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Growth Promotion in *Arabidopsis thaliana* Induced by Fungal Endophytes Isolated from Plants Growing in Extreme Habitats

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ABSTRACT

Plants and microbes have co-evolved in nature over the past few years for their better adaptation. This intricate symbiotic relationship is captured in the hologenome concept, which emphasizes that the combined genome of plants and their associated microbial partners, function as a single evolutionary unit. It highlights the possibility of using beneficial microorganisms, including fungal endophytes, to increase plant growth and productivity by habitat-adapted symbiosis mechanism. According to earlier research, fungal endophytes activate specific physiological traits in crops. However, the specific ways in which these endophytes benefit plants and underlying mechanisms remain unclear. In this context, the objective of this study was to utilize the model system *Arabidopsis thaliana* to explore possible mechanisms of plant-endophyte interaction. Eight fungal endophytes (K-23, LAS-6, N-14, P-10, P-37, PJ-9, SF-5 and V4-J) previously isolated from extreme habitats were re-examined in terms of their colony morphology. Based on the leads from previous studies, five fungal endophytes (LAS-6, N-14, P-10, PJ-9 and SF-5) were selected and exposed for *In vitro* co-cultivation with new host *A. thaliana*. The highest colonisation percentage was observed for the SF-5 (67%) followed by P-10 (33%), LAS-6 (27%) and N-14 (33%), whereas a lower colonisation percentage was observed for PJ-9 (20%). Additionally, these endophytes showed growth promotion activity by improved photosynthetic leaf area, root dry weight and shoot dry weight in fourteen days old *Arabidopsis* seedlings. The identification of trait-specific endophytes and incorporation of hologenome-enrichment approach can serve as a sustainable and eco-friendly strategy for crop improvement.

Keywords : Fungal endophytes, Plant growth promotion, *Arabidopsis thaliana*

As the global population continues to surge, agricultural land dwindles and climate change poses significant challenges to crop production, there is an urgent need to explore innovative strategies to enhance crop growth and yield (IPCC, 2022). In recent times, various strategies, including genetic modification, mutational selection and targeted breeding, *etc.,* have been employed to incorporate traits from wild systems to improve the yield (Shen *et al.,* 2018; Ma *et al.,* 2023 and Singha & Singha, 2024). However, the success rate has been limited, and potential drawbacks, such as the inadvertent loss of beneficial genes and significant impacts on biodiversity, exist (Phillips, 2008 and Jacobsen *et al.,* 2013). Traditional genetic breeding approaches have largely exhausted the potential for yield improvements, necessitating the exploration of alternative methods. Therefore, novel approaches are being used to manipulate plants in an eco-friendly manner. In this context, the utilization of external, eco-friendly supplements has garnered attention as a means to meet the growing food demands sustainably.

One such approach is the manipulation of hologenome. The term 'hologenome' refers to the collective genetic material of an organism and the symbiotic microorganisms associated with it (Jefferson 1994). The concept emphasizes that the genomes of the microorganisms that live in or on an organism, as well as the genome of the organism itself, may interact and contribute to the traits and adaptations of the latter. The host organism (plant) and its associated microbial communities (in the apoplast) as a functional unit, play a crucial role in plant growth, adaptation and ecological interactions (Zilber-Rosenberg and Rosenberg, 2008).

The apoplastic organisms, the endophytes, are primarily fungi and bacteria, that reside without causing apparent harm to the plants. There are reports to suggest that plants have adapted to stressful environments by forming symbiotic associations with endophytes (Delaux and Schornack, 2021). The habitat-adapted symbiosis by endophytes represents a fascinating phenomenon with significant implications for plant health and ecosystem dynamics. By incorporating endophytes adapted to specific environmental conditions, crops can potentially exhibit enhanced stress resilience (Lata *et al.,* 2018). Endophytic fungi mainly rely on the apoplastic fluid to seek nutrients and develop mutualistic relationships with plants (Bacon and White, 2000; Kusari *et al.,* 2012 and Gouda *et al.,* 2016). As endophytes have facilitated better plant growth and development under stressful environments, it appears to be an interesting strategy if they can be employed to alleviate crop growth to increase agricultural production. Thus, understanding the mechanisms behind this endophyte-mediated growth promotion will open new opportunities for their commercial application in crop production. There is a possibility that endophytes can better crop growth even under normal conditions as they appear to provide external and additional resources for plants through their symbiotic associations (Rodriguez *et al.,* 2008; Sangamesh *et al*., 2018; Chitnis *et al*., 2020 and Dhanyalakshmi *et al.,* 2023).

