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RESEARCH ARTICLE

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Divulgence into Mung Bean Growth Promotion Potential of Endophytes Isolated Across Different Geographical Regions of India

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ABSTRACT

Background: Endophytic fungi in symbiotic association with their host plant are well known to improve plant growth and reduce the adverse effects of abiotic stresses. Therefore, fungal endophytes are beginning to receive increased attention in an effort to find growth-promoting strains that could be applied to enhance crop yield and quality.

Methods: In our study, the plant growth promoting activities of endophytic fungi isolated from various parts of India have been revealed and investigated. Fungal isolates were identified using molecular taxonomical methods, while their plant growth promoting abilities were evaluated in plate assays and *in vitro* studies. Altogether, seven strains were selected, representing the genera *Fusarium, Chaetomium* and *Ulocladium*.

Result: Six of the isolates possessed phosphate solubilization activities, four secreted siderophores and four showed amylase activity, while all of them were able to produce indoleacetic acid (IAA). In order to investigate the effect of endophytes on plant growth, all seven fungi were selected concerning their potential ability to promote growth in mung bean. The results indicated that microbial endophytes selected from different geographical regions living originally in the plant host are able to influence the growth and fitness parameters of other plants through their promotion effects and could be used as inoculants to establish a sustainable crop production system.

Key words: Amylase activity, Endophytic fungi, IAA (indole-acetic-acid) production, Mung bean, Phosphate solubilisation, Siderophore production.

INTRODUCTION

A long-term approach to organic and inorganic fertilizers besides pesticides is urgently needed to enhance crop production. In particular, these applications have a detrimental effect on soil quality and cause environmental degradation. Innovative strategies based on microbial inoculation are currently garnering more attention as a means of reducing the negative consequences of conventional agricultural processes. Microorganisms and plants work together in a symbiotic relationship that is advantageous to both parties. The influence of plant-microbe symbiosis on plant health and growth, which successfully improves agricultural attributes and enhances soil quality and nutrient cycling, is more significant (Sahu *et al.,* 2017). The close relationship between plant growth-promoting endophytes (PGPEs) and plant tissues facilitates the exchange of nutrients and enzymes within the plant. It is believed that endophytic microorganisms produce growth-promoting hormones that are distributed in plant tissues to foster plant growth (Sharma *et al.,* 2023). Indole-3-acetic acid (IAA) is such hormone that plays specific roles in plant growth by promoting root development, enhancing nutrient uptake and increasing crop yield by reducing synthetic chemicals and fostering sustainable agriculture practices.

Moreover, endophytes can solubilize inorganic phosphates and produce siderophores (Kabir *et al.,* 2023). Siderophores are organic compounds that are produced by organisms during iron-limiting conditions. Previous studies showed that plants can utilize microbial siderophores for iron acquisition. Microbial siderophores supplemented into iron-deficient tomato plants led to higher yields and increased chlorophyll as well as iron content in leaves (Radzki *et al.,* 2013). Several endophytes that are linked

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with plants can solubilize insoluble inorganic phosphates, making them potentially accessible for plant absorption. Several crop plants treated with phosphate-solubilizing endophytes have shown positive growth responses (Passari *et al.,* 2015). Without exhibiting symptoms, microbial endophytes penetrate plant tissues where they compete with other microbial pathogens for the same ecological niches. Hence, the established link between plants and endophytes promotes plant growth. Fungal endophytes also produce amylase enzymes that catalyze the hydrolysis of polysaccharide internal glycosidic linkages producing sugar monomers that strengthen the plant at the expense of reserve stores (Byregowda *et al.,* 2022). These endophytes can enhance nutrient uptake, improve seed germination and protect against pathogens, ultimately leading to healthier, more robust seeds. Understanding their role enables farmers to select and utilize endophytes that positively influence seed quality, resulting in improved crop performance and yield. Additionally, this knowledge can lead to reduced reliance on chemical fertilizers and pesticides and promote sustainable and cost-effective agriculture.

Mung bean [*Vigna radiata* (L.) R.Wilczek] is India's third-largest pulse crop, accounting for almost 16% of the country's total pulse area. Despite being a valuable crop, mung bean yield has remained flat due to drought stress which affects all phases of crop growth (Pratap *et al.,* 2020). Drought stress reduced mung bean yields by 51 per cent to 85.50 percent (Zare *et al.,* 2013). As a result, the current study's premise is that microbial endophytes isolated from plants that have acclimated to harsh environments are viable bio-inoculants having traits that promote plant growth. The main focus of this study is on the isolation, molecular characterization and identification of fungal endophytes, as it is crucial to identify beneficial microorganisms within plant tissues, contributing to enhanced plant growth and seed quality. More specifically, verification for plant growth-promoting properties of these microbial isolates such as IAA, siderophore production, P solubilization and amylase activity was evaluated to test their influence on the seedling growth of mung bean plants.

MATERIALS AND METHODS

The laboratory experiment was conducted in 2021-22 at the Department of Seed Science and Technology, University of Agricultural Sciences, GKVK, Bangalore.

