The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
20(3): 385-396, 2025 www.thebioscan.com

PLANT GROWTH PROMOTION BY HALOTOLERANT *ASPERGILLUS* STRAINS

1. **SUVARNA DNYANESHWAR PATIL**
2. **DR. ARPANA HEMRAJ JOBANPUTRA**
3. **DR. NAZIYA M. A. REHMAN-Corresponding author**
4. **UMANATH GULABRAO FULPAGARE**
5. **MS. SAROJINI RANGARAO DESHMUKH**
6. **JAYASHRI VITHHAL BORASE**

1. Suvarna Dnyaneshwar Patil, Research centre:PSGVPM's S.I.P. Arts, G.B.P. Science, S.T.K.V. Sangh Commerce College, Shahada. Dist: Nandurbar, Maharashtra, Email ID: suvarna1210@gmail.com
2. Prof. Dr. Arpana Hemraj Jobanputra, PSGVPM's S.I.P. Arts, G.B.P. Science, S.T.K.V. Sangh Commerce College, Shahada. Dist: Nandurbar, Maharashtra. Email: arpanahjobanputra@gmail.com
3. Dr. Naziya M. A. Rehman, Dr.BAMU, Sambhaji Nagar. 431004, MH.INDIA. Email- nazirehman786@gmail.com
4. Umanath gulabrao fulpagare, PSGVPM's S.I.P. Arts, G.B.P. Science, S.T.K.V. Sangh Commerce College, Shahada.
5. Dist: Nandurbar, Maharashtra. Email: umeshfulpagare25 @gmail.com
6. Ms. Sarojini Rangarao Deshmukh, PSGVPM's S.I.P. Arts, G.B.P. Science, S.T.K.V. Sangh Commerce College, Shahada. Dist: Nandurbar, Maharashtra. Email- sarojinigaikwad1974@gmail.com
7. Jayashri Vithhal Borase, Research centre: PSGVPM's S.I.P. Arts, G.B.P. Science, S.T.K.V. Sangh Commerce College, Shahada. Dist: Nandurbar, Maharashtra. Email- Jayashriborase84@gmail.com

DOI: 10.63001/tbs.2025.v20.i03.pp385-396

KEYWORDS
Halotolerant PGPR, Plant growth promotion, Microbial bioinoculants, Aspergillus species, Saline agriculture
Received on:

23-05-2025

Accepted on:

18-06-2025

Published on:

31-07-2025

ABSTRACT

This study aimed to isolate, characterize, and evaluate halotolerant rhizospheric fungi from saline soils in Maharashtra, India, for their plant growth-promoting potential. Soil samples from four ecologically distinct sites—Lonar Lake, Mehrun Lake, Girgaon Coastal Region, and Surat Coastal Region—were analyzed for physicochemical properties (pH, EC, texture). Fungal isolates were obtained via serial dilution and plating on PDA with chloramphenicol, followed by halotolerance screening (2–14% NaCl). Among 20 isolates, 18 exhibited significant salt tolerance, with *Aspergillus versicolor* (F17) and *Aspergillus proliferans* (F20) showing the highest growth at 14% NaCl. Morphological, physiological, and molecular characterization (ITS sequencing) confirmed their identities.

The isolates demonstrated multiple plant growth-promoting traits, including phosphate solubilization (F17: 11 cm halo; F20: 5 cm), zinc solubilization (F17: 12 cm halo; F20: 3 cm), ammonia production (F20: 7.8 µg/mL; F17: 7.2 µg/mL), nitrogen fixation (F20 > F17), EPS production (F17: 1300 µg/mL; F20: 1200 µg/mL), and IAA synthesis (F20: 7.8 µg/mL; F17: 7.2 µg/mL). In pot and seed germination assays, both fungi significantly enhanced wheat and maize growth compared to controls. F17-treated plants showed higher biomass (950 mg maize; 855 mg wheat fresh weight), while F20 improved root and shoot elongation (4.5 cm plant height in wheat).

These findings highlight the potential of *Aspergillus versicolor* (F17) and *Aspergillus proliferans* (F20) as biofertilizers for saline agriculture due to their halotolerance, nutrient solubilization, and plant growth promotion.

INTRODUCTION

Soil salinity is a growing global concern, affecting over 800 million hectares of arable land worldwide and posing serious challenges to agricultural productivity and ecological stability (FAO, 2021). In salt-affected soils, native microorganisms—particularly rhizospheric fungi—play a crucial role in supporting plant growth through nutrient solubilization, phytohormone production, and enhancement of osmotic stress tolerance

(Egamberdieva *et al.*, 2017). Among these, halotolerant fungi have emerged as promising bioresources due to their ability to persist in high-salinity environments while contributing to plant health and soil remediation.

Fungal genera such as *Aspergillus* and *Penicillium* are frequently reported from saline habitats and are recognized for their metabolic versatility and adaptability (Nazareth & Gonsalves, 2014). These fungi produce a wide array of extracellular enzymes and bioactive metabolites that enable them to thrive under salt stress, while simultaneously offering benefits to host plants. *Aspergillus versicolor*, in particular, has demonstrated the ability to synthesize hydrolytic enzymes like amylases and cellulases under saline conditions, underscoring its relevance in biotechnological and agricultural applications (Prasad *et al.*, 2011).

Despite their potential, systematic investigations of halotolerant fungi from saline ecosystems in India remain limited. The coastal and inland saline regions of Maharashtra, including Lonar Lake, Mehrun Lake, the Girgaon Coastal Region, and the Surat Coastal Region, represent underexplored microbial niches with the potential to harbor unique stress-adapted fungal communities. Previous studies have highlighted the importance of such organisms in bioremediation, biofertilizer development, and enzyme production, particularly in relation to sustainable agriculture in marginal lands (Raghukumar, 2008; Mapelli *et al.*, 2013).

The present study was therefore designed with the following objectives:

1. To isolate and characterize halotolerant rhizospheric fungi from four ecologically distinct saline habitats in Maharashtra.
2. To evaluate their salt tolerance under increasing concentrations of NaCl and KCl.
3. To assess their biotechnological potential through physiological, biochemical, and molecular profiling.

By addressing the functional diversity and adaptive traits of halotolerant fungi, this research contributes to the broader goal of leveraging microbial resources for sustainable agriculture and environmental resilience in salt-affected regions.

2.1. Collection and Isolation of Salt-Tolerant Rhizospheric Fungi

2.1.1. Soil Sample Collection

Soil samples were collected from four ecologically distinct locations across Maharashtra, India—namely, Lonar Lake, Mehrun Lake, Girgaon Coastal Region, and Surat Coastal Region. Sampling was performed using a sterilized hand auger, extracting soil from a depth of 0–20 cm. Special care was taken to avoid disturbing plant root systems. Bulk soil adhering to roots was carefully removed, and the rhizospheric soil—closely associated with the root surface—was collected by gentle shaking. Residual particles were brushed off using sterile tools. The soil samples were labelled on-site, placed in sterile polyethylene bags, and transported to the laboratory under cold conditions (4°C) to preserve microbial viability until fungal isolation.

2.1.2. Preliminary Analysis of Soil Samples

Collected soil samples were analyzed for key physical and chemical properties, including texture, color, pH, and electrical conductivity (EC). EC was measured using a standard saturated paste method, where a soil-deionized water mixture was prepared and the supernatant extracted. The conductivity of the solution was then recorded using a digital EC meter. These parameters provided baseline data on the saline conditions of the sampling sites, which are critical for understanding fungal halotolerance.

2.1.3. Isolation of Rhizospheric Fungi

Rhizospheric fungi were isolated using serial dilution and plating techniques. Ten grams of each soil sample were suspended in 90 mL of sterile saline and subjected to serial dilutions. From appropriate dilutions, 100 µL aliquots were spread onto Potato Dextrose Agar (PDA) plates supplemented with chloramphenicol (50 µg/mL) to suppress bacterial growth. The plates were incubated at 28 ± 2°C for 4–7 days. Emergent fungal colonies were sub-cultured repeatedly onto fresh PDA plates to obtain pure isolates. Control plates without soil inoculum were used to check for media sterility.

