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Exploring the Potential of Endophytic Fungi in Enhancing the Growth of Cultivated Pigeonpea

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Abstract

Endophytes represent an important group of organisms that have an intimate symbiotic relationship with their hosts and mostly colonize intercellular spaces. Asymptomatic association and beneficial traits coveted on plants attracted interest to study them as a potential source of plant benefiters. The present investigation explores the potential of endophytic fungi isolated from wild pigeonpea [*Cajanus scarabaeoides* (L.) Thouars] as biofertilizer and biostimulant to enhance the growth of cultivated pigeonpea variety (ICP 8863). A total of 34 fungal endophytes were isolated from wild pigeonpea leaf and stem tissues and screened for phosphate, potassium, zinc solubilization and siderophore production. Twelve endophytes demonstrated significant biofertilization potential by exhibiting positive response to the qualitative assays and were further evaluated for their plant growth-promoting effects *in planta*. All the endophyte-treated plants showed improved growth parameters compared to control. But the endophyte-designated SF6 exhibited the highest germination rate at 88.88 per cent, along with substantial improvements in plant height (81.96cm), number of branches (12.33), number of leaves (47.33) and dry matter accumulation (133.20g/plant). Further, phytohormone profiling of SF6 using High-Performance Liquid Chromatography (HPLC) revealed the presence of growth-promoting hormones *viz.,* indole acidic acid, cytokinin, gibberlic acid and abscisic acid. Molecular characterization through internal transcribed spacer (ITS) sequencing identified SF6 as a *Fusarium* species. The findings suggest that SF6 holds significant promise as a biofertilizer and biostimulant, offering potential applications in sustainable agriculture to improve crop resilience and productivity.

Keywords : *Cajanus scarabaeoides*, Endophytic fungi, Biofertilization, Biostimulation, Phytohormone

The term 'endophyte' derived from the Greek words 'endon,' meaning inside and 'phyton,' meaning plant. It was originally postulated by De Bary (1866). Endophytes are microorganisms (fungi, bacteria, protozoa, virus or algae) that live part of their life or complete th]eir life cycle inside living plants without causing negative symptoms (Saikkonen *et al.,* 2004). The distribution of endophytes is ubiquitous and has been reported in almost all tissues of plants including leaves, stems, roots, flowers and fruits and form symbiotic relationships with their host plants. The diversity of endophytes within a single plant species can be remarkable with some plants hosting over 100 different endophytic species. This high biodiversity suggests a complex and dynamic interaction between plants and their endophytic communities, shaped by environmental factors and the evolutionary history of the host plant (Liu *et al.,* 2017).

Pigeonpea is a leguminous crop known for its adaptability to various soil types and climatic

conditions. Wild varieties of pigeonpea, often found in uncultivated regions, can harbor unique endophytes due to their exposure to diverse environmental stresses. These endophytes potentially offer novel traits for plant growth promotion and stress resistance which can be harnessed to improve crop resilience and productivity (Lacava *et al.,* 2022).

Plant growth-promoting endophytes (PGPE) display three disparate mechanisms in PGP activity *viz.,* phyto stimulation, biofertilization (directly) and biocontrol (indirectly) (Baron and Rigobelo, 2022). PGPE augment plant growth by modulating phytohormones (gibberellin, auxin, cytokinin and ethylene), nutrient availability (phosphorus solubilization, siderophore production, nitrogen fixation, etc.) and are involved in bioremediation of heavy metals (Afzal *et al.*, 2019). Thus, they act as biostimulants and biofertilizers (Baron and Rigobelo, 2022).

Fossil evidence indicates that the relationship among plants and symbionts has been persistent throughout the evolutionary history of land plants (Delaye *et al.,* 2013). This enduring association highlights the fundamental role of endophytes in plant evolution and adaptation. Exploring endophytes from diverse and resilient plants such as wild pigeonpea presents exciting opportunities to enhance agricultural sustainability and resilience. By harnessing endophytes' natural biodiversity and functional capabilities, researchers can unlock new possibilities for sustainable agriculture and environmental management (Fadiji and Babalola, 2020 and Baron & Rigobelo, 2022).

This study aims to evaluate the significance of endophytes isolated from wild pigeonpea in improving the cultivated variety of pigeonpea.

Material and Methods

Isolation of Endophytes

Endophytes were isolated from wild pigeonpea, *Cajanus scarabaeoides* (L.) Thouars by following a standard protocol (Arnold *et al.,* 2000) in aseptic condition under laminar airflow which is illustrated in Fig. 1. Briefly, segments of leaf and stem tissues, each measuring 1 cm, were subjected to a thorough washing with distilled water. Subsequently, these explants underwent surface sterilization, involving a 1-minute treatment with 70 per cent (v/v) ethanol, followed by 30-second sterilization with 1 per cent (v/v) sodium hypochlorite and concluding with another 1-minute treatment using 70 per cent (v/v) ethanol. Finally, all the leaf and stem segments were