Isolation, purification and microscopy of endophytic fungi

All pure cultures of fungal endophytes were preserved at the School of Ecology and Conservation Laboratory, Department of Crop Physiology, UASB, GKVK, Bangalore. A few fungal endophytes from the library were chosen to study their potential in plant growth promotion. Earlier for the isolation of endophytic fungi, plant samples (root, leaves and stem) were collected from different parts of India *i.e.*, Kargil (J and K), Pangong Tso, Thar desert, Tamil Nadu, Karnataka and Kerala. The collection of endophytes from these regions offers a unique ecological niche with diverse plant species that could harbor a wide range of endophytic fungi with distinct properties. Plant samples were surface sterilized (70% ethanol for 2 min, 1% perchloric acid for 30 s). After sterilization, the final washing was done with double-distilled water. Similarly, sterilized plant parts were sliced into small pieces and deposited on potato dextrose agar media plates. The fungal colonies that had developed from plant parts were carefully picked and cultivated at 25°C. After 7 days of incubation, various colonies of endophytic fungal strains with different morphological features were taken into consideration. Each strain was carefully sub-cultured on PDA plates to get their respective pure culture and the microscopic features of fungal colonies was obtained using the method as described by Shamly *et al.* (2014).

Molecular identification of fungi

Fungal isolates were molecularly recognized using universal primers ITS-1 and ITS-4 to amplify their ITS region of 18S rDNA. For molecular identifications, the total gDNA of each selected fungal isolate was extracted by a modified method of Lacap *et al.* (2003). Then, the ITS region was amplified using ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) primers (White *et al.,* 1990). The obtained FASTA sequences were analyzed by MEGA11 software. Phylogenetic relatedness among the fungal species obtained from different habitat plants was determined by employing a Neighbor-Joining method (Saitou and Nei, 1987) and evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). The consensus sequence data of these species were submitted to the Gene Bank database and accession numbers were obtained (Table 1).

Screening of endophytes for plant growth-promoting attributes

The phosphorus solubilization potential of the endophytes was checked using the method followed by Pikovskaya, 1948. Fungal endophytes were checked for siderophore-producing ability by universal CAS assay (Louden *et al.,* 2011). The phytohormone IAA, an indole compound was detected by following the method delineated by Gordon and Weber (1951). The fungal isolates were screened for amylolytic activity by a starch hydrolysis test on a starch agar plate (Aneja, 2007).

Evaluation of the endophytes for their ability to improve seedling growth promotion and impart drought tolerance to the mung bean

To create drought stress, different concentrations (5%, 10%, 15%, 20%, 25%) of PEG-8000 solution were prepared in sterile distilled water (Muddarsu and Manivannan, 2017). These concentrations were corresponding to the osmotic potential of (-0.47 MPa, -1.45 MPa, -2.93 MPa, -4.92 MPa, -7.41 MPa) respectively. LC_{50} value was calculated by Probit analysis using statistical software IBM SPSS Statistics 20 (https://www.ibm.com/in-en/analytics/spss-statistics-software) and found that -3.36 MPa PEG-800 was a lethal dose. Therefore, one set of the germination sheets was treated with a lethal dose (Fig 8) of PEG (Muddarsu and Manivannan, 2017) to create drought stress and incubated at 28±2°C for 7 days. Another set was treated with sterile distilled water and maintained as control.

The endophytes were evaluated for their ability to promote growth and impart drought tolerance to mung bean seedlings. The seeds of mung bean were surface (Arnold *et al.,* 2000) sterilized and germinated for 48 hours. Five-day-old fungal colony cultures were used for inoculum preparation by washing the mycelial mat with sterile distilled water. Pre-germinated seeds were treated with the mycelia suspension (2×10^6 cfu/ml) for 3 hours along with control (treated with sterile distilled water). The seedlings were then transferred to moistened (distilled water for control and PEG solution for drought stress) germination sheets (Sangamesh *et al.,* 2018).

Scanning electron microscope (SEM)

The samples were analysed at the Central Instrumentation Facility, UAS, GKVK, Bangalore. Mycelial discs (5 mm dia) of five-day-old endophytic fungal culture grown on nylon membrane in PDA plates were used (Chen *et al*., 2016) for visualizing the mycelial and spore morphology under SEM in SE2 mode (ZEISS; EVO18).

Statistical analysis

The experiments were carried out in a completely randomized design in the laboratory experiments. Standard errors were calculated for each parameter in each treatment. Statistical analyses were performed in the R statistical environment. Statistical comparisons were done by one-way ANOVA tests followed by a post-hoc Tukey analysis; the same letters above the graph are statistically indistinguishable at 95% confidence.

RESULTS AND DISCUSSION

Morphological characterization of endophytic fungi

The morphological characterization of fungal isolates showed that all sporulating fungi had spores with varying morphology. The colony of fungal culture on PDA plates and morphotype of endophytic fungal isolates are represented in Fig 1. K23-FE had either micro or macroconidia. Microconidia were single-celled, hyaline, nonseptate, ovoid. Macroconidia were four to five septate, slightly curved, tapered at the apex and found similar to *Fusarium* sp.