2.1.4. Screening for Halophilic Potential

To assess salt tolerance, the purified fungal isolates were cultured on PDA medium amended with increasing concentrations of NaCl—2%, 4%, 6%, 8%, 10%, 12%, and 14%. Control plates without added NaCl were included to evaluate baseline growth. Each fungal inoculum was standardized by using a 5 mm mycelial disc taken from the actively growing margin of a 5-day-old culture. Inoculated plates were incubated at 28°C for 5–7 days. Growth was monitored visually and by measuring colony diameter. Isolates demonstrating consistent and substantial growth at NaCl concentrations above 6% were classified as halotolerant fungi and selected for further analysis.

2.2. Identification and Characterization of Fungal Isolates

2.2.1. Morphological and Cultural Characterization

Initial identification of salt-tolerant fungal isolates was performed based on macroscopic and microscopic features. Cultural characteristics—including colony color, texture, margin, elevation, and sporulation—were recorded from PDA plates. Microscopic examination was conducted using lactophenol cotton blue staining to observe structures such as conidia, hyphae, and spore-bearing organs under a compound microscope.

2.2.2. Physiological and Biochemical Profiling

The ability of each isolate to grow under varied environmental conditions was assessed to support their classification as halophilic fungi. Fungal isolates were grown in media containing NaCl concentrations ranging from 1% to 14%, and at temperatures from 20°C to 50°C, to determine their physiological adaptability.

2.2.3. Molecular Identification

For precise taxonomic identification, genomic DNA was extracted from selected fungal isolates using a standard CTAB-based method. The Internal Transcribed Spacer (ITS) region of ribosomal DNA was amplified using universal fungal primers (ITS1 and ITS4). PCR products were sequenced, and the resulting sequences were compared with existing fungal sequences in the NCBI GenBank database using the BLAST tool. Closest matches were used to determine the species-level identity of the halotolerant fungal isolates.

2.3. Plant growth promoting activities of isolates-

2.3.1. Phosphate solubilisation- A qualitative assay for phosphate solubilisation was conducted using Pikovskaya medium supplemented with tricalcium phosphate (Pikovskaya, 1948). Pikovskaya medium was prepared with varying salt concentrations: 0 mM, 50 mM, 100 mM, and 150 mM. The prepared medium was then poured into sterile plates. Control plates without bacterial inoculation were included for each salt concentration to account for abiotic changes in the medium. All experiments were conducted in triplicates to ensure reproducibility and reliability of the results, and the average values of the measurements were considered for analysis. The experimental plates were spot-inoculated with the bacterial cultures and incubated at 30°C for 48 hours. The formation of a clear halo zone around the culture spot (figure 1) signifies the phosphate solubilisation ability of the isolate. The halo zones were measured in millimetres and recorded.

2.3.2.Zinc solubilisation- A qualitative assay was conducted to evaluate the zinc solubilizing ability of fungal isolates F17 and F20 using modified Tris minimal agar medium supplemented with 0.1% zinc oxide (ZnO) as the insoluble zinc source. Both fungal isolates were spot-inoculated at the center of individual sterile plates under aseptic conditions, and a control plate without inoculation was maintained for comparison. The inoculated plates were incubated at 28 ± 2°C for 5 to 7 days. Following incubation, clear halo zones were observed around the fungal colonies of both F17 and F20, indicating their zinc solubilizing potential. The diameter of the colony and the surrounding clear zone was measured to determine the zinc solubilization index (ZSI), calculated as the ratio of the total diameter (colony + halo zone) to the colony diameter.

2.3.3. Ammonia production-Ammonia production was assessed in a test tube containing 20.0 mL of sterile peptone water at pH 7.2. The test tube was inoculated with a 1.0% (v/v) inoculum and incubated at 30°C with shaking at 120 rpm on an orbital shaker for 144 hours. The amount of NH_3 produced was measured at intervals from 24 to 144 hours. Ammonia estimation was

performed using a spectrophotometric method as outlined by Cappucino and Sherman (1992). One milliliter of culture supernatant was mixed with 0.1 mL of Nessler's reagent, and the final volume was adjusted to 5.0 mL with ammonia-free distilled water. Ammonia production was indicated by a color change from yellow to brown.

2.3.4. Nitrogen fixation- The nitrogen-fixing potential of fungal isolates was assessed using nitrogen-free Jensen's medium supplemented with 0.002% methylene blue as a redox indicator. The medium was prepared with 20 g/L glucose, 0.1 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.2 g/L $CaCO_3$, 0.005 g/L $FeSO_4 \cdot 7H_2O$, 0.002 g/L $Na_2MoO_4 \cdot 2H_2O$, and 15 g/L agar, adjusted to pH 7.0. Fungal isolates previously grown in Potato Dextrose Broth for 3-5 days were inoculated onto the solidified medium using 5 mm mycelial discs, with uninoculated plates serving as negative controls. The plates were incubated at 28±2°C for 5-7 days under aerobic conditions. Growth on the nitrogen-free medium was interpreted as a positive indication of nitrogen fixation capability. The reduction of methylene blue from blue to colorless around fungal colonies provided additional evidence of nitrogenase activity, as this color change indicated the creation of anaerobic microenvironments suitable for nitrogen fixation.

2.3.5. Exopolysaccharides (EPS) Production- Fungal isolates F17 and F20 were inoculated into Luria-Bertani (LB) broth supplemented with 4% glucose to evaluate their exopolysaccharide production. The EPS produced by these fungal cultures was extracted and quantified using the phenol-sulfuric acid method as described by Dubois *et al*. (1956). Briefly, the fungal cultures were incubated and subsequently centrifuged at 10,000 × g for 15 minutes at 4°C to remove the cell biomass. The resulting supernatant, containing the soluble EPS, was treated with three volumes of cold absolute ethanol and incubated at 4°C for 24 hours to allow polysaccharide precipitation.

The precipitated EPS was then recovered by centrifugation, washed with 70% ethanol to eliminate residual impurities, and dried. The dried EPS was dissolved in distilled water, and 1 mL of the solution was mixed with 1 mL of 5% (w/v) phenol, followed by the rapid addition of 5 mL of concentrated sulfuric acid. The mixture was allowed to stand at room temperature for 30 minutes for color development. The absorbance was measured at 490 nm using a UV-Visible spectrophotometer. A standard calibration curve was prepared using glucose solutions of known concentrations to estimate the total carbohydrate content in the EPS samples derived from F17 and F20.

2.3.6. Indole acetic acid production (IAA)- To test IAA production, the isolates were evaluated using a colorimetric method adapted from Sarker and Al-Rashid (2013). Each bacterial isolate was cultured in 5 mL of LB Broth supplemented with 0.1% tryptophan and incubated at 28°C for 72 hours. A control group was prepared with only the growth medium, without any inoculation. Following incubation, the cultures were centrifuged at 3,000g for 10 minutes to separate the supernatant from the cell pellet. IAA concentration in the culture supernatant was estimated using Salkowski reagent (Gordon and Weber 1951). To prepare the Salkowski reagent, a solution was made by adding 2 mL of 0.5 M $FeCl_3$ to 49 mL of distilled water, followed by the careful addition of 49 mL of 70% perchloric acid. For the assay, 2 mL of the Salkowski reagent was mixed with 1 mL of the supernatant and allowed to react at room temperature for 25 minutes, during which a pink color developed in the presence of IAA. The absorbance of the reaction mixture (300 µL) was measured at 530 nm using a Spectramax Microplate reader. A standard curve of IAA was prepared with concentrations of 0, 5, 10, 20, 50, and 100 µg/mL. The concentration of IAA in the supernatant was calculated by comparing the absorbance readings to the standard curve. Each sample was tested in triplicate, and the entire procedure was repeated twice to verify the accuracy and consistency of the results.

2.4. Pot experiment- The experiment involved surface-sterilizing maize and wheat seeds with 0.2% $HgCl_2$, followed by rinsing and soaking them in fungal spore suspensions (1×10^7 spores/mL) of *Aspergillus versicolor* (F17) or *Aspergillus* sp. (F20) for 5 hours. Control seeds were treated with sterile water. The inoculated seeds were sown in autoclaved soil in pots arranged in a randomized block design with three replicates. To ensure fungal-specific adaptations, spore suspensions were standardized (unlike bacterial cultures), and sterility checks were implemented to avoid contamination. Plants were grown in a polyhouse (20–25°C) with weekly sterile irrigation. Growth parameters (height, fresh/dry weight) were recorded at 15, 25, 35, and 45 days after sowing (DAS).