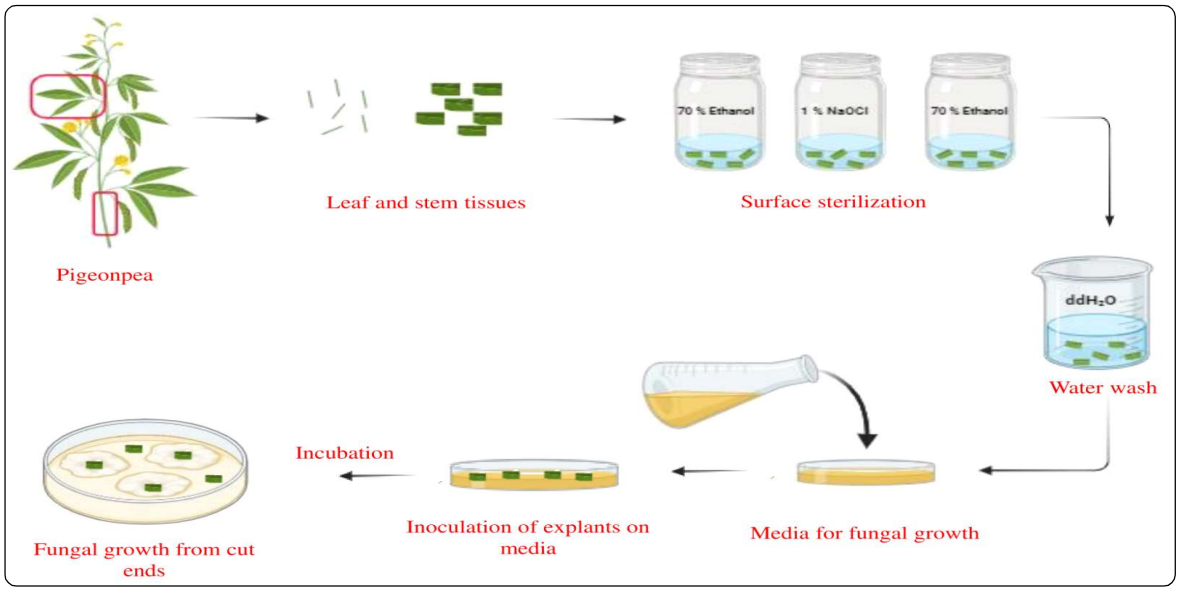

Fig. 1 : Illustration of isolation of endophytes from pigeonpea under aseptic condition

rinsed thoroughly with sterile distilled water and air-dried.

The processed leaf and stem segments were placed over different media *viz.,* potato dextrose media (PDA), Richards synthetic media (RSM) and Sabouraud dextrose agar (SDA) in aseptic conditions. The inoculated plates were incubated for 5-7 days and observed for endophytic growth from cut ends. To check epiphytic contamination, the processed explants were imprinted on media (Schulz *et al.,* 1993).

The coding of endophytes was done by considering the first letter of the explant (L-leaf; S-stem) and the endophytic organism (F-fungi).

Colonization Frequency of Endophytes

Four explants of each stem and leaf tissue were placed on different media and three replications were maintained. A total of 36 explants of each stem and leaf tissue were evaluated for their colonization. The per cent colonization frequency (CF) was calculated by following the formula (Suryanarayanan *et al.,* 2003)

$$Cf\,\% = \frac{\text{Number of segements colonized by endophytes}}{\text{Total number segements analysed}} \times 100$$

Morphological Differentiation of Isolated Endophytes

The isolated endophytes were characterized phenotypically by macroscopic studies where the mycelial color, colony pigmentation, texture, elevation, margin, form were evaluated by fungal descriptions of Ellis (1976) and Barnett and Hunter (1998). The growth rate was calculated by considering the time taken by the fungus to cover entire Petri plate *viz.,* (1) very fast= within 5 days; (2) fast= 5-10 days; (3) medium= 10-15 days; (4) slow= 15-20 days.

Screening the Isolated Endophytes for Biofertilization Activity

All the isolated endophytes were qualitatively tested for their ability to solubilize phosphorus, potassium and zinc and for siderophore production. The evaluations were conducted using established methods and specific media: Pikovskaya medium for phosphorus solubilization (Jasim *et al.,* 2013), Aleksandrow medium for potassium (Hu *et al.,* 2006), a minimal medium with ZnO for zinc (Fomina *et al.,* 2005) and Chrome Azurol S (CAS) solid medium for siderophore production (Schwyn and Neilands, 1987).

The *in-vitro* tests involved placing fungal plugs onto the respective media plates and incubating them at 30°C for 7 days. The development of the clear zones around the fungal plugs was taken as an indication of the solubilization of phosphorus, potassium and zinc. However, for siderophore production, the presence of an orange zone around the colony was a positive indication.

***In planta* Evaluation of Endophytes as Biostimulants**

The cultivated variety of pigeonpea, ICP 8863 was used for *in planta* studies. The suspension of respective endophytes was prepared with a concentration of 2×10^6 CFU/mL and the same was used for overnight seed-priming. Germination per cent of the primed seeds was observed using a blotter paper test and germination per cent was calculated after 2-3 days. Primed seeds were also sown in pots (2 kg capacity) under glasshouse conditions (Nandan, 2022). After 12 weeks of sowing, observations were taken on plant height (cm), number of branches, number of leaves and dry matter accumulation (g/plant).

Determination of Fungal-derived Phytohormones by High-Performance Liquid Chromatography (HPLC)

The endophytes demonstrating biostimulant and biofertilizer properties were selected for further phytohormone profiling using High-Performance Liquid Chromatography (HPLC). Each endophytic fungus was initially cultured on potato dextrose agar for 7 days at 30°C. Subsequently, a 5 mm disc of the fungal culture was inoculated into 100 mL of potato dextrose broth and incubated at 30°C for 14 days.

Uninoculated media served as the control. For indole-3-acetic acid (IAA) quantification, the potato dextrose broth was supplemented with 5 mg/mL of tryptophan, following the protocol by Tien *et al.* (1979).

After 14 days, the culture filtrate from each flask was filtered using Whatman No. 42 filter paper. The pH of the filtrate was adjusted to a range of 2.5 to 3.0 by adding 0.1 N HCl to enhance their solubility in organic solvents.The extraction process was conducted according to the method described by Rachev *et al.* (1993). An equal volume of diethyl ether was added to the acidified supernatant and the mixture was incubated overnight at 4°C to facilitate phase separation. The organic phase was discarded, and the solvent phase was collected and evaporated using a rotary vacuum evaporator at 40°C and 10 rpm. The resulting residue was dissolved in HPLC-grade acetonitrile / methanol (2 mL).

HPLC analyses were conducted in the Department of Agricultural Microbiology, UAS, GKVK, Bengaluru, on a Shimadzu instrument (Prominence - I, LC-2030C) equipped with a UV detector (LC-2030 UV detector) and fitted with a C18 reverse phase HPLC column (Shim-pack GIST C18, Dimension 250 x 4.6 mm, particle size 5 µm). The column temperature was maintained at 30 °C for all samples, with additional specific conditions as outlined below.