K-26 had flat mycelium and produced white with very light pink-purple aerial mycelium. The reverse side of the colonies was light yellowish-brown to aurantium. Microconidia were oval to cylindrical with 0 to 1 septa and were produced on monophialides and found similar to *Fusarium* sp.

P-10 had colonies that were white and cottony and, on the underside, they were reddish-brown. The conidiophores branched into thin, elongated monophialides that produce conidia. The macroconidia were slightly curved, hyaline, broad and had 3-4 septa. Microconidia were oval or cylindrical, hyaline, smooth and lack septa and looked like *Fusarium* sp.

P-37 conidia ranged from obovoid to short ellipsoidal, with colors of golden brown to blackish brown, roughened with 1–5 oblique or longitudinal septa and 1–5 lateral septa. Conidiophores were pale brown, erect and multicelled and was matching with *Ulocladium* sp.

SF-5 and V4-J produced white colonies and at the agar base, they progressively changed to a pale cream color. The mycelium was smooth, branching, cylindrical and septate. Conidiophores were cylindric in shape. While macroconidia were curved with short apices pedicellate basal cells, hyaline and 3-4 septate, microconidia were round to oval in shape. Terminally produced chlamydospores were approximately spherical, hyaline, singly or in chains. Their traits matched those of *Fusarium* sp.

LAS-6 had an olivaceous to the grey-green color colony while the under surface was tinged with yellow. Asci emerged from the ascomata and were club-shaped and ascospores were lemon-shaped. The ascospores were broadly limoniform and triangle-shaped in a side view, brown when mature and was matching with *Chaetomium* sp (Fig 1,2).

Molecular characterization of endophytic fungi

All seven endophytic fungal isolates obtained were subjected to molecular characterization by amplifying the rDNA region using Internal Transcriber Sequence (ITS) primers. The sequences obtained for fungal isolates had similarities ranging from 98 to 100%. Many sequences had similarities

Table 1: Molecular identification based on sequencing of ITS region of eight endophytic fungal isolates.

Sample code	Query length (bp)	Blast search result	Query cover (%)	Identity (%)	Homologue accession number
K23-FE	503	*Fusarium equiseti*	100	99.40	MG664730
K-26	525	*Fusarium redolens*	100	99.62	OM746868
P-10	534	*Fusarium solani*	100	99.63	OM746866
P-37	496	*Ulocladium* sp.	99	99.00	OR064527
SF-5	484	*Fusarium equiseti*	100	99.79	MH593375
V4-J	483	*Fusarium equiseti*	100	99.59	MN170565
LAS-6	512	*Chaetomium madrasense*	100	99.80	KX668854

above 99% and were assigned species identity. Among seven endophytes, the genus *Fusarium* was found to be dominant.

The ITS partial gene sequence of all fungal isolates K23-FE, K-26, P-10, P-37, SF-5, V4-J and LAS-6 showed >99 percent homology with isolates of mother culture having accession numbers MG664730, OM746868, OM746866, OR064527, MH593375, MN170565 and KX668854 available in the NCBI database (Fig 3 and Table 1).

Screening plant growth promoting activities of endophytes

The PGP activities of fungal endophytes that could directly or indirectly promote plant growth were evaluated. The phosphate solubilizing ability of each fungal isolate was analyzed. Except for LAS-6 (*Chaetomium* sp.), all other fungal isolates were capable of forming halo zones around fungal colonies and thus confirming phosphate solubilization (Fig 4). The siderophore producing potential of all seven fungal isolates were examined. Except SF-5, V4-J and LAS-6 all other isolates (K23-FE, K-26, P-10 and P-37) produced siderophores forming orange zone around mycelium as evidenced by positive reaction in CAS agar plate test (Fig 5). These compounds facilitate iron uptake in a variety of environments thus promoting plant growth.

The endophytic fungal isolates were screened for amylase activity. Among seven isolates, P-10, K-26, SF-5, P-37 and V4-J have given positive results as they have hydrolyzed starch by the production of amylase in starch agar medium, thus forming clear zones around fungal mycelium (Fig 6). The ability to biosynthesize IAA was observed in all fungal isolates (Fig 7). Quantitatively IAA production in the presence of L-Tryptophan ranged from 13.36±0.15 to 39.43±1.05 $\mu g\ ml^{-1}$. Isolate P-10 showed the highest production of IAA of 39.43±1.05 $\mu g\ ml^{-1}$ followed by K-26 (26.03±0.30 $\mu g\ ml^{-1}$) and V4-J (24.40±1.35 $\mu g\ ml^{-1}$).

Effect of endophytes on seedling length of mung bean

All seven fungal isolates obtained from the culture collections were used to evaluate their ability to modulate early seedling growth in normal and drought stress conditions in drought-sensitive genotypes of mung bean (KKM-3). For inducing drought stress conditions, the pre-germinated seeds of mung bean were subjected to LC_{50}, -3.36 MPa (Fig 8). All selected seven endophytes showed significantly increased seedling length in mung bean (KKM-3) under unstressed conditions and under PEG-8000 induced drought stress conditions except LAS-6 (*Chaetomium madrasense*). However, mung bean enriched with P-37 (27.77, 16.65 cm), K-26 (26.63, 14.88 cm), P-10 (27.43, 13.90 cm), K23-FE (27.26, 15.51 cm), V4-J (27.16, 13.78 cm) and SF-5 (26.45, 13.92 cm) significantly increased seedling growth when compared to control (23.43, 11.62 cm) in both unstressed and drought stressed conditions respectively.