2.4.1. Seed germination assay- A seed germination assay was conducted to evaluate the effects of two fungal isolates, *Aspergillus versicolor* (F17) and *Aspergillus* sp. (F20), on the germination and early seedling growth of maize (Hybrid 9444) and wheat (local variety). Seeds of both crops were first surface-sterilized by immersing them in 0.2% $HgCl_2$ for 2 minutes, followed by three rinses with sterile distilled water. The sterilized seeds were then soaked in fungal spore suspensions (1×10^7 spores/mL, prepared from 5-day-old PDA cultures) of F17 and F20 for 5 hours at room temperature, while control seeds were treated with sterile distilled water. After inoculation, the seeds were placed on sterile, moistened Whatman filter paper arranged in a double-folded manner, with ten seeds per replicate. The paper towels were carefully rolled to maintain seed-moisture contact and transferred into sterile ziplock bags for incubation in a growth chamber at 20-25°C. The experiment included three replicates per treatment for both crops, with moisture maintained by periodic addition of sterile distilled water. Germination was monitored daily, and on days 5 and 7, germination percentage, root length, shoot length, and seedling vigor index were recorded.

2.5. Results and discussion- A total of 120 microorganisms, comprising 100 bacterial and 20 fungal isolates, were obtained from soil samples collected from four locations in Maharashtra, India: Lonar Lake, Mehrun Lake, Girgaon Coastal Region, and Surat Coastal Region. Characteristics of soil samples collected are given in table 1.

Characteristics of soil →	Location of soil collection	Colour of soil	Texture of soil	pH of soil	EC of soil (dS/m)
1.	Lonar Lake	Brown	Loamy	7.30	2.7
2.	Mehrun Lake	Black	Clayey	7.22	4.1
3.	Girgaon Coastal Region	Black	Clayey	7.30	1.8
4.	Surat Coastal Region	Brown	Clayey	7.00	11

Table 1: Details of sample collected from various sites

Sr. No	Isolated Microorganisms	Salt Tolerance Activity			
		12% NaCl	14% NaCl	12% KCl	14% KCl
1	F1	+	+	+	+
2	F2	+	+	+	+
3	F3	+	+	+	+
4	F4	+	+	-	-
5	**F5**	**+**	**+**	**-**	**-**
6	F6	+	+	+	+
7	**F7**	**+**	**+**	**+**	**+**
8	F8	-	-	-	-
9	F9	+	+	+	+
10	F10	+	+	+	+
11	F11	+	+	-	-
12	F12	+	+	-	-
13	F13	+	+	+	+
14	F14	-	-	+	+
15	F15	+	+	-	-
16	F16	+	+	+	+
17	**F17**	**+**	**+**	**+**	**+**
18	F18	+	+	+	+
19	F19	+	+	+	+
20	**F20**	**+**	**+**	**+**	**+**

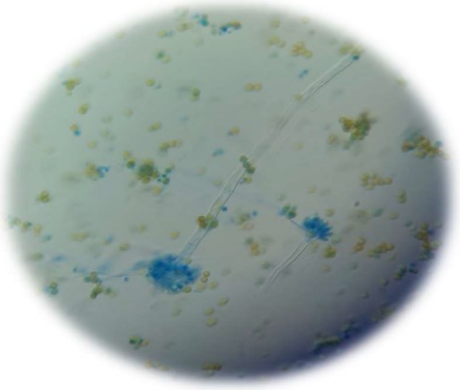
Table 2: Details of fungal isolates collected from various sites

Out of 20 isolates, the majority (18 strains) demonstrated significant salt tolerance, showing positive growth at both 12% and 14% NaCl concentrations. Additionally, many of these isolates also tolerated 12% and 14% KCl, although a few (such as F4, F5, F11, F12, and F15) did not grow under potassium salt stress. Notably, strains F7, F17, and F20 exhibited the highest halotolerance (table 2), showing consistent growth under all tested salt conditions (NaCl and KCl up to 14%), indicating their strong potential as halotolerant fungi. Only two isolates, F8 and F14, failed to grow at any of the tested salt concentrations, suggesting salt sensitivity.

The ability of rhizospheric fungi to tolerate high salinity is a key adaptive trait, particularly in stress-prone environments such as saline soils. In the present study, 18 out of 20 fungal isolates demonstrated growth at both 12% and 14% NaCl concentrations, indicating broad halotolerance. Similar findings were reported by El-Sayed *et al.* (2015), where *Aspergillus* and *Penicillium* species isolated from saline environments showed growth at salt concentrations exceeding 10%. Furthermore, the growth of several isolates (e.g., F7, F17, and F20) under both NaCl and KCl stresses up to 14% highlights their robust osmotolerance, aligning with the work of Raghukumar (2008), who observed comparable dual-salt tolerance in marine-derived fungi.

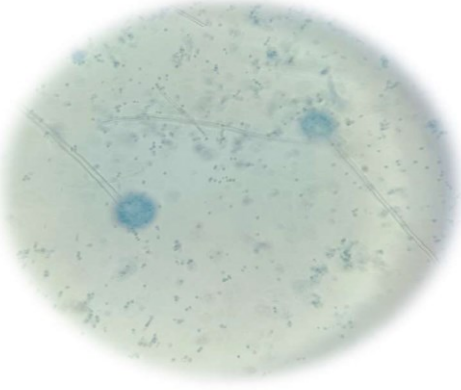
Notably, isolates F7, F17, and F20 were the most halotolerant, thriving under all tested salinity levels, suggesting potential utility in biotechnological applications like saline soil remediation and biofertilizer development under saline conditions (Mapelli *et al.*, 2013). In contrast, isolates F8 and F14 failed to grow under any tested salt stress, indicating their sensitivity and possible restriction to non-saline niches. These findings reinforce the ecological significance of fungal halotolerance and its variation among species and strains, as also observed in previous studies focusing on extremophilic fungi (Nazareth & Gonsalves, 2014).