Phytohormone	Solvent	Wave length (nm)	Flow rate (mL/min)
IAA	Methanol:water (80:20)	270	1.0
GA	Methanol:water (70:30)	208	0.8
Cytokinin	Methanol:water (80:20)	240	1.0
ABA	Acetonitrile:Acetic acid 0.5 % (80:20)	254	0.8

Characterization of Efficient Endophyte

Molecular characterization of efficient endophyte was done by DNA isolation by the established Cetyl (hexadecyl) Trimethyl Ammonium Bromide (CTAB) method. (Csaikl *et al.,* 1998). Selected fungal endophyte was inoculated into a conical flask containing 100 mL potato dextrose broth (PDB) media and incubated for five days at a controlled temperature of 27±1 °C. The resulting mycelial mat was harvested, dried in air and subsequently utilized for genomic DNA isolation.

Further, amplification of the internal transcribed spacer (ITS) region of genomic DNA was done by using universal primers ITS1 - F (52 CTTGGTCATTTAGAGGAAGTAA 32) and ITS4-R (52 TCCTCCGCTTATTGATATGC 32). The PCR amplification was performed in a 25 µL reaction mixture containing 8.75 µL of PCR Master Mix (Genes2Me^{R} REF- MM101D), 1.25 µL of both forward and reverse primers and 2.5 µL of DNA template. The PCR was conducted using a Proflex PCR thermal cycler (Carlsbad, California, United States). The amplification protocol included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 60 seconds with a final extension at 72°C for 8 minutes. The amplified products were subjected to electrophoresis on a 1 per cent (w/w) agarose gel to confirm the specific region's amplification (Nandan, 2022).

The PCR products were submitted to Eurofins Genomics for sequence analysis. Following sequencing, the obtained sequences were compared against those available in the Gen Bank database using the Basic Local Alignment Search Tool (BLASTn) provided by NCBI. Sequences exhibiting the highest similarity to our endophytic isolate were selected and sequence identity matrices were created using the BioEdit Sequence Alignment Editor (Version 5.0.9). Additionally, a phylogenetic tree was constructed using MEGA X software (Kumar *et al.,* 2018) to elucidate the evolutionary relationships between sequence of our endophyte isolate and other related sequences.

Statistical Analysis

The experiment was laid out in the completely randomized design (CRD) and data obtained from

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laboratory and pot experiments were statistically analyzed using WASP: 2.0 (Web Agri Stat Package 2) statistical tool (www.icargoa.res.in/wasp2/index.php) and means were analysed by Duncan's Multiple Range Test (DMRT).

Results and Discussion

Isolation and Colonization of Fungal Endophytes

Leaf and stem segments of wild pigeonpea, *Cajanus scaraboides* were processed for fungal endophyte isolation by inoculating onto the different media. Following an incubation period of 5 to 7 days, mycelial growth was observed emerging from the cut ends of inoculated plant segments. A total of 34 fungal endophytes were isolated from 72 tissue segments with an equal distribution of 36 segments each from leaf (20; Plate 1) and stem (14; Plate 2) samples.

The colonization frequencies of endophytic fungi from wild pigeonpea ranged from 69.44 per cent and 83.33 per cent. Among the leaf and stem sample segments,

Plate 1 : Endophytes isolated from leaf tissues of pigeonpea after 7-10 days of inoculation

