAA production as the salt concentration increased. IAA, a plant hormone, is a natural auxin produced by PGPF. The microbes isolated from salt-affected soils can tolerate salt stress. Numerous investigations have illustrated the role of microorganisms in triggering indole-3-acetic acid (IAA) signaling pathways and augmenting the endogenous IAA levels in plants grown in salt stressed areas.

Microbial phytohormones modulate physiological processes such as root cell growth and tissue differentiation and boost rhizosphere microbes. Murali66 isolated Penicillium janczewskiyi and reported that it can inhibit Rhizoctonia solani, a plant pathogen that causes stem rot, through IAA production. Various fungal endophytes, including Aspergillus flavus, Aspergillus niger, Fusarium oxysporum, Penicillium corylophilum, Paecilomyces Formosus and Penicillium funiculosum have been documented for their ability to promote plant growth and produce indole-3-acetic acid (IAA) as reported by Deng and Cao.74 Additionally, Muhammad75 highlighted the capacity of Penicillium roqueforti to synthesize IAA, thereby assisting host plant species in coping with stressful saline conditions.

The tested PGPF isolates produce significant amounts of EPS even at higher NaCl concentration in F36 (3.54 g/ml) followed by F45 (2.1 g/ml) and F62 (1.56 g/ml) isolates; however, EPS production decreases with increasing salt concentration in other tested isolates. EPS constitute a minor fraction (0.1 to 1.5%) of the total soil organic matter. Their unique water-holding and cementing properties play a vital role in the formation and stabilization of soil aggregates and regulation of nutrient and water flow across the plant roots.76 EPS is added to the soil as either sloughed off plant parts and root exudates of intact plants or capsular and slime materials secreted by soil microbes. EPS, or exopolysaccharides, forms a binding association with sodium ions, consequently alleviating the impact of elevated soil salinity. The exopolysaccharides produced by microorganisms play a crucial role in mitigating salt stress by preserving the Na+/K+ balance, thereby aiding plants in surviving adverse soil conditions.

Osemwegie78 listed different types of EPS produced by common fungal species such as Botryosphaeran sp. (Botryosphaeran) Aureobasidium pullulans (Pullulan), Schizophyllum commune (Schizophyllan), and Aspergillus fumigatus (Galactosaminoglucon). Abbas66 reported that the EPS produced by PGP rhizobacteria have a significant role in plant growth during salinity stress conditions; they achieve this by producing hydrophilic compounds, in addition, they are involved in formation of a coating around the rhizosphere root.

Molecular Characterization of Selected PGPF
The isolates possessed significant salt tolerance; the PGPF activity under maximum salinity was identified using 18S rRNA sequencing for the identification of fungi and identified as Penicillium citrinum (MF170859), Trichoderma viride F1 (MF170862), Trichoderma viride F2 (MF170858), Acremonium borodinense (MF170860), and Periconia varicolor (MF170861). To the best of our knowledge, this is the first report on the isolation of salt-tolerant PGPF from the rhizosphere of agroforestry tree species. Nazareth and Marbaniang79 isolated a halotolerant Penicillium sp. from mangroves and salterns,
recorded their resistance towards heavy metals. All Penicillia showed extreme halo tolerance, and they are able to grow in the absence of salt as well. Plants inoculated with *Trichoderma* isolates exhibit tolerance against biotic and abiotic stresses, through increased nutritional uptake, enhanced root length, and induced protection against oxidative stress. Adedayo and Babalola explained the importance of plant growth promoting fungi such as *Aspergillus flavus*, *Actinomucor elegans*, *Gliocladium virens*, *Penicillium digitatum*, *Podospora bulbillosa*, *Trichoderma* sp., and Arbuscular mycorrhizal fungi, they are ecofriendly and enhance the yield of crops through promoting the growth of the roots and shoots and increasing the germination of seeds and the production of chlorophyll for photosynthesis, resulting in high crop yields. Dukare reported the plant growth promoting traits of the rhizosphere fungi, *Trichoderma* species (T. *viride*, T. *harzianum*) and *Penicillium chrysogenum*. Plant cell walls are stimulated by PGPF; this helps avoid nutrient loss during abiotic stress conditions.

**Conclusion**

The global population is estimated to be 7.8 billion. This number is expected to rise to 9.7 billion by 2050. More than half of the lands are affected by salinity typically due to secondary salination, and most of these lands are not suitable for agriculture production. Agroforestry is one of the important alternative income sources for farmers, who own salt-affected lands in the study locations. Agroforestry can improve and restore soil quality and bio-diversity, enhance nutrient cycling, increase carbon sequestration, and reduce climate pressure in degraded lands. With increasing demand for industrial wood, fuel, and timber, plantation forestry, especially those outside the forest area has gained importance. The high salinity and low soil fertility in tropical regions results in poor plant growth and mortality of newly-transplanted tree seedlings. PGP microbes are bio-inoculants, which are promising in forestry for increasing the survival and growth of tree species in saline lands and in other problematic soils.

In this study, we aimed to isolate and identify the native salt-tolerant PGPF from agroforestry plantations in saline soils at different study sites. However, there was only limited data on the presence of free-living rhizosphere soil fungi. We isolated five promising salt-tolerant PGPF and evaluated the possibility of their application as potential bioinoculants that could be cost-effective, more economical than the application of synthetic chemical fertilizers. Once these stress-tolerant bio-inoculants are established in the roots of selected tree seedlings, they can further multiply in the roots and supply the essential nutrients to the host plants in the salt-affected areas, in addition, they can help plants survive better in stressed areas. This study provides valuable insights into the diversity status of salt-tolerant fungal species from agroforestry areas in Karur district; however, it has certain limitations associated with sample collection. The samples were collected only from one particular district and the sample size was small. These limitations highlight the need for further research using a larger sample size to enhance the validity and generalizability of the findings. By addressing these limitations, future studies can contribute to a more comprehensive understanding of the large diversity of fungal isolates and their PGP activity.

**Authors’ Contribution**

Saranya Devi K - analysis and interpretation of results, manuscript preparation. Mohan V- study conception and design, final review of manuscript.

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**Conflict of Interest**

Authors have declared that no competing interests exist.
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