Studies indicate that endophytes also play a crucial role in promoting early seedling growth in rice, green gram, soybean and cowpea (Vasanthakumari *et al*., 2019 and Ayesha *et al*., 2022). Recent reports suggest that these endophytes can improve the photosynthetic performance of plants by increasing internal CO_2 (Ci) concentration through their respiratory metabolism (Suryanarayanan *et al*., 2022) and contrary to this it is also argued that endophytes can minimise the photosynthetic limitation by increasing the triose phosphate utilisation and ribulose-1, 5-bisphosphate (RuBP) regeneration; Rho *et al*., 2020 and Bangari & Nataraja, 2023). There are compelling evidences to argue that endophytes impart stress tolerance in major crops such as rice (Sampangi Ramaiah *et al*., 2020 and Manasa *et al*., 2020), cucumber (Moghaddam *et al*., 2021), tomato (Pallavi & Nataraja, 2022) and maize (Zhang *et al*., 2018 and Siddiqui *et al*., 2022).

Numerous reports indicate the positive influence of endophytes on plant growth promotion. However, despite efforts to decipher the communication patterns between endophytes and their host (Sampangi Ramaiah *et al.,* 2019), the fundamental mechanisms underlying plant-endophyte interactions remain largely unexplored. Because of complex interactions, it would be ideal to use model system *A. thaliana* (Michal *et al*., 2011) for fruitful output within a short period. *A. thaliana* provides an added advantage in carrying out experiments due to the availability of ample bioresources, complete genome sequence information, and a rapid life cycle of approximately 45 days (TAIR, *https://www.Arabidopsis.org*). This model system also helps in studying the host-specificity of endophytes, a fundamental aspect of utilizing endophytes in commercial crops. The present study investigates the ability of the eight habitat-adapted fungal endophytes isolated from extreme habitats to colonize the new host *A. thaliana*. We demonstrate that the select endophytes colonize in model plants and enhance growth by activating growth traits.

TABLE 1

Habitat information of the fungal endophytes used in the study

Fungal strains	Plant location/habitat	Latitude (° N)	Longitude (° E)	Altitude (m) Above mean sea level (AMSL)
K-23	Kargil (J&K) mountains	34°34'223 ° N	76°72 573 ° E	2750
SF-5	Tamil Nadu coast	11.1271° N	78.6569° E	253
N-14	Namika La mountains	34°23'00° N	76°27'34° E	3832
LAS-6	Thar desert	27.4695° N	70.6217° E	250
P-10, P-37	Pangong Tso mountains	33°432 2.743 ° N	78°532 29.083 ° E	4250
PJ-9	Bellary, Dryland	15.3173° N	75.7139° E	610
V4-J	Pokkali soils	9.9667° N	76.3168°E	49.6

MATERIAL AND METHODS

Collection of the Endophytic Fungi

Endophytic fungi were collected from the fungal repository of the School of Ecology and Conservation Laboratory, Department of Crop Physiology, University of Agricultural Sciences, Bengaluru, India (kindly donated by Prof. R. Uma Shaanker). The collection comprises six *Fusarium* species designated as K-23 (*Fusarium incarnatum),* SF-5 (*Fusarium equiseti*), N-14 (*Fusarium* sp.), P-10 (*Fusarium* sp.), PJ-9 (*Fusarium* sp.), V4-J (*Fusarium chlamy dosporum)* and additionally, it includes two distinct species: LAS-6 (*Chaetomium globosum)* and P-37 (*Ulocladium dauci)*. These fungal endophytes were originally isolated from the wild plants thriving in extreme climatic conditions in India as indicated in Table 1.