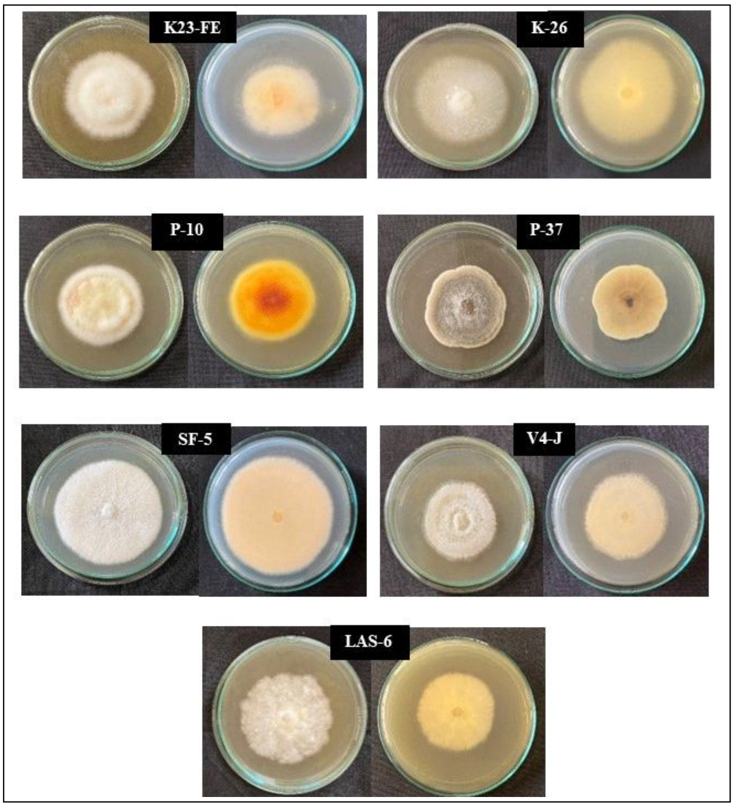


Fig 1: Fungal endophytes used in the study (front and reverse view) and its colony characteristics.

Morphological identification using scanning electron microscope (SEM)

The endophyte P-37 (*Ulocladium* sp.), which performed best under *in-vitro*, was further morphologically characterized using a scanning electron microscope. SEM image of P-37 (*Ulocladium* sp.) showed that spores are multi-celled due to their transverse and longitudinal septation, have an obovoid (narrowest at the base) rough-walled shape and most of them become verrucose. Spores appeared with uniform, knobby surfaces and with a bud at one end (Fig 9).

Early vigor in plants plays a pivotal role in determining overall crop yield and success. This characteristic offers several advantages, including a competitive edge as vigorous plants establish themselves quickly and outcompete weeds and neighboring plants for essential resources like sunlight, water and nutrients. With this respect, endophytes play a key component of a plant's microbiome that plays a crucial role in enhancing early seedling vigor. The endophytes are noteworthy for their ability to colonize without apparent symptoms and provide habitat-adapted fitness advantages to genetically distant hosts. Endophytes also can transfer habitat-specific stress tolerance to plants through a process known as Habitat Adapted Symbiosis. It has been hypothesized that host fungal endophytes provide plant fitness to extreme habitats through intergenomic epigenetic mechanisms to allow the plant to tolerate specific stresses (Rodriguez *et al.,* 2008). According to some reports, plants adapted to harsh habitats typically harbor endophytes that are highly tolerant to extreme conditions through mutualistic association and can improve non-host growth and physiological status (Sangamesh *et al.,* 2018; Sampangi-Ramaiah *et al.,* 2020). Plant growth-promoting endophytes stimulate the growth of seedlings through various mechanisms, including the production of phytohormones, such as auxins and gibberellins, which promote root and shoot elongation. Additionally, they can solubilize essential nutrients, fix atmospheric nitrogen and protect plants from harmful