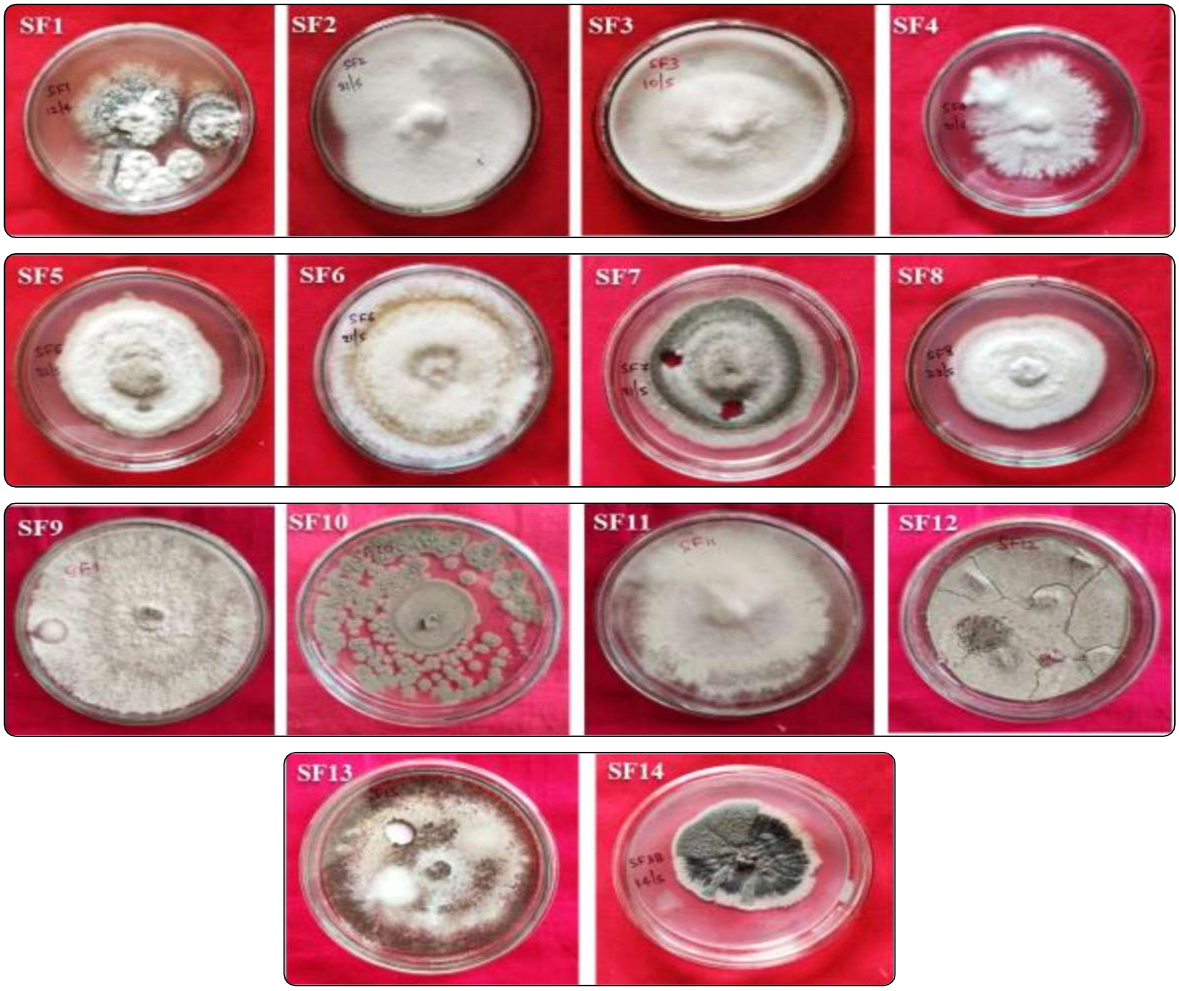

Plate 2 : Endophytes isolated from stem tissues of pigeonpeaafter 7-10 days of inoculation

the leaf samples demonstrated the higher colonization frequency (83.33%) in the plant samples processed (Table 1).

The isolated endophytes were categorized based on their morphological characteristics, as detailed in the accompanying Table 2. A comprehensive study of fungal endophytes inhabiting wild pigeonpea (*Cajanus scaraboides*) identified a diverse fungi community within leaf and stem tissues. This research aimed to elucidate the ecological roles and interactions of these endophytes within their host plant. The colonization frequencies observed, ranging from 69.44 to 83.33 per cent, suggest a high prevalence of endophytic fungi within *C. scaraboides*. Notably, leaf

TABLE 1

Colonization frequency of isolated endophytes from different tissues of pigeonpea

Plant part	No. of segments placed on media	No. of segments colonized by endophytes	No. of endophytes emerged	Colonization frequency (%)
Leaf	36	30	20	83.33
Stem	36	25	14	69.44

TABLE 2

Morphological characterization of isolated endophytes from different tissues of pigeonpea

Isolate code	Mycelium colour	Pigmentation	Form	Elevation	Margin	Surface texture	Growth rate
LF1	Blackish grey	Nil	Filamentous	Umbonate	Filiform	Appressed	Medium
LF2	Dark grey	Nil	Filamentous	Umbonate	Undulate	Slightly Fluffy	Slow
LF3	White	Nil	Rhizoid	Flat	Lobate	Slightly Fluffy	Medium
LF4	White	Light green	Irregular	Flat	Undulate	Appressed	Medium
LF5	Whitish black	Black	Irregular	Raised	Entire	Fluffy	Fast
LF6	White	Nil	Filamentous	Convex	Lobate	Fluffy	Fast
LF7	White	Nil	Circular	Flat	Entire	Slightly Fluffy	Fast
LF8	White	Pink	Circular	Umbonate	Entire	Slightly Fluffy	Fast
LF9	Black	Nil	Irregular	Umbonate	Lobate	Appressed	Slow
LF10	Light Grey	Nil	Circular	Raised	Curled	Slightly Fluffy	Fast
LF11	Grey	Black	Circular	Raised	Entire	Slightly Fluffy	Fast
LF12	White	Nil	Rhizoid	Flat	Filiform	Appressed	Fast
LF13	White	Nil	Filamentous	Flat	Filiform	Appressed	Medium
LF14	White	Nil	Rhizoid	Flat	Filiform	Appressed	Very Fast
LF15	Blackish grey	Nil	Irregular	Raised	Undulate	Slightly Fluffy	Very Fast
LF16	Whitish black	Light yellow	Irregular	Raised	Undulate	Slightly Fluffy	Very Fast
LF17	Grey	Nil	Circular	Raised	Entire	Slightly Fluffy	Very Fast
LF18	Whitish black	Black	Circular	Raised	Entire	Slightly Fluffy	Fast
LF19	White	Nil	Circular	Flat	Undulate	Slightly Fluffy	Fast
LF20	Whitish black	Black	Irregular	Raised	Undulate	Slightly Fluffy	Medium
SF1	Whitish grey	Nil	Filamentous	Umbonate	Filiform	Slightly Fluffy	Medium
SF2	White	Nil	Circular	Umbonate	Entire	Slightly Fluffy	Fast
SF3	White	Nil	Circular	Umbonate	Entire	Slightly Fluffy	Very Fast
SF4	White	Nil	Rhizoid	Flat	Lobate	Slightly Fluffy	Medium
SF5	White	Black	Irregular	Convex	Undulate	Fluffy	Fast
SF6	White	Light orange	Filamentous	Raised	Entire	Fluffy	Fast
SF7	Grayish black	Black	Circular	Convex	Curled	Fluffy	Fast
SF8	White	Black	Circular	Umbonate	Undulate	Fluffy	Medium
SF9	White	Nil	Rhizoid	Flat	Lobate	Slightly Fluffy	Very Fast
SF10	Dark grey	Nil	Irregular	Flat	Entire	Appressed	Very Fast
SF11	White	Nil	Filamentous	Umbonate	Filiform	Slightly Fluffy	Medium
SF12	Light grey	Black	Circular	Flat	Entire	Appressed	Medium
SF13	White	Nil	Filamentous	Flat	Entire	Slightly Fluffy	Fast
SF14	Blackish grey	Nil	Filamentous	Umbonate	Filiform	Slightly Fluffy	Slow

segments exhibited the highest colonization frequency compared to stem segments.