Microscopic Observations of the Endophytic Fungi Morphology

For microscopic examination of fungus morphology, the slide culture technique was employed with slight modification of the method by Harris (1986) and Rosana *et al*. (2014). Approximately 1cm potato dextrose agar (PDA) square blocks were prepared and positioned in the middle of the slide. Subsequently, a sterile needle was utilized to inoculate endophytic fungi towards the four corners of the PDA block. Another slide was then pressed on top to ensure adhesion and the entire setup was placed in sterile petri dishes, followed by incubation at 28°C (Prakash and Bhargava, 2016). The growth of the fungi was observed 72 hours after incubation at various magnifications using the ZEISS imaging system (Carl Zeiss™ Axio Vert.A1 Inverted Microscope).

Co-cultivation of the Fungal Endophytes with Arabidopsis

Growth Condition of Endophytic Fungus : Mycelial discs from the mother culture were used to grow the endophytic fungi by placing them in petri dishes containing Potato Dextrose Agar (PDA) medium.

Growth Condition of A. thaliana : *A. thaliana* (Col) seeds were surface sterilized by Vapor-phase Sterilization with sodium hypochlorite and hydrochloric acid for 45 minutes (Lindsey *et al.,* 2017) and 20-30 seeds were placed in petri dishes containing half-strength Murashige and Skoog media (pH 5.7-5.8) without hormones, along with solidifying agent 0.8 per cent (w/v) agar (Murashige and Skoog 1962). Petri dishes were incubated for 48 hours at 4°C for seed stratification to ensure uniform germination. After the cold treatment, the petri dishes were incubated in the plant growth chamber (ARALAB plant growth chamber, serial no 2714) with 16/8 hours light and dark period, 22/24°C temperature and 65 per cent relative humidity.

Co-cultivation of A. thaliana and Endophytic Fungi : For *in vitro* co-cultivation, a modified Plant Nutrient Media (PNM) medium was used (Michal *et al*., 2011). Mycelial discs from 4-week-old endophytic fungi were placed in petri dishes containing PNM media and incubated for 72 hours in the dark at 28°C, for control conditions PDA disc without endophytic fungus was placed. Twelve seedlings were taken per treatment with three replications each. Next, 10-12 days old *Arabidopsis* seedlings were placed on endophyte-inoculated PNM plates for co-cultivation and observations were recorded after one week with four biological replicates per replication and a total of three replications per treatment. Root dry weight (mg plant^{-1}) and shoot dry weight (mg plant^{-1}) were recorded after oven-drying the samples at 50°C for two days.

Leaf Area Measurement

The leaf area of *Arabidopsis* seedlings was measured by the non-destructive method. The photographs of individual *Arabidopsis* seedlings along with the measuring scale were manually captured from the top a week after co-cultivation. The individual photographs were processed using image analysis (Image J) software and images were converted to 8-bit format, the leaf area was estimated (Kokorian *et al*., 2010).

Re-isolation of the Endophytic Fungi from *A. thaliana*

To confirm the colonization efficiency of the endophytic fungi, the leaf, stem and root tissues were cut into 0.5cm segments and surface sterilized with 70 per cent (v/v) ethanol for 50 seconds followed by sequential sterilization with 0.1 per cent (v/v) NaOCl for 60 seconds and 70 per cent (v/v) ethanol for 30s with intermittent rinse using sterile distilled water four to five times. (Arnold *et al*., 2000). To ensure the effectiveness of the surface sterilization the cut segments were then imprinted on the PDA plates (Schulz *et al*., 1993). Later the surface sterilized plant segments were placed on PDA plates supplemented with antibacterial antibiotic ingredient streptomycin sulfate (50-100 µg/ml) and incubated for five days at room temperature (27-28°C) (Suryanarayanan, 1992). The fungus emerged from the explants was isolated and pure cultured on fresh PDA plates using a sterile needle. The pure cultures were compared with their respective mother cultures for colony appearance, spore and hyphae structures using a Zeiss Fluorescence microscope (Carl Zeiss™ Axio Vert. A1 Inverted Microscope) (Domsch *et al*., 1980 and Arx Von, 1981).