2.5.1.Morphological and Cultural Characterization-



2. Culture F17

Figure 1-Morphology of F17 under microscope



3. Culture F20

Figure 2-Morphology of F20 under microscope

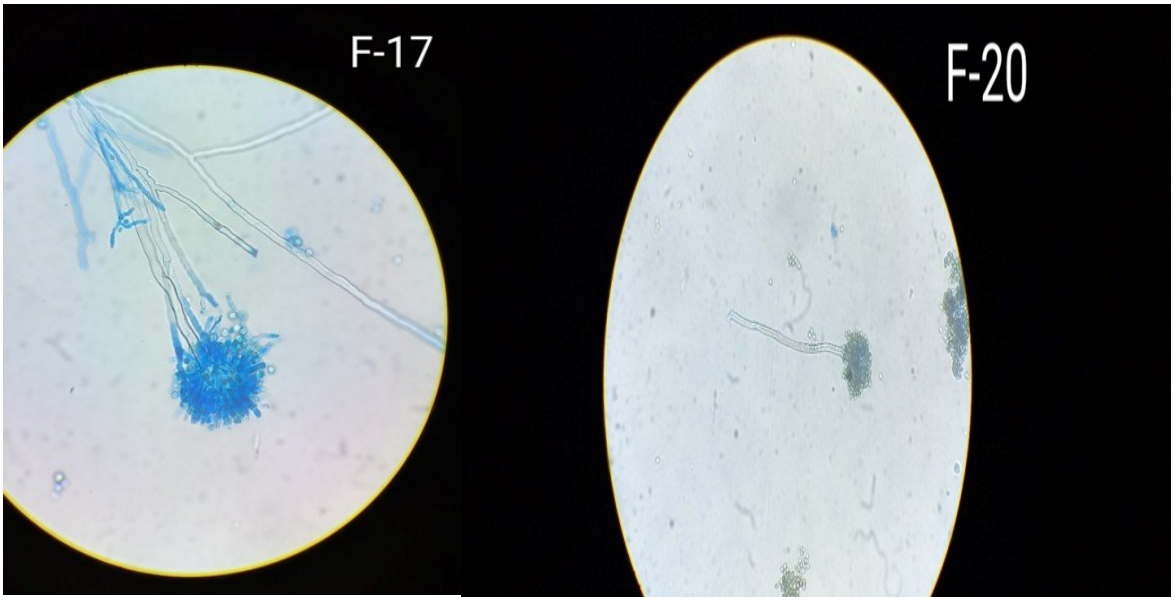


Figure 3-Morphology of F17 and F20 under microscope

Characteristic	Observation	
	F20	F17
Colony Color	White initially; becomes greenish to bluish-green with age	Brownish grey to pinkish brown
Texture	Velvety to powdery; slightly wrinkled in older cultures	Cottony
Reverse Colony Color	Pale yellow to orange	Yellow olive brown to dark brown
Colony Margin	Regular to slightly lobed; well-defined	Irregular
Elevation	Flat to low raised	Elevated center
Sporulation	Abundant; produces dense conidial heads	Abundant on CYA
Hyphae	Septate, hyaline, branched	Septate, hyaline (transparent)
Conidiophores	Arise from foot cells; smooth-walled and relatively short	Smooth-walled, brown; 150–300 µm long, 4–6 µm wide
Vesicles	Globose to subglobose; biseriate arrangement	Pyriform (pear-shaped), 9–13 µm in diameter; biseriate
Conidia	Spherical, rough-walled, arranged in long unbranched chains	Globose, 2.4–2.8 µm; coarsely roughened to echinulate
Staining	Lactophenol cotton blue highlights hyphae and reproductive structures clearly	Positive violet reaction with Ehrlich's reagent

Table 3: Morphological characteristics of F17 and F20

In the comparative analysis (table 3), isolate F20 initially exhibited white colonies that turned greenish to bluish-green with age, while F17 developed brownish grey to pinkish brown colonies. The texture of F20 was velvety to powdery and became slightly wrinkled in older cultures, whereas F17 showed a cottony (floccose) texture. The reverse colony color in F20 was pale yellow to orange, in contrast to the yellow olive brown to dark brown observed in F17. Colony margins in F20 appeared regular to slightly lobed and well-defined, while F17 showed irregular margins. F20 colonies were flat to low raised, but F17 had an elevated center. Both isolates sporulated abundantly; however, F20 produced dense conidial heads, and F17 showed abundant sporulation on Czapek Yeast Extract Agar. Microscopically, both had septate, hyaline hyphae, though F20's were branched. The conidiophores in F20 arose from foot cells, were smooth-walled and relatively short, whereas in F17, they were smooth-walled, brown, and measured 150–300 µm in length. Vesicles in F20 were globose to subglobose with a biseriate arrangement, while those in F17 were pyriform and 9–13 µm in diameter. Conidia of F20 were spherical, rough-walled, and arranged in long unbranched chains, whereas F17 had globose, coarsely roughened to echinulate conidia, measuring 2.4–2.8 µm. Staining with lactophenol cotton blue in F20 highlighted hyphae and reproductive structures clearly, while F17 gave a positive violet reaction with Ehrlich's reagent.

Parameter	Test Range/Conditions	Observation

		F20	F17
Salt Tolerance (NaCl %)	0% to 14%	Growth up to 14%; optimal at 0%–4%; poor above 12%	Growth up to 14%; optimal at 0%–4%; poor above 12%
Temperature Range (°C)	20°C to 50°C	Grows from 20°C to 45°C; optimal at 28°C–30°C; no growth at 50°C	Grows from 20°C to 45°C; optimal at 28°C–30°C; no growth at 50°C
pH Tolerance	pH 4.5 to 9.0	Growth across range; optimal at pH 6.0–7.0	Growth across range; optimal at pH 6.0–7.0
Amylase Activity	Starch hydrolysis on agar	Positive (clear halo after iodine staining)	Positive
Protease Activity	Casein hydrolysis on agar	Variable (strain-dependent)	Negative
Lipase Activity	Tributyrin agar	Positive (zone of clearance)	Positive
Cellulase Activity	CMC (carboxymethyl cellulose) agar	Positive (halo after Congo red staining)	Positive
Catalase Activity	Reaction with 3% H_2O_2	Strong positive (immediate bubbling)	Negative

Table 4: Physiological and biochemical characteristics of F17 and F20

Both isolates F20 and F17 demonstrated growth in NaCl concentrations up to 14% (table 4), with optimal growth observed between 0% and 4%, and poor growth above 12%. They grew well within a temperature range of 20°C to 45°C, with optimal growth at 28°C–30°C; neither isolate grew at 50°C. Both strains tolerated a pH range of 4.5 to 9.0, showing optimal growth at pH 6.0–7.0. Amylase activity was positive in both strains, indicated by clear halos after iodine staining. Protease activity was variable in F20 and negative in F17. Lipase activity was positive in both, confirmed by clear zones on tributyrin agar. Cellulase activity was also positive in both isolates, as shown by halos after Congo red staining. However, a key difference was observed in catalase activity: F20 showed a strong positive reaction with immediate bubbling upon exposure to hydrogen peroxide, while F17 tested negative.

2.5.2. Molecular Identification

Molecular characterization of the fungal isolate F7 was conducted through 18S rRNA gene sequencing. Genomic DNA was extracted using the HiPurA Fungal DNA Purification Spin Column Kit and amplified using universal fungal primers NS1 and NS4, yielding an amplicon of approximately 1012bp. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on an Applied Biosystems 3500xL Genetic Analyzer.

The obtained sequence was subjected to BLASTn analysis against the NCBI GenBank database. The F7 isolate showed 99.70% sequence identity (1009/1012 bp) to the 18S rRNA gene of *Aspergillus versicolor* (Accession No. NG_067623.1). Other close hits included *Aspergillus flavipes* (99.31%, NG_063230.1) and *Aspergillus terreus* (99.31%, NG_064804.1). The BLAST results confirmed that isolate F7 belongs to the genus *Aspergillus*, with strong homology indicating its closest relationship to *A. versicolor*. Phylogenetic analysis further supported this identification. A neighbor-joining tree constructed using MEGA11 revealed that isolate F7 clusters closely with reference sequences of *A. versicolor*, forming a distinct clade with high bootstrap support. These findings confirm that F7 is a halotolerant strain of *Aspergillus versicolor*.