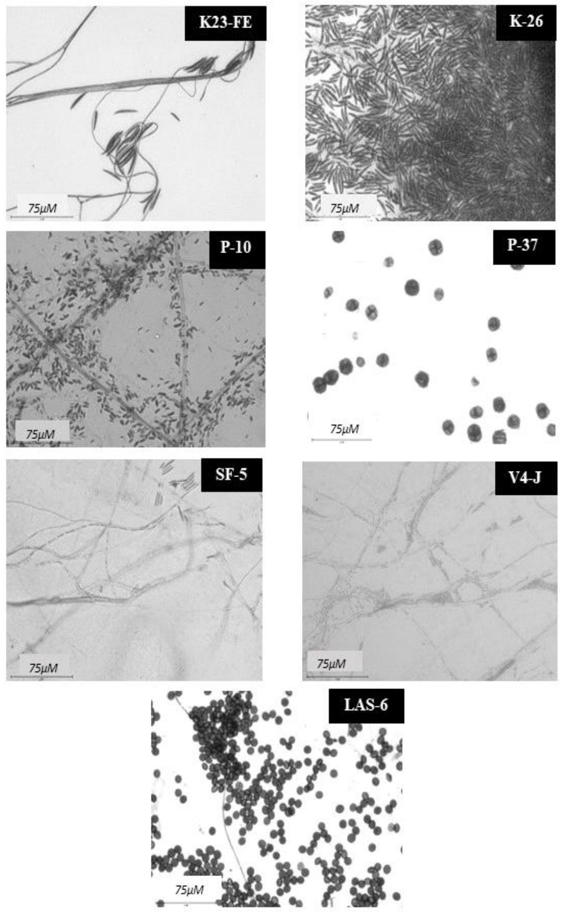
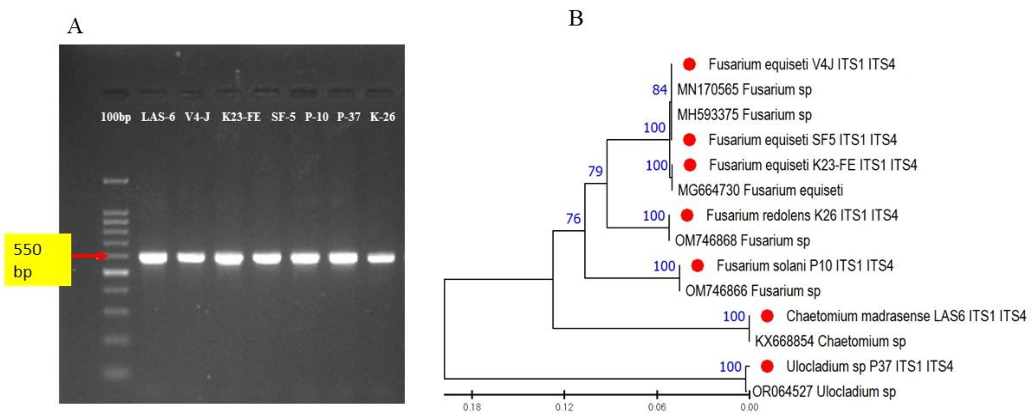


Fig 2: Morphological characteristics (spore, conidia and mycelial pattern) of fungal endophytes.



Sequences of eight fungal isolates along with their ITS reference sequence from NCBI. The tree was constructed with Bootstrap values of 1000 replicates. The isolates whose sequences have been submitted in NCBI GenBank are highlighted with a red colour symbol.

Fig 3: The results of molecular biological identification.

pathogens, ultimately contributing to improved nutrient availability and overall seedling vigor. Their presence can lead to longer and healthier seedlings, enhancing plant growth and development. Endophytes have been shown to increase seedling length by 10 to 20% or more compared to control groups under favorable conditions. However, these results can be highly variable and the magnitude of the increase may differ significantly from one experiment to another.

In this study, we characterized the microbial properties of all endophytes which are associated with plant growth promotion. In order to comprehend the potential underlying processes for these roles, we used *in-vitro* microbiological techniques to observe the phenotypic functioning of these endophytes associated with plant growth promotion. The major goal of this study was to determine which endophytes are capable of promoting mung bean plant growth. The formation of IAA, the solubilization of phosphate, the production of siderophores and amylase activity were among the additional growth-promoting activities that these strains were evaluated for.

IAA biosynthesis was detected in all endophyte strains that were examined. Nevertheless, endophytes isolated from a range of other plant species were reported with comparable results (Videira *et al.,* 2012). Plant exudates commonly include L-tryptophan (Hardoim *et al.,* 2008). The need for an external precursor may be a result of the host and symbiont's positive interactions, in which the microbe

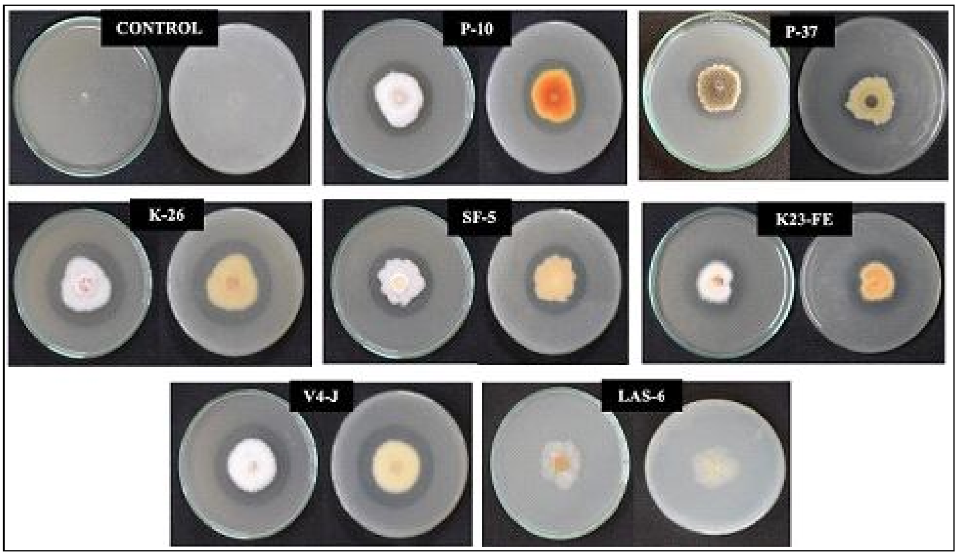


Fig 4: The phosphate solubilization activity of endophytic fungi on Pikovskaya's media forming halo zones around mycelial growth.