Per cent Colonization (%)

The extent of colonization was measured using the morphological method by counting the fungal emergence from the cut ends of the explants *i.e.* number of explants colonized by fungus to the total number of explants placed (colonization frequency) and multiplying it by 100 (Lawson *et al.,* 2014).

Molecular Identification of the Endophytic Fungi

Genomic DNA was isolated from the endophytic fungi by the cetyltrimethyl ammonium bromide (CTAB) method (Rogers and Bendich, 1994) and polymerase chain reaction (PCR) was carried out to amplify the Internal Transcribe Sequences (ITS) region of genomic DNA using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) as forward and reverse primers respectively (Martin and Rygiewicz, 2005). The PCR product amplified was purified and sequenced by the Sanger sequencing method. The FASTA sequence was BLASTn searched in the NCBI GenBank database (www.ncbi.nlm.nih.gov). Based on the maximum homology and per cent similarity, the identity was assigned to endophytes using the criterion described by Higgins *et al*., 2007. The phylogenetic analysis was carried out using the Clustal W plugin from MEGA software, version 11.0 (Kumar *et al*., 2016). Phylogenetic relatedness was determined by employing a UPGMA (unweighted pair group method with arithmetic mean) analysis method

(Stefan Van Dongen and Winnepenninckx, 1996) with 1000 bootstrap replications (Felsenstein, 1985).

Statistical Analysis

All the collected data sets were presented as mean ± standard error (SE), with a minimum of three samples per genotype serving as biological replicates and a Completely Randomised Design (CRD) was employed. The multiple comparison was done by employing Tukey's honestly significant difference (HSD) test. The data analysis was carried out using R software version 4.2.2 and all the graphs were drawn using ggplot2 package of R studio.

Results and Discussion

Identification and Re-confirmation of the Endophytic Fungi

The microscopic observation was done for all eight endophytes (K-23, LAS-6, N-14, P-10, P-37, PJ-9, SF-5 and V4-J). Results revealed that six out of eight endophytes belong to the *Fusarium* species. Among these, K-23 (*Fusarium incarnatum)* has an elevated network of mycelia, a fast-growth habit while the SF-5 (*F. equiseti*) showed soft white mycelia. Both the endophytes possess micro and macroconidia. Whereas, N-14 (*Fusarium* sp.) was sporulating and showed white mycelia with a red tinge on top and bottom of the petri dishes and P-10 (*Fusarium* sp.) produced red colour spores at the bottom of the plate with white to yellowish mycelia on top, PJ-9 (*Fusarium* sp.) showed a floccose white fungal mat and had a very thin mycelia and V4-J (*F. chlamydosporum)* has hyaline or light colour hyphae with septa and possesses thin filament-like structures with dull white mycelia as reported earlier (Walsh *et al*., 2004). The two other endophytes, LAS-6 belongs to *Chaetomium globosum* possess brown ascospores with black color spores and P-37 (*Ulocladium dauci)* was a slow-growing fungus with *Alternaria*-like colony morphology with pale brown conidiophores (Gannibal, 2018) (Fig. 1). LAS-6 was earlier identified as *Chaetomium* species has produced the fruiting body called perithecia covered by long hairs (Sangamesh *et al*., 2018).