Figure4 -Phylogenetic tree of F17

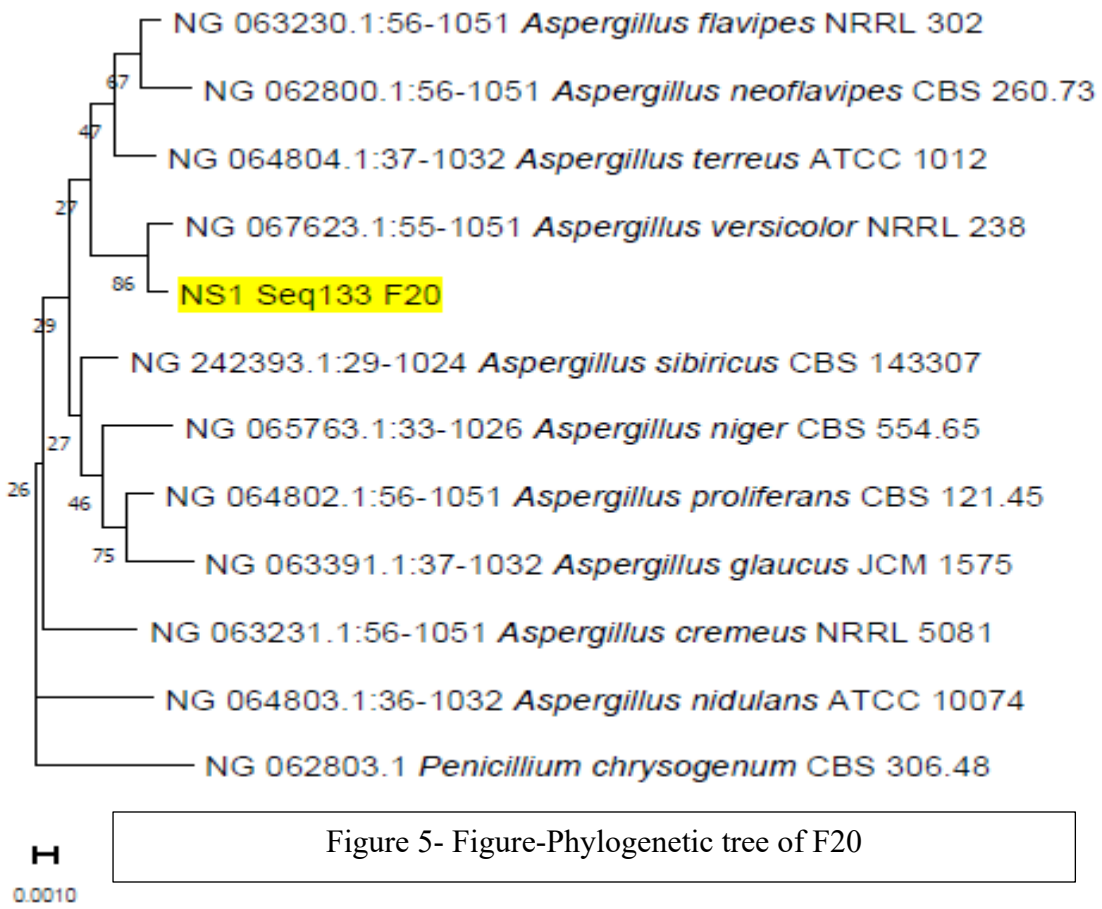


Figure 5- Figure-Phylogenetic tree of F20

The morphological and microscopic traits observed in isolate F20 are in strong agreement with previous descriptions of *Aspergillus versicolor*, a well-known filamentous fungus commonly encountered in soil and extreme environments. The characteristic colony progression from white to bluish-green, velvety to powdery texture, and yellow-orange pigmentation on the reverse are consistent with classical descriptions of *A. versicolor* provided by Raper and Fennell (1965) and elaborated by Samson *et al*. (2007). Microscopic features such as biseriate conidiophores terminating in globose vesicles, along with rough-walled conidia arranged in long, unbranched chains, further affirm the identity of the isolate and reflect typical taxonomic markers used for species differentiation within the genus *Aspergillus* (Klich, 2002).

Physiologically, isolate F20 exhibited moderate halotolerance, with optimal growth at 0–4% NaCl and limited tolerance up to 12%. Although *A. versicolor* is not considered an extreme halophile, its ability to grow under elevated salt concentrations aligns with findings by Nazareth and Gonsalves (2014), who demonstrated the halotolerant nature of many *Aspergillus* species in saline and coastal ecosystems. The isolate also showed mesophilic growth, thriving between 20°C and 45°C and optimally at 28–30°C, which is typical for *A. versicolor* strains isolated from temperate and tropical habitats (Frisvad & Larsen, 2016). Additionally, its broad pH adaptability (4.5–9.0) highlights the ecological plasticity of the species, supporting its ability to colonize diverse soil environments (Pitt & Hocking, 2009).

Biochemically, the strain demonstrated strong hydrolytic enzyme production, including amylase, lipase, and cellulase activities. Such enzymatic capabilities are of industrial relevance and have been previously reported in *A. versicolor* isolated from marine and terrestrial substrates (Prasad *et al*., 2011). Although protease production was variable, the robust catalase activity observed—indicated by rapid oxygen release upon exposure to hydrogen peroxide—is a typical oxidative stress response trait of *Aspergillus* spp., facilitating survival in reactive oxygen-rich environments (Abdel-Rahim & Mahmoud, 2008). These physiological and biochemical features underline the biotechnological potential of *A. versicolor*, particularly in applications involving saline soils or enzymatic biotransformation under stress conditions.

In the analysis, the isolate F17 (NS1 NS4RC Seq133) clustered closely with *Aspergillus proliferans* (CBS 121.45), supported by a relatively high bootstrap value of 77, indicating strong confidence in this grouping. This placed F17 in the same clade as *A. proliferans* and *A. glaucus*, suggesting a close genetic relationship. Other *Aspergillus* species such as *A. clavatus*, *A. terreus*, *A. flavipes*, and *A. niger* were placed in separate branches, showing distinct evolutionary divergence. *Colletotrichum gloeosporioides* served as the outgroup, clearly separated from the *Aspergillus* genus. Overall, this phylogenetic analysis confirmed that isolate F17 shared the highest sequence similarity and evolutionary proximity with *Aspergillus proliferans*.

2.5.3. Phosphate solubilisation- The phosphate solubilization assay demonstrated distinct capabilities between the two fungal isolates. F17 exhibited strong phosphate solubilization activity (figure 6), producing a clear halo zone of 11 cm on Pikovskaya's medium, indicating efficient secretion of organic acids and/or phosphatases to release soluble phosphates from tricalcium phosphate. In contrast, F20 showed moderate activity, forming a smaller 5 cm halo zone, suggesting comparatively lower phosphate-solubilizing efficiency. These results aligned with previous studies where certain *Aspergillus* strains displayed superior phosphate solubilization due to enhanced organic acid production (Sharma *et al*., 2013). The significant difference in

halo sizes between F17 and F20 highlighted strain-specific variations in phosphate solubilization mechanisms, possibly linked to genetic differences in acid synthesis pathways (Whitelaw, 2000). The robust performance of F17 under saline conditions suggested its potential as a biofertilizer candidate for saline-affected soils, consistent with findings on halotolerant phosphate-solubilizing fungi (Narsian *et al.*, 2010). Further molecular characterization could elucidate the specific genes responsible for F17's high activity.

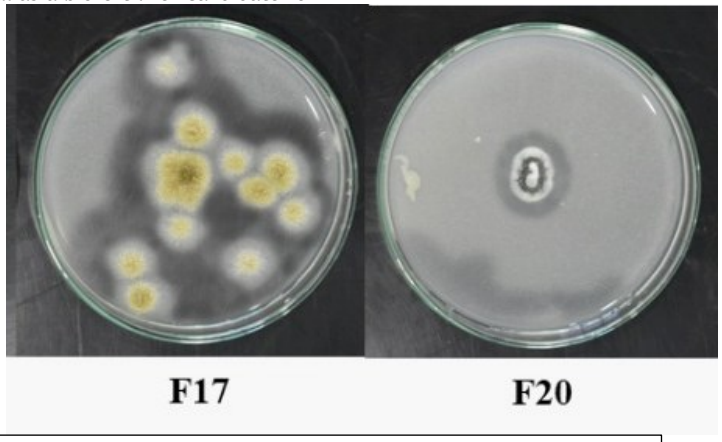


Figure 6-Phosphate solubilization zone of F17 and F20

2.5.4. Zinc solubilisation- In the present study, the qualitative zinc solubilization assay revealed a distinct difference in the solubilization efficiency between the two fungal isolates, F17 and F20. Isolate F17 produced a prominent clear halo zone of 12 cm in diameter around the colony (figure 7), whereas F20 formed a comparatively smaller halo of 3 cm. This indicates that F17 exhibited a significantly higher zinc solubilizing potential than F20 under identical conditions.

The formation of a clear halo around fungal colonies on zinc oxide (ZnO)-amended media is indicative of the conversion of insoluble zinc into soluble forms, primarily through the production of organic acids such as gluconic, citric, and oxalic acids (Saravanan *et al.*, 2007). These acids reduce the pH of the surrounding medium, chelating the zinc ions and making them available for plant uptake. The larger solubilization zone observed for F17 suggests a more efficient acid production or a more aggressive enzymatic mechanism compared to F20.

Zinc solubilizing fungi play a vital role in sustainable agriculture by improving zinc bioavailability in soils, particularly in zinc-deficient areas. Similar observations have been reported by Sharma *et al.* (2012), where *Aspergillus* species showed varying capabilities of zinc solubilization, and those producing larger halos were more effective in enhancing zinc uptake by plants.