The observed variation in colonization frequencies between leaf and stem tissues underscores the differential ecological niches occupied by endophytes within the host plant. Such differences could be linked to the specific micro environments and nutrient availability in leaves versus stems. For instance, Gond *et al.* (2012) reported the highest colonization of endophytes in leaves than in stems, possibly due to the larger surface area of the leaf relative to the stem

and the presence of stomata in leaves, which facilitate the entry of fungal mycelium, likely leading to more extensive colonization. Leaves, being directly exposed to the external environment, may offer a more favorable habitat for fungal endophytes owing to the availability of nutrients and favorable micro climatic conditions, which can influence endophyte colonization and proliferation (Liu *et al.,* 2019). Additionally, Goveas *et al.* (2011) observed that the low frequency of stem endophytes is probably due to the more nutrient-rich environment provided by the leaves for fungal growth with a variety of organic compounds available for fungal metabolism. Despite this preference, stem tissues also exhibit appreciable colonization. This dual colonization highlights the significance of leaf and stem tissues as distinct habitats for endophytic fungi in wild pigeonpea ecosystems.

Qualitative Analysis of Biofertilization Activity of Endophytes

The study investigated various plant growth-promoting traits in fungal endophytes *viz.,* siderophore production, phosphate, potassium and zinc solubilization. Qualitative analysis revealed that all 34 endophytic isolates established phosphate solubilization, as evident by production of clear zones around their colonies. However, not all isolates have exhibited positive results for the other parameters.

Among the isolates, only 12 (LF4, LF6, LF7, LF11, LF19, SF2, SF3, SF6, SF8, SF9, SF11 and SF13) isolates showed positive responses across all tested parameters. These isolates displayed clear zones around their colonies indicating phosphate, potassium, and zinc solubilization and an orange zone denoting siderophore production. This qualitative assessment, depicted in the binary category plot (Fig. 2), highlights the variability in plant growth-promoting capabilities among the fungal endophytes isolated from the study.

The universal ability among the isolates to solubilize phosphate suggests that these fungi could be instrumental in enhancing soil phosphorus availability, which is a critical trait for plant growth (Singh *et al.,* 2020). This lines with the report published by many researchers who witnessed the improvement in phosphorus acquisition by endophytic fungal inoculation (Ortega-Garcia *et al.,* 2015 and Baron *et al.,* 2018). While phosphate solubilization was universal among the isolates, the solubilization of potassium and zinc was more selective. The ability to solubilize potassium and zinc is significant since these micronutrients are essential for various plant physiological processes, including enzyme activation and protein synthesis (Nalini and Mutturaj, 2023). These 12 isolates that demonstrated the ability to solubilize both potassium and zinc, in addition to phosphate, exhibit a broad spectrum of nutrient-mobilizing properties, which was previously shown by Ravi *et al.* (2022) in *chrysanthemum-Fusarium haematococcum* (root endophyte) ecosystem and Tripathy *et al.* (2022) in rice-endophytic fungal communities. This suggests that these isolates could be particularly effective in biofertilization, improving overall nutrient uptake and plant health. Some isolates exhibited siderophore production, implying that these fungi can enhance iron availability (Eslahi *et al.,* 2020) thereby supporting plant growth in iron-deficient soils.

Thus, these endophytes can convert elements from unavailable to available forms for plant uptake by releasing their compounds to the soil through root exudates, thereby boosting plant growth. The abilities of 12 isolates (LF4, LF6, LF7, LF11, LF19, SF2, SF3, SF6, SF8, SF9, SF11 and SF13) to solubilize phosphate, potassium and zinc solubilization, along with siderophore production, are valuable for developing biofertilizers in future. Their multifunctional capabilities can address multiple nutrient limitations simultaneously, promoting more robust plant growth.

Evaluation of Selected Endophytes for Biostimulant Activity

Twelve endophytes, which demonstrated positive biofertilizer activity, were selected for further *in planta* growth promotion analysis. Seeds were treated with each of these endophytes and germination rates were monitored. At 12 weeks post-sowing, plant height (cm), number of branches per plant, number of leaves per plant and dry matter ccumulation (g/plant) were measured.