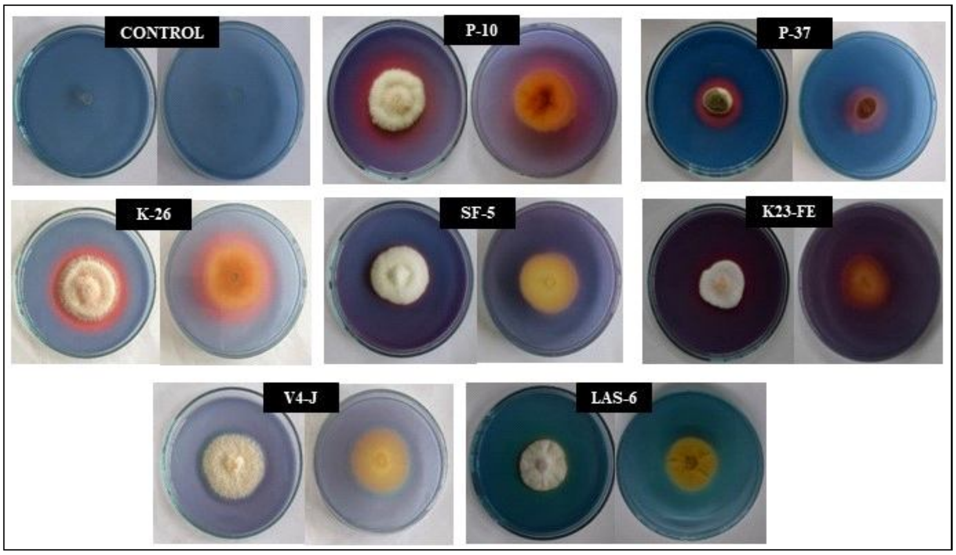


Fig 5: Siderophore production activity by fungal isolates forming orange halo zones through $FePO_4$ solubilization around mycelial growth.

transforms a host metabolite into an enzyme that helps the host to thrive (Kravchenko *et al.,* 2004).

Among the endophytes, the majority of them solubilized phosphorus in PVK medium containing insoluble phosphorus. Several previous studies have shown that endophytes can solubilize inaccessible soil phosphorus into bioavailable forms, providing plants with phosphorus resources to grow and develop (Oteino *et al.,* 2015). Thus, inorganic phosphate solubilization is one of the major mechanisms of plant growth promotion by plant-associated fungi (Varga *et al.,* 2020). In addition, multiple endophyte strains showed signs of producing siderophores. To accumulate iron from surroundings, microbes or plants make siderophores, which are organic molecules that chelate iron. Microbial siderophores have been shown to promote plant growth and alleviate iron shortage symptoms in a variety of crop plants (Ahmed and Holmström, 2014; Saha *et al.,* 2016). Whereas the production of extracellular enzymes like amylase plays a significant role in the decay of organic matter and plant growth promotion. Many plant beneficial fungi produce extracellular enzymes through an indirect mechanism for plant growth promotion (Gupta *et al.,* 2015). The production of hydrolytic enzymes by endophytes emerges as important for the colonization of plant roots and the movement of fungi into the interior of plants rendering help in plant growth (Choubane *et al.,* 2016).

Utilization of endophytes for seed biopriming has scores of benefits in the field of agriculture and seed technology. Available evidence has shown the positive influence of biopriming on seed quality parameters and plant growth promotion. However, studies on seed priming with endophytes on seedling growth parameters in mung bean are lacking and/or are very few in number. On the contrary, seed biopriming studies are mostly limited to popular biocontrol agents such as *Trichoderma viride*, *T. harzianum* and *Pseudomonas fluorescens* being used for biopriming in many of the crops (Swain *et al.,* 2021). The findings revealed that seedling growth of mung bean enhanced upon priming with endophytes both under unstressed and drought stress conditions (Fig 10,11).

The present study attempted to explore the endophytic fungal biota associated with plants adapted to extreme conditions and evaluated for their plant growth promoting activities along with their ability to confer drought tolerance to mung bean seedlings. The enhanced plant growth under normal and drought stress due to endophyte treatment shows that endophytes from adverse habitats can impart drought stress tolerance to mung bean in a habitat-specific manner through symbiotic association.