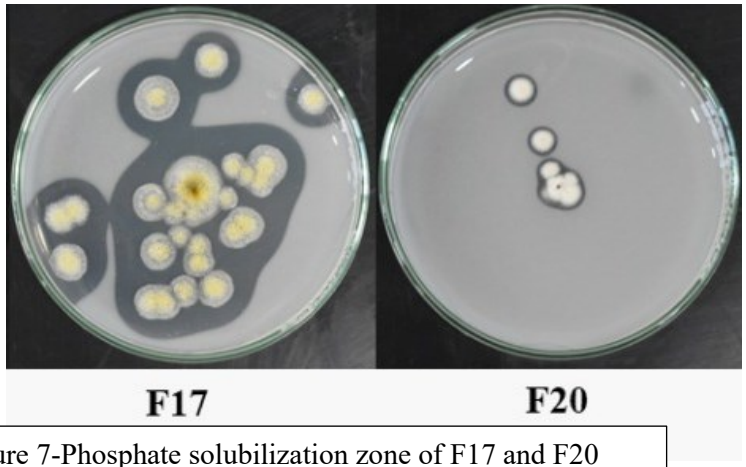
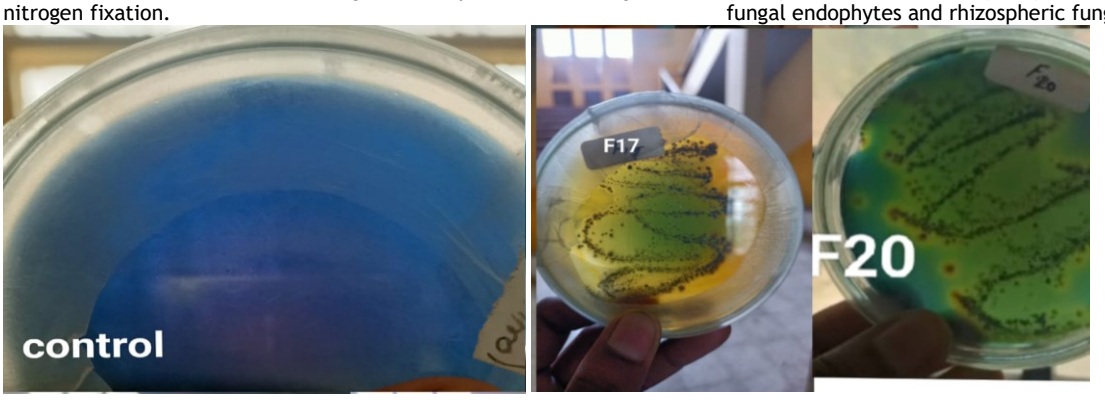


Figure 7-Phosphate solubilization zone of F17 and F20

2.5.5. Ammonia production-The results (figure 8) suggested that F20 had greater potential to release ammonia, thereby contributing more effectively to nitrogen availability in the rhizosphere. Ammonia-producing fungi are considered beneficial in agriculture as they enhance soil fertility and promote plant growth. These observations are consistent with earlier studies by Harikrishnan *et al.* (2014) and Joseph *et al.* (2007), who reported variable ammonia production among *Aspergillus* and other plant growth-promoting fungi, with higher producers being more effective in improving soil nitrogen content and crop productivity.

Figure 8- Ammonia production by F17 and F20

2.5.6. Nitrogen fixation-The plate inoculated with F17 turned yellowish-orange, while the plate with F20 exhibited a distinct green to yellow-green color (figure 9). These color shifts suggested that both isolates were capable of fixing atmospheric nitrogen into bioavailable forms. The color change results from acidification due to ammonia or organic acid production during nitrogen fixation. Among the two, F20 showed a more prominent green zone, indicating stronger nitrogen fixation potential compared to F17. This observation supports the hypothesis that some *Aspergillus* species may possess the enzymatic machinery necessary for nitrogen fixation, consistent with reports by Deshwal *et al.* (2011) and Sahoo *et al.* (2013), who noted similar activity in fungal endophytes and rhizospheric fungi.



2.5.7. Exopolysaccharides (EPS) Production-

Figure 9- Nitrogen fixation test (Control, F17 and F20)

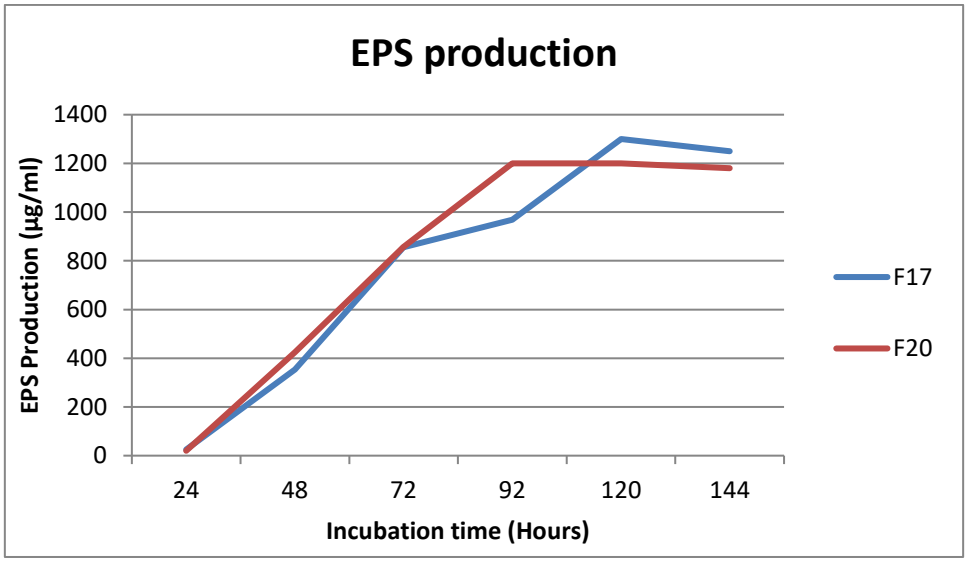


Figure 10-EPS production by F17 and F20

The exopolysaccharide (EPS) production by fungal isolates F17 and F20 was monitored over a period of 144 hours (figure 10). Both isolates demonstrated a steady increase in EPS production from 24 to 120 hours. F17 showed a slightly delayed onset compared to F20, but surpassed F20 at 120 hours, reaching a peak production of approximately 1300 µg/mL. F20 exhibited an earlier saturation in EPS yield, stabilizing around 1200 µg/mL after 96 hours and maintaining that level with slight decline till 144 hours. In contrast, F17 showed a continuous rise in EPS until 120 hours, followed by a minor decline by 144 hours.

EPS production by microorganisms is influenced by environmental factors, including nutrient availability, incubation time, and strain-specific metabolic capacity. In the current study, both F17 and F20 fungi demonstrated significant EPS-producing potential, with F17 ultimately achieving higher yield than F20. The initial rapid EPS accumulation by F20 suggests a more efficient early metabolic activation, while F17 appeared to maintain biosynthesis for a longer duration, leading to greater final accumulation.

These findings are consistent with earlier reports by Suresh Kumar *et al*. (2007) and Rani *et al*. (2018), who also observed that fungal EPS production generally peaks between 96–120 hours of incubation, after which depletion of carbon sources or accumulation of toxic metabolites can lead to reduced production. Similarly, Degefu *et al*. (2013) reported variations in EPS yield across fungal strains, highlighting the strain-specific nature of EPS biosynthesis.

EPS plays a crucial role in biofilm formation, soil aggregation, and stress tolerance in fungi, and the robust production seen in F17 suggests its potential application in agriculture and biotechnological processes where biofilm formation or stress mitigation is required.

2.5.8.Indole acetic acid production (IAA)- The IAA production assay revealed significant differences between the two fungal isolates over the incubation period (figure 11). F20 demonstrated higher IAA production, reaching a maximum concentration of 7.8 µg/mL, while F17 showed comparatively lower production, peaking at 7.2 µg/mL. Both isolates exhibited a gradual increase in IAA levels during the initial hours of incubation, followed by stabilization or decline, suggesting potential substrate depletion or metabolic regulation.