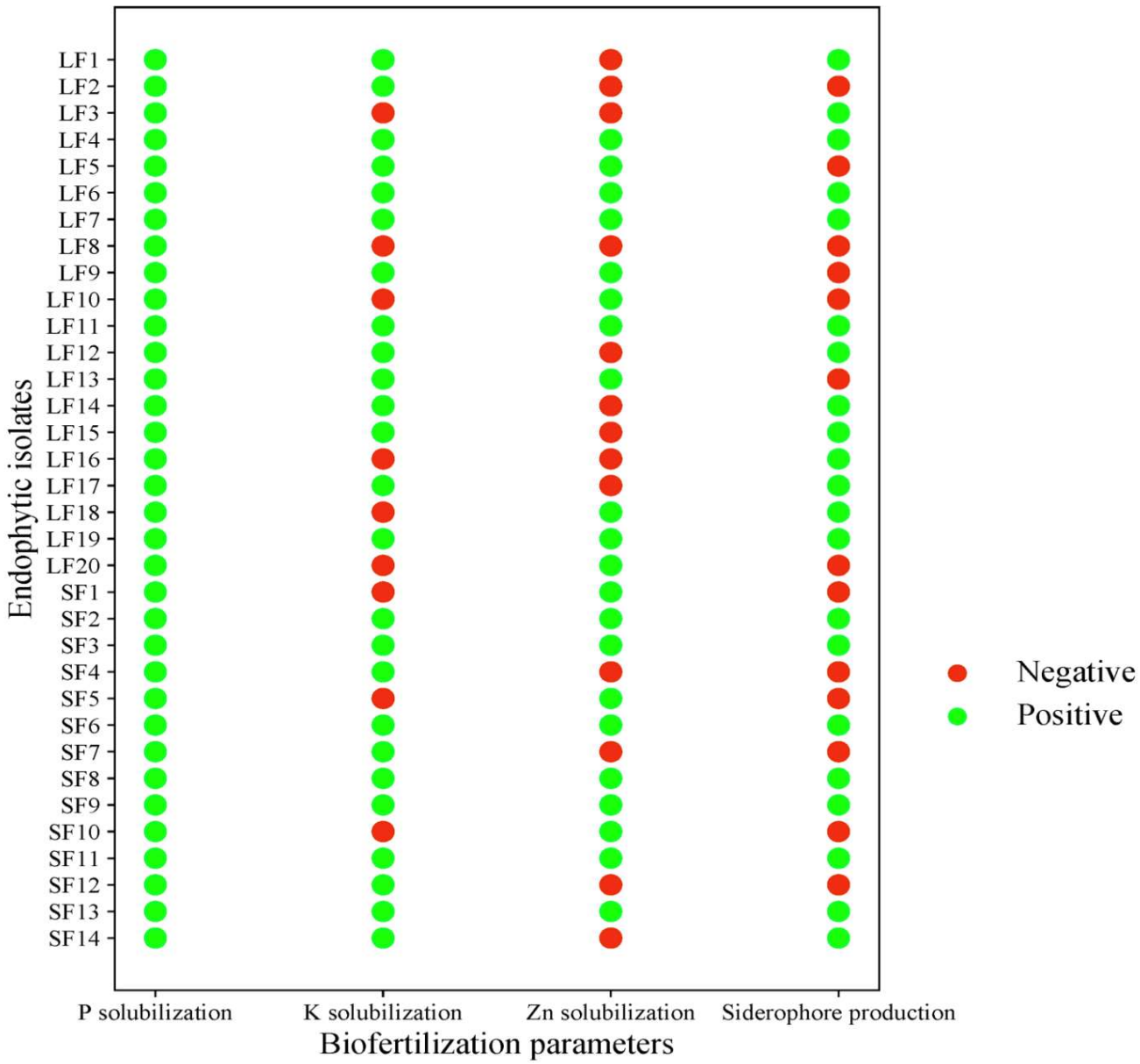

Fig. 2 : Binary category plot representing biofertilization parameters expressed by isolated endophytes

All endophyte-treated seeds exhibited higher germination percentages compared to the control, which had a germination rate of 62.22per cent (Table 3). The SF6-treated seeds showed the highest germination rate upto 88.88 per cent, followed by SF8-treated seeds (82.22%).

Consistent with the germination results, other growth parameters *viz.,* plant height, number of branches, number of leaves and dry matter accumulation showed a significant increase in the endophyte-treated plants (Table 3). Notably SF6-treated plants exhibited a significant increase in plant height (81.96 cm), number of branches per plant (12.33), number of leaves per plant (47.33) and dry matter accumulation (133.20) compared to the plants in control treatment (Table 3). The enhancements of these growth parameters highlight the potentiality of SF6 as an effective biostimulant.

TABLE 3

Role of endophytes on biostimulation of plant growth

Treatments	Isolates	Plant height (cm)	No. of branches	Number of leaves	Dry matter accumulation (g/plant)	Germination per cent (%)
T1	LF4	75.56 fg	8.66 de	37.66 de	110.55 e	68.88 ef
T2	LF6	72.06 i	8.33 ef	34.33 gh	107.27 fg	66.66 fg
T3	LF7	76.20 ef	9.33 cde	32.33 i	106.19 g	77.77 bc
T4	LF11	77.13 de	10.00 bc	35.33 fg	110.52 e	68.88 ef
T5	LF19	76.33 ef	8.66 de	36.33 ef	109.92 e	71.10 def
T6	SF2	78.93 c	8.66 de	34.33 gh	107.81 f	77.77 bc
T7	SF3	74.53 g	9.66 bcd	40.66 c	118.61 c	75.55 cd
T8	SF6	81.96 a	12.33 a	47.33 a	133.20 a	88.88 a
T9	SF8	80.00 b	10.66 b	42.66 b	124.47 b	82.22 b
T10	SF9	75.86 f	9.33 cde	36.33 ef	108.05 f	68.88 ef
T11	SF11	73.26 h	9.00 cde	38.33 d	115.31 d	73.33 cde
T12	SF13	77.63 d	9.66 bcd	33.33 hi	107.45 fg	68.88 ef
T13	Control	69.26 j	7.33 f	30.33 j	97.78 h	62.22 g

Note: Values are mean (±SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT (p>0.05)

In planta growth promotion analysis provide convincing evidence of the potential endophytes which showed enhancement of plant growth and development through various mechanisms. The higher germination percentage in endophyte-treated seeds compared to the control indicates the positive influence of these microorganisms on seed viability and early growth stages (Mathur *et al.,* 2022). The findings align with the earlier reports by Lalngaihawmi *et al.* (2018) on the potency of endophytic *Aspergillus niger*, showing an 88.52 per cent improvement in rice seed germination, indicating a positive impact on seed health. Notably, the substantial increase in germination rates, particularly with the SF6 isolate (88.88%), suggests that this endophyte may produce growth-promoting substances such as phytohormones, which likely enhance seed germination and vigor. A study conducted by Yamaguchi *et al.* (2001) found that gibberellin biosynthesis intermediates are transported between cells to produce active gibberellins, which are crucial for seed germination. They activate the embryo, weaken the endosperm, mobilize food reserves (Davies and Slack, 1981) and counteract abscisic acid, thus enhancing seed germination.

Further analysis at 12 weeks post-sowing showed that endophyte treatment not only improved germination rates but also positively impacted other growth parameters. Plants treated with SF6 isolate exhibited the greatest increase in plant height compared to the control, indicating probable enhanced cell elongation and division contributing towards improved vertical growth. This could be attributed to the endophyte's ability to increase the availability of nutrients and possibly phytohormones produced by the endophytes (Abhinandana *et al.,* 2024). Additionally, a notable increase in the number of branches and leaves per plant was also observed in the SF6-treated group. This indicates that the endophyte might stimulate lateral growth and foliage development, potentially through improved nutrient solubilization and availability (Neekshitha and Earanna, 2023). Increased branching and leaf production are associated with a higher

capacity for photosynthesis and resource acquisition, which in turn, supports greater biomass production.

The observed increase in dry matter in plants treated with endophytes signifies enhanced biomass accumulation, a crucial indicator of overall plant health and productivity. Many reports say that this enhancement is likely due to improved nutrient availability (Tulja *et al.,* 2022), increased photosynthetic efficiency from a larger leaf area and the endophytes' overall stimulation of plant metabolic processes (Rana *et al.,* 2020). These findings underscore the potential of these endophytes, especially SF6, as effective biostimulants for improving plant growth and productivity. Finally, our findings confirm previous reports on the promotion of growth by fungal endophytes (Khan *et al.,* 2011 and 2013).