Endophytes are found inside the tissues of plants; this close association promotes mutualism between endophytes and plants. Endophytes have several advantages that have a substantial impact on plant growth because they operate as makers of different bioactive substances through a variety

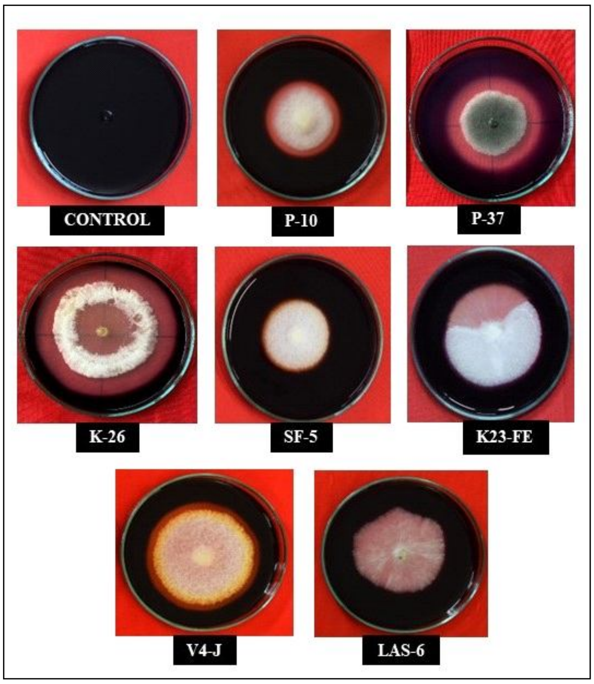
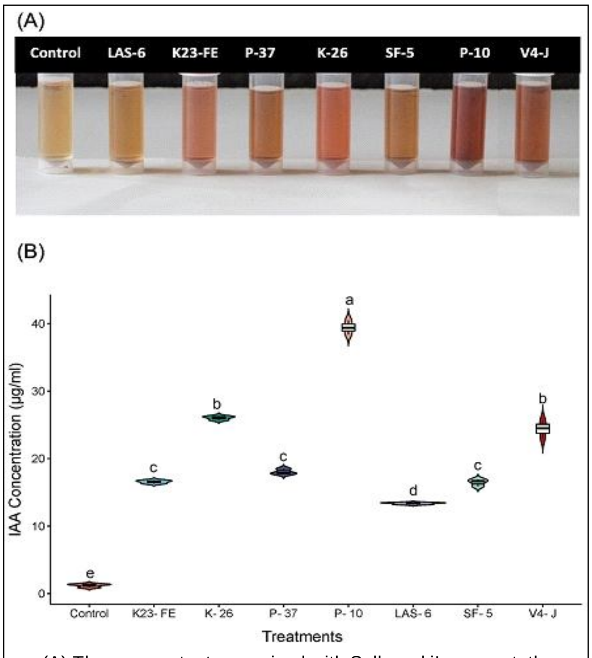


Fig 6: Amylase activity of fungal isolates showing zone of starch hydrolysis.



(A) The supernatant was mixed with Salkowski's reagent; the appearance of red indicated IAA production (tube 1: un-inoculated; tube 2-9: endophytes inoculated). (B) IAA was estimated in culture filtrate of all endophytes and expressed as µg/ml. Mean±SE (n=3) with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over violin boxplot indicate standard error over mean.

Fig 7: IAA production by fungal endophytes.

of processes. Under normal and drought-stress conditions in an *in vitro* experiment, endophytes-inoculated plants showed better seedlings than uninoculated plants. Endophytes isolated from plant and their inoculation enhanced the growth of other plants was previously found, where the root length of IR-64 paddy seedlings treated with

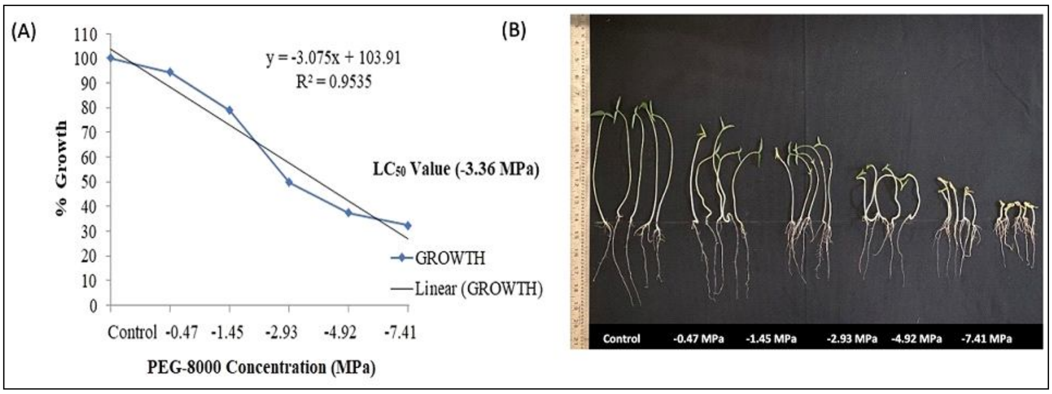
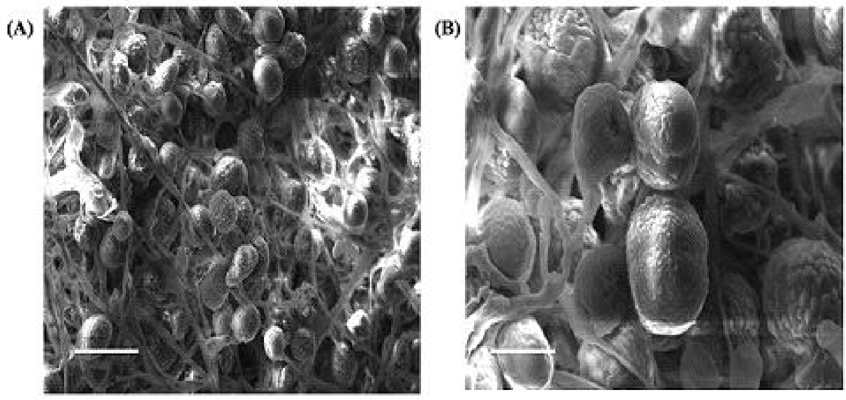
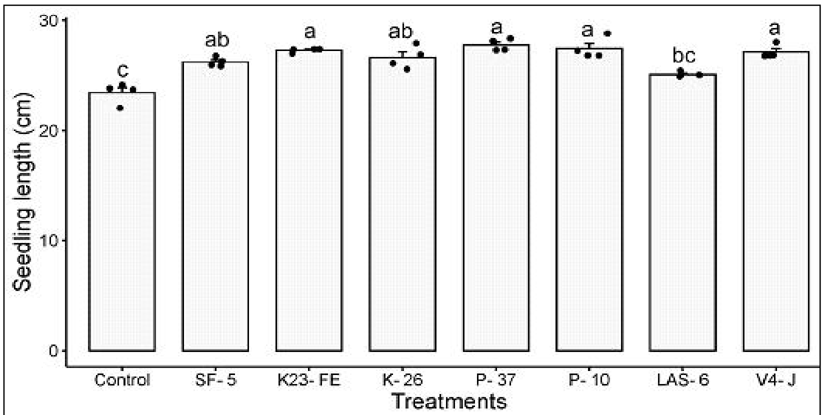