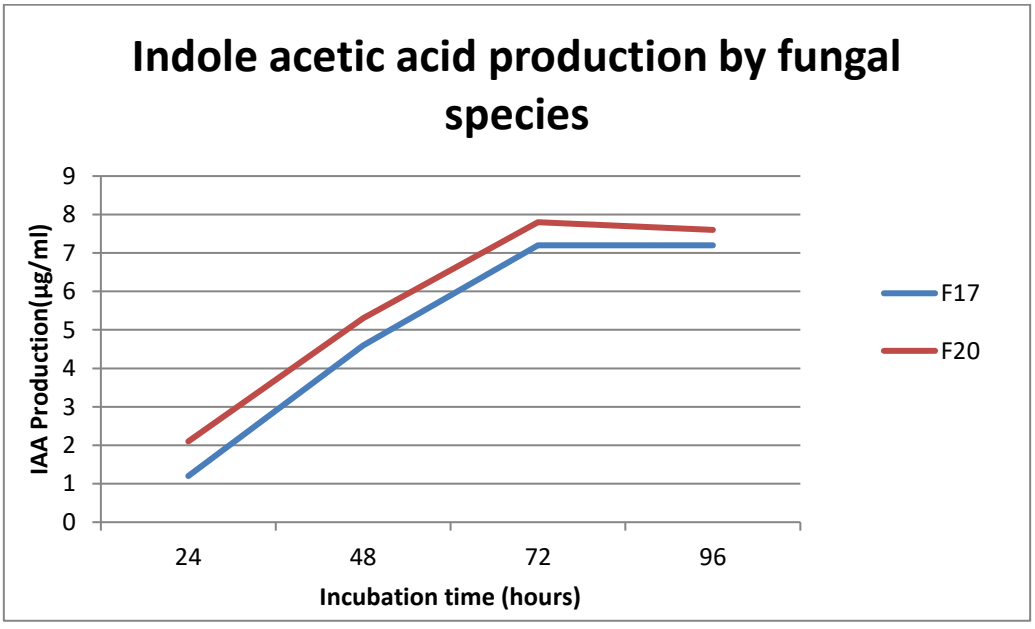


Figure 11- IAA production by F17 and F20

These results align with previous studies on *Aspergillus* species, where IAA production was linked to tryptophan-dependent pathways involving enzymes such as indole-3-pyruvate decarboxylase (IPDC) (Tsavkelova *et al.*, 2012). The superior performance of F20 correlates with findings by Khan *et al*. (2016), who reported that halotolerant fungi from saline soils often exhibit enhanced phytohormone production as an adaptive trait. The lower yield in F17 may reflect strain-specific variations in tryptophan metabolism or differences in secretory efficiency, as observed in other fungal systems (Numponsak *et al.*, 2018).

The ability of these isolates to produce IAA under saline conditions suggests their potential role in plant growth promotion in stress-prone environments. Similar to *A. versicolor* strains studied by Rahmoune *et al*. (2017), F17's robust IAA production could enhance root elongation and nutrient uptake in host plants, making it a promising candidate for biofertilizer development. Further molecular characterization of IAA biosynthesis genes (e.g., *iaaM*, *ipdC*) could elucidate the mechanistic basis for these observed differences.

2.5.9.Pot assay and seed germination assay-In the pot assay, fungal treatments with F17 and F20 influenced various growth parameters of maize and wheat seedlings compared to the untreated control. F17-treated maize and wheat plants exhibited the highest fresh weights (950 mg and 855 mg, respectively) and dry weights (310 mg and 315 mg, respectively), indicating robust biomass accumulation. Additionally, F17 enhanced root fresh and dry weights in both crops, particularly in wheat, where values reached 260 g (fresh) and 120 g (dry), suggesting improved nutrient and water absorption. In contrast, F20 treatment resulted in comparatively lower plant fresh weights but slightly higher plant dry weight in maize (380 mg), implying greater dry matter accumulation. F20 also increased the number of leaves in maize (4), suggesting stimulation of vegetative growth. The control plants displayed the lowest values across all parameters, confirming the beneficial role of fungal inoculation. Overall, F17 demonstrated superior performance in promoting overall plant biomass, while F20 showed moderate improvement, especially in shoot development and leaf number.

	Plant fresh weight (mg)	Plant dry weight (mg)	Root fresh weight (g)	Root dry weight (g)	Number of leaves

	Maize	Wheat	Maize	Wheat	Maize	Wheat	Maize	Wheat	Maize	Wheat
F17	950	855	310	315	240	260	150	120	3	3
F20	630	610	380	300	170	165	85	105	4	3
Control	600	350	200	200	220	180	80	90	2	2

Table 5: Wheat and Maize Bioassay on Growth Promotion by Inoculation with *F17 and F20*

In the seed germination assay, both F17 and F20 fungal treatments resulted in 100% germination for wheat and maize seeds, indicating a strong positive influence on seed viability and early growth. In contrast, the control group showed slightly lower germination rates at 95% for both crops. This suggested that fungal inoculation enhanced seed germination efficiency compared to untreated seeds.

	Seed germination percentage		
	F17	F20	Control
Wheat	100	100	95
Maize	100	100	95

Table 6: Seed germination percentage of Wheat and Maize

In the pot assay, both fungal isolates F17 and F20 significantly enhanced wheat seedling growth parameters compared to the untreated control (table 7). F20 consistently showed the highest improvements across all metrics. The average plant height in the F20-treated group was notably greater (approximately 3.9–4.5 cm) compared to the control (1.5–1.7 cm), while F17-treated plants showed moderate growth (1.6–2.9 cm). Root lengths were also significantly enhanced by both isolates, with F20 promoting longer roots (1.6–1.9 cm) than F17 (0.5–1.6 cm), in contrast to shorter roots in the control group (0.6–0.9 cm). Similarly, shoot lengths increased under fungal treatment, especially with F20 (up to 2.6 cm), followed by F17 (1.0–1.5 cm), compared to the control (1.8–2.0 cm). These results indicated that both F17 and F20 had plant growth-promoting effects, with F20 demonstrating superior performance in stimulating early seedling development.

Wheat Seed number	Plant height (cm)			Root length (cm)			Shoot length (cm)		
	F17	F20	Control	F17	F20	Control	F17	F20	Control
1	1.8	3.9	1.5	0.6	1.8	0.8	1.2	2.1	
2	1.7	4.1	1.5	1.6	1.7	0.9	1.1	2.4	
3	1.8	4.3	1.5	0.8	1.8	0.8	1.0	2.5	
4	2.0	4.5	1.6	0.7	1.9	0.7	1.3	2.6	
5	2.3	4.2	1.7	0.9	1.8	0.6	1.4	2.4	
6	2.5	3.5	1.5	1.0	1.6	0.7	1.5	1.9	
7	2.9	3.5	1.6	0.7	1.7	0.7	1.2	1.8	
8	1.6	3.7	1.5	0.5	1.8	0.7	1.1	1.9	
9	1.7	3.9	1.6	0.6	1.9	0.8	1.1	2.0	
10	1.9	3.5	1.5	0.8	1.6	0.9	1.1	1.9	

Table 7: Wheat Seed germination assay details

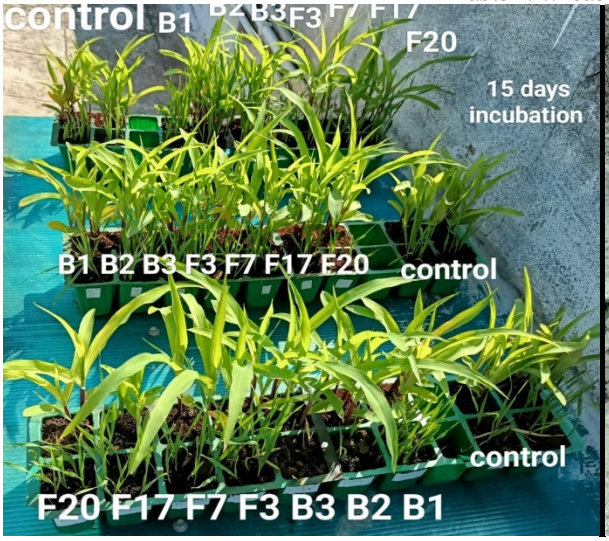


Figure 12 -Wheat pot assay

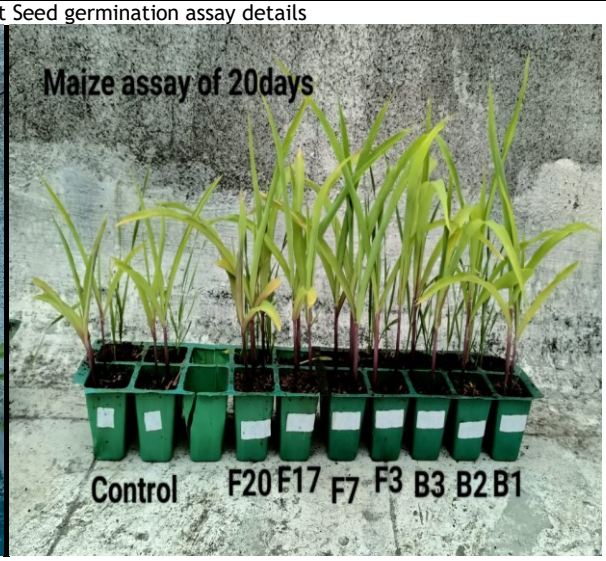


Figure 13-Maize Pot assay

The results of the pot assay revealed that both fungal isolates, F17 and F20, promoted wheat seedling growth, with F20 demonstrating a more pronounced effect on plant height, root length, and shoot length compared to F17 and the untreated control. These findings align with earlier reports on the plant growth-promoting potential of *Aspergillus* species.