Phytohormone Profiling of Efficient Endophyte

The endophyte SF6, recognized for its potential as a biofertilizer and biostimulant was investigated for its ability to produce phytohormones using high-performance liquid chromatography (HPLC). This analytical technique is effective for quantifying phytohormones produced by microorganisms, which is crucial since even low concentrations of these hormones can significantly influence plant growth and development (Ma *et al.,* 2008). Indole-3-acetic acid (IAA), a common auxin, is known for regulating various aspects of plant development. Many microorganisms produce IAA as a secondary metabolite by utilizing L-tryptophan (L-TRP) found in root exudates. In this study, the biologically active phytohormones IAA, gibberellin (GA), cytokinin and abscisic acid (ABA) synthesized by the endophytic isolate SF6 were quantified *via* HPLC, with the results displayed in a balloon plot (Fig. 3).

In vitro studies have been shown that few microorganisms can produce small amount of auxins even in the absence of L-TRP; however, in the presence of this precursor, significantly higher quantities of auxins are produced (Zahir *et al.,* 2010). The current results indicated that SF6, exhibited the highest IAA production when cultured in a medium supplemented with L-tryptophan, compared to fungi grown in a standard medium. This underscores the role of plant roots excrete organic compounds, including L-TRP, which can be utilized by plant growth-promoting microorganisms for IAA synthesis under natural conditions (Mano and Nemoto, 2012).

Gibberellins (GAs) form a large family of phytohormones with GA1, GA3, GA4 and GA7 being

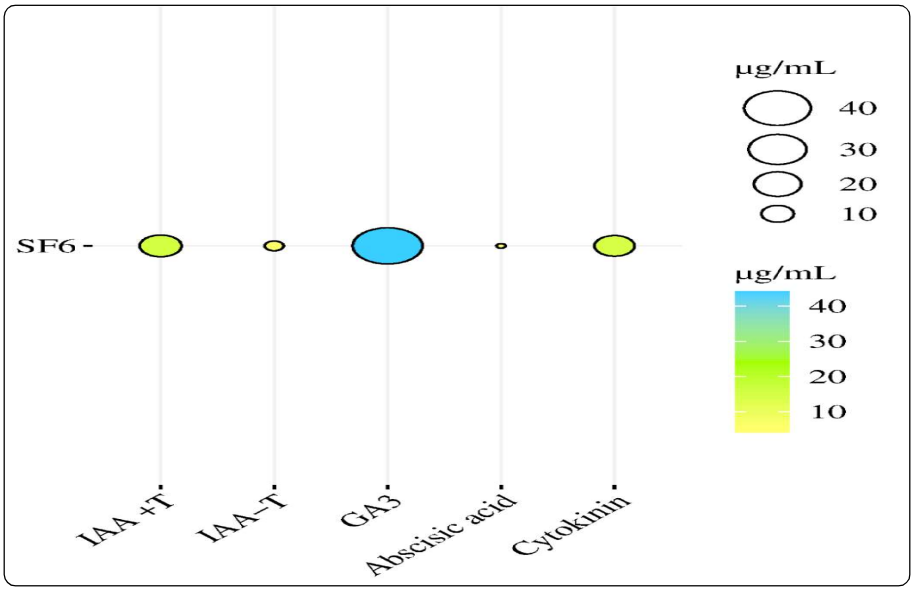

Fig. 3 : Ballon plot representing the phytohormone production by efficient endophytic isolate (SF6)

the most biologically active members. Among them, GA3 is the most prevalent form. Gibberellins produced by various organisms can promote stem elongation, alter seed dormancy and enhance leaf and fruit senescence as well as influence flowering and other physiological processes through interactions with other phytohormones (Yamaguchi, 2008). In this study, SF6 demonstrated the capability to produce approximately 40 µg/mL of GA3, which was the highest concentration among the phytohormones examined.

SF6 also produced cytokinin at a concentration of approximately 20 µg/mL, comparable to the amount of IAA produced in the presence of L-tryptophan. Cytokinins are essential for promoting cell division (cytokinesis) in plant roots and shoots, thereby facilitating tissue expansion and growth. They also delay leaf senescence by maintaining chlorophyll content and promoting nutrient mobilization, which extends the plant's photosynthetic activity and productivity (Li *et al.,* 2021).

Additionally, SF6 produced a small concentration of abscisic acid (1 µg/mL), consistent with other studies that have reported the inhibitory effects of abscisic acid on plant growth and development. Abscisic acid, known for its growth-inhibitory properties, interacts antagonistically with other phytohormones such as auxins, gibberellins and cytokinins (Brook bank *et al.,* 2021). ABA typically acts as a brake on growth processes and promotes stress responses, whereas other hormones foster growth and development under favorable conditions.

In summary, SF6, as an endophytic isolate, has demonstrated significant potential in producing various phytohormones critical for plant growth and stress responses. These findings underscore its potential utility as a biofertilizer and biostimulant in agricultural applications, enhancing plant growth and resilience in varying environmental conditions.

Molecular Characterization of an Endophyte

The identification of endophytic fungi is crucial for understanding their roles in plant ecosystems and their potential applications in agriculture and biotechnology. The endophytic fungus SF6 was studied to determine its genetic identity using molecular techniques. This process involved several steps, including genomic DNA extraction, PCR amplification, sequencing and sequence analysis using bioinformatics tools.

Genomic DNA was successfully extracted from a pure culture of the endophytic fungus SF6 using the cetyl trimethy lammonium bromide (CTAB) method. This extracted DNA served as a template for PCR amplification of the internal transcribed spacer (ITS) region, a standard genetic marker for fungal identification. Specific primers targeting conserved regions flanking the ITS region facilitated the amplification of an approximately 550 base pair fragment (Fig. 4b), consistent with the expected size of the ITS region in many fungi. The amplified ITS region was subsequently sequenced to reveal the nucleotide sequence of the DNA. This sequence was then queried using a BLASTn search against the NCBI GenBank database.