Fig 8: (A) and (B) are effect of different concentrations of PEG-8000 on seedling growth of mung bean variety KKM-3.



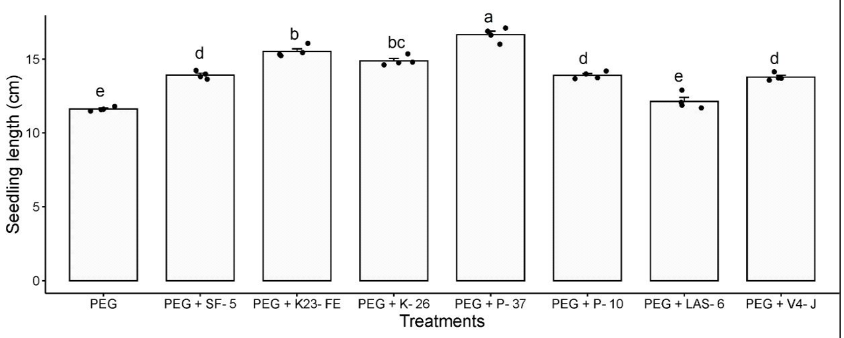
Scale bar: (A) 20 µm and (B) 10 µm.

Fig 9: SEM image of spores of best fungal isolate that performed well in mung bean, (A) and (B) Ulocladium sp.



Mean±SE (n=40) with dissimilar alphabets are significantly different at Tukey's p≤0.05. Lines

Fig 10: Seedling length of mung bean (KKM-3) colonized with different fungal isolates under unstressed conditions.



Mean±SE (n=40) with dissimilar alphabets are significantly different at Tukey's p≤0.05. Lines over barplots indicate standard error over mean.

Fig 11: Seedling length of mung bean (KKM-3) colonized with different fungal isolates under PEG-8000 induced drought stress conditions.

LAS-4 (*Aspergillus* sp.) and SAP-3 (*Aspergillus* sp.) fungal endophytes significantly increased compared to non-treated control plants under PEG induced drought stress (Sangamesh *et al.,* 2018). Utilizing microbial consortiums with varied endophytic isolates that have different PGP features may be able to be integrated and increase plant growth by utilizing various mechanisms at various stages of the plant life cycle (Li *et al.,* 2016). Obviously, endophytes isolated in this study exhibited multiple routes to improve plant growth.

However, the effect of an endophyte on a particular plant host may be variable (Bastias *et al.,* 2021) depending on the endophytic species, the host genotype and the environmental conditions. Here, in the present study, each endophytic isolate behaved differently in a crop system studied. This could be due to the differences in plant-endophyte interactions and host system may have unique signaling molecules or mechanisms.

CONCLUSION

The utilization of microbial symbionts in crop cultivation offers an ecologically sound and sustainable way of farming. The results from *in vitro* assays suggest that endophytes isolated from plants adapted to extreme environments have the potential to increase plant growth, possibly through the mechanisms of amylase activity, IAA production, phosphate solubilization and siderophore production. The most recent developments in molecular biology and functional genomics will contribute to the improvement of our knowledge of plant-endophyte interactions. The combination of many microbial strains into a single consortium can benefit crop plants in various ways. To fully utilize the plant growth-promoting potential of endophytes, additional research on the results of plant-endophyte interactions in various ecological situations and under field conditions would be helpful.

Conflict of interest

All authors declare no conflicts of interest related to the research and no financial or personal relationships that could potentially bias the research.

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