For instance, Bhagat *et al*. (2019) demonstrated that *Aspergillus niger* significantly enhanced root and shoot growth in wheat by producing phytohormones and solubilizing essential nutrients. Similarly, Gopalakrishnan *et al*. (2011) observed that *Aspergillus terreus* improved biomass and root architecture in chickpea under greenhouse conditions, attributing these effects to phosphate solubilization and siderophore production.

The observed increase in root and shoot lengths in F20-treated plants suggests enhanced nutrient uptake, possibly due to improved solubilization of micronutrients such as zinc and phosphate—activities already confirmed in F20 during qualitative assays. Comparable results were reported by Vassileva *et al*. (2000), who demonstrated that fungal strains capable of solubilizing phosphate and producing organic acids contribute significantly to improved plant vigor and nutrient availability in the rhizosphere.

Moreover, the stimulatory effects on plant height and development might also be linked to secondary metabolite production or exopolysaccharide-mediated root colonization. Radhakrishnan *et al*. (2017) emphasized the importance of microbial EPS in facilitating root adherence, moisture retention, and nutrient transport in the rhizosphere, which ultimately translates to improved plant growth.

Based on the overall analysis of all experimental results, both fungi demonstrated plant growth-promoting potential, F17 emerged as the more effective bio-inoculant, particularly for enhancing biomass, root health, and overall vigor in both wheat and maize. Its ability to boost fresh and dry weights, along with consistent performance across multiple parameters, makes it more suitable for use in sustainable agriculture as a plant growth-promoting fungus (PGPF).

REFERENCES

- Abdel-Rahim, E. A., & Mahmoud, S. Y. (2008). Catalase activity of some fungal strains. *Mycobiology, 36*(3), 145–150. https://doi.org/10.4489/MYCO.2008.36.3.145
- Bhagat, N., Verma, A., & Pande, V. (2019). Plant growth promoting potential of *Aspergillus niger* and *Trichoderma viride* in wheat (*Triticum aestivum*). *International Journal of Agriculture and Biology, 21*(3), 571–576.
- Degefu, T., Wolde-Meskel, E., & Frostegård, Å. (2013). Multilocus sequence analysis reveals high genetic diversity of rhizobia that nodulate common bean (*Phaseolus vulgaris* L.) in Ethiopia. *Systematic and Applied Microbiology, 36*(3), 141–148.
- Egamberdieva, D., Wirth, S., Bellingrath-Kimura, S. D., Mishra, J., & Arora, N. K. (2017). Salt-tolerant plant growth promoting rhizobacteria for enhancing crop productivity of saline soils. *Frontiers in Microbiology, 8*, 1201. https://doi.org/10.3389/fmicb.2017.01201
- Egamberdieva, D., Wirth, S. J., Shurigin, V. V., Hashem, A., & Abd-Allah, E. F. (2017). Endophytic bacteria improve plant growth, symbiotic performance of chickpea (*Cicer arietinum* L.) and induce suppression of root rot caused by *Fusarium solani*. *Plant and Soil, 410*(1–2), 123–136. https://doi.org/10.1007/s11104-016-2982-7
- El-Sayed, W. S., El-Sayed, M. H., & Rady, M. M. (2015). Isolation and characterization of halotolerant fungi from salt marshes and their potential for plant growth promotion. *Mycobiology, 43*(1), 25–36.
- Food and Agriculture Organization. (2021). *Global map of salt-affected soils*. http://www.fao.org/soils-portal/en/
- Frisvad, J. C., & Larsen, T. O. (2016). Extrolites of *Aspergillus versicolor* and related species. *Frontiers in Microbiology, 7*, 1231. https://doi.org/10.3389/fmicb.2016.01231
- Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L. L., & Krishnamurthy, L. (2011). Evaluation of plant growth-promoting potential of rhizosphere fungi from chickpea. *World Journal of Microbiology and Biotechnology, 27*(2), 261–265.
- Harikrishnan, H., Shanmugaiah, V., Balasubramanian, N., & Gomathinayagam, S. (2014). Ammonia production ability of plant growth promoting *Aspergillus* species isolated from rice rhizosphere. *Journal of Basic Microbiology, 54*(S1), S63–S74.
- Joseph, B., Patra, R. R., & Lawrence, R. (2007). Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *International Journal of Plant Production, 1*(2), 141–152.
- Klich, M. A. (2002). *Identification of common Aspergillus species*. Centraalbureau voor Schimmelcultures.
- Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, A., Guesmi, A., Ouzari, I., Daffonchio, D., & Borin, S. (2013). Potential for plant growth promotion of rhizobacteria associated with *Salicornia* growing in Tunisian hypersaline soils. *BioMed Research International, 2013*, 248078. https://doi.org/10.1155/2013/248078
- Narsian, V., Samaha, S. M., & Patel, H. H. (2010). Rock phosphate solubilization by *Aspergillus niger* under saline conditions. *Journal of Agricultural Science, 2*(4), 147–154. https://doi.org/10.5539/jas.v2n4p147
- Nazareth, S., & Gonsalves, V. (2014). Halotolerant fungi from mangroves and their potential industrial applications. *Mycology, 5*(4), 172–182. https://doi.org/10.1080/21501203.2014.939582
- Pitt, J. I., & Hocking, A. D. (2009). *Fungi and food spoilage* (3rd ed.). Springer. https://doi.org/10.1007/978-0-387-92207-2
- Prasad, R., Vishwakarma, S. K., & Jain, R. K. (2011). Extracellular enzyme activity of *Aspergillus versicolor* isolated from the soil of coal mining areas. *Advances in Applied Science Research, 2*(2), 110–116.
- Radhakrishnan, R., Hashem, A., & Abd_Allah, E. F. (2017). Exopolysaccharides from plant growth-promoting fungi enhance plant productivity under stress conditions: A review. *Microbiological Research, 198*, 30–39.
- Raghukumar, C. (2008). Marine fungal biotechnology: An ecological perspective. *Fungal Diversity, 31*, 19–35.
- Rani, M. U., Appaiah, K. A. A., & Renu, A. (2018). Production, optimization, and characterization of exopolysaccharide from endophytic fungi. *3 Biotech, 8*, 102.
- Raper, K. B., & Fennell, D. I. (1965). *The genus Aspergillus*. Williams & Wilkins.
- Samson, R. A., Hoekstra, E. S., & Frisvad, J. C. (2007). *Introduction to food- and airborne fungi* (7th ed.). CBS Fungal Biodiversity Centre.
- Saravanan, V. S., Subramoniam, S. R., & Raj, S. A. (2007). Assessing *in vitro* solubilization potential of different zinc solubilizing bacterial (ZSB) isolates. *Brazilian Journal of Microbiology, 38*(1), 145–148.
- Sharma, A., Johri, B. N., Sharma, A. K., & Glick, B. R. (2012). Plant growth-promoting bacterial endophytes. In D. K. Maheshwari (Ed.), *Plant growth and health promoting bacteria* (pp. 195–221). Springer.
- Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013). Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus, 2*(1), 587. https://doi.org/10.1186/2193-1801-2-587
- Suresh Kumar, A., Mody, K., & Jha, B. (2007). Bacterial exopolysaccharides–a perception. *Journal of Basic Microbiology, 47*(2), 103–117.
- Vassileva, M., Azcon, R., Barea, J. M., & Vassilev, N. (2000). Solubilization of rock phosphate by immobilized *Penicillium* and *Aspergillus niger*. *Bioresource Technology, 73*(1), 21–27.
- Whitelaw, M. A. (2000). Growth promotion of plants inoculated with phosphate-solubilizing fungi. *Advances in Agronomy, 69*, 99–151. https://doi.org/10.1016/S0065-2113(08)60948-7