The analysis revealed that the ITS sequence of SF6 showed maximum identity with sequences from *Fusarium* species (Fig. 4a), a genus known for its diverse range of species, many of which are significant as plant pathogens, endophytes or producers of bioactive compounds (Toghueo, 2020 and Ahmed *et al.,* 2023). Endophytic *Fusarium* can enhance plant growth by producing phytohormones, such as auxins and gibberellins or by facilitating nutrient uptake. They may also confer resistance to biotic and abiotic stresses, including pathogen attack and drought, through the production of secondary metabolites and other bioactive compounds (Wei *et al.,* 2019 and De-Lamo & Takken, 2020).

The findings of this study underscore the potential of endophytic fungi from wild pigeonpea as valuable bioresource for sustainable agriculture. The ability of these fungi to enhance plant growth through nutrient solubilization and phytohormone production positioning them as promising candidates for the development of eco-friendly biofertilizers and biostimulants. Precisely, the isolate SF6, identified as

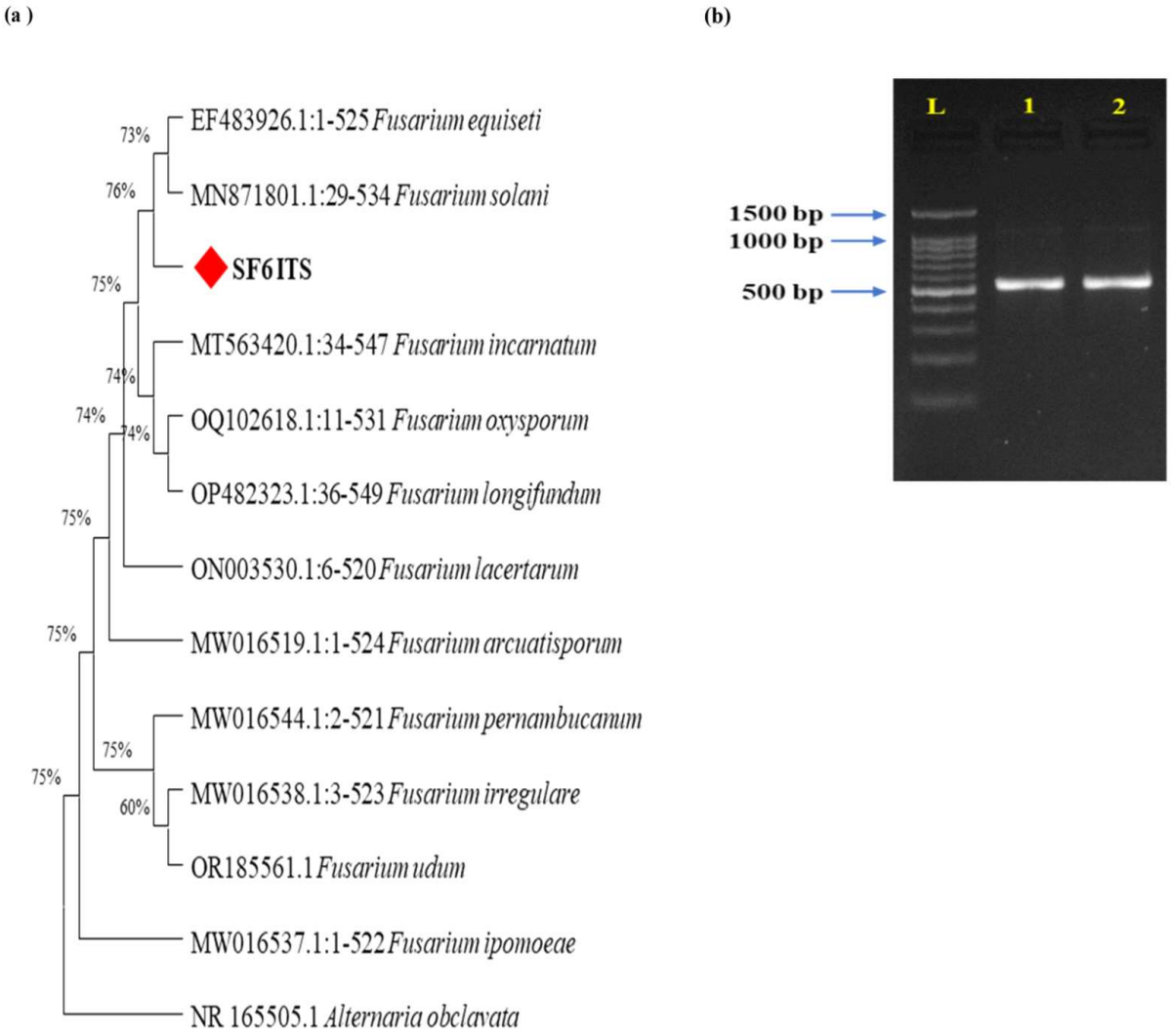

Fig. 4 : (a) Phylogenetic tree of endophytic fungi, SF6 constructed by a neighbor-joining method using ITS housekeeping gene primer and respective gel picture (b) (L - Ladder; 1&2 - Fungal DNA sample)

Fusarium sp., shows a considerable capacities due to its multifaceted plant growth-promoting activities. These activities are attributed to its distinctive morphological features, including fluffy mycelial growth (which enhances plant colonization), pigmentation (associated with secondary metabolite production) and a rapid growth rate (facilitating high multiplication within plants). Further studies should be focused on large-scale field trials to validate the efficacy of SF6 and other promising isolates under diverse agro-climatic conditions. Investigation into the mechanisms underlying the biostimulant and biofertilization effects of these endophytes could provide deeper insights into their interactions with plants and soil ecosystems. By exploring the potential of these endophytes in biocontrol applications against plant pathogens could be enhance their utility in integrated pest management strategies. In conclusion, this research highlights the significant potential of endophytic fungi from wild pigeonpea as a tool for enhancing crop resilience and productivity, contributing to more sustainable and resilient agricultural practices. The identification and characterization of these endophytes offers a new avenue for improving crop management and addressing global agricultural challenges.

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