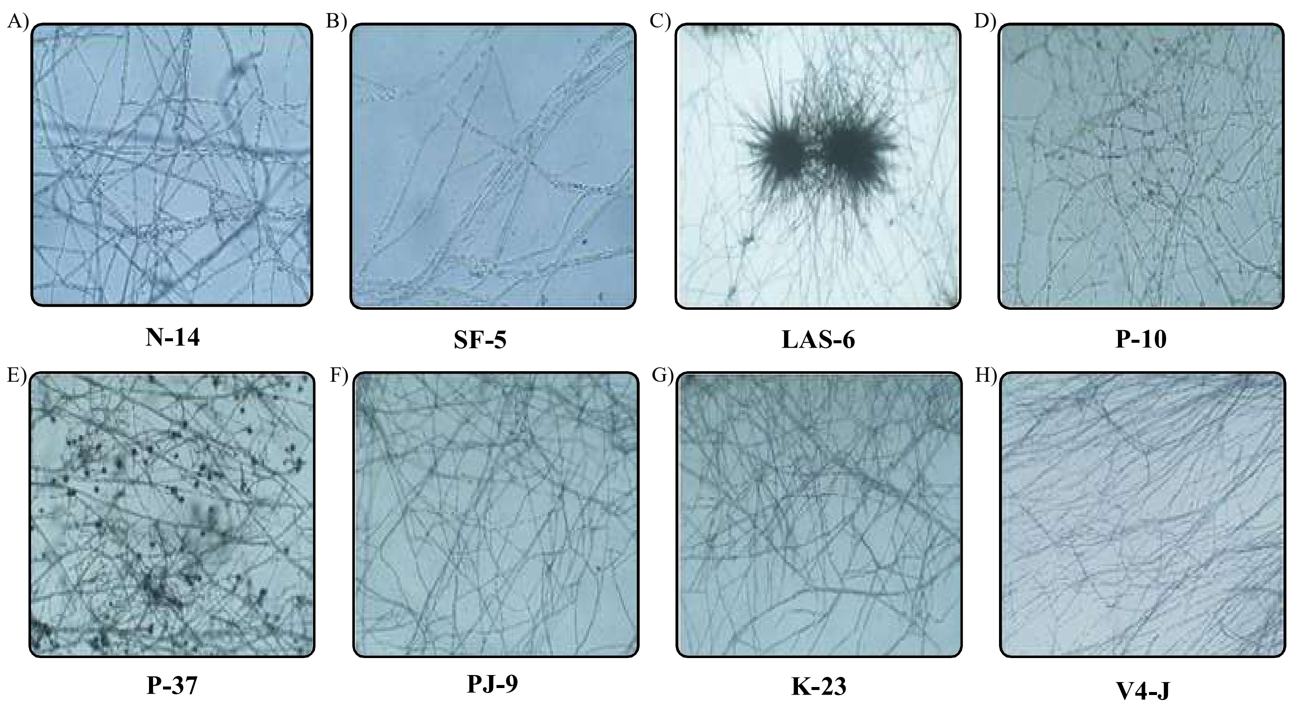

Fig. 1 : Microscopic observation of the endophytic fungi at 40x magnification. Mycelial structures of a) N-14, b) SF-5, c) LAS-6, d) P-10, e) P-37, f) PJ-9, g) K-23 and h) V4-J

Re-isolation of the Endophytic Fungi

Based on the leads from previous experiments in rice and tomato (Sangamesh *et al.,* 2018 and Pallavi & Nataraja, 2022), in this study an attempt has been made to understand the potential of five fungal endophytes (LAS-6, N-14, P-10, PJ-9 and SF-5) in new host *A. thaliana*. (Fig. 4). The colonisation percentage among the fungal endophytes differed significantly. The highest colonisation percentage was observed with the SF-5 treatment, reaching 67 per cent, while the lowest was observed with the PJ-9 treatment at 20 per cent. The other fungal endophytes showed moderate colonization percentage, with P-10 and N-14 both at 33 per cent and LAS-6 at 27 per cent. The control plants exhibited a zero per cent colonization rate, confirming the absence of any foreign organism contamination (Fig. 2a). *Fusarium* strains have been reported with a hallmark sign with tissue specificity causing vascular wilts (Alabouvette & Couteaudier, 1992 and Wang *et al*., 2020). However, the select fungi did not show a pathogenicity in the present study. Also, the majority of *Fusarium* endophytes have been shown to colonize roots (Fang *et al.,* 2019 and Zhang *et al.,* 2015). Further, the re-isolated endophytes were compared with the mother culture in terms of their morphology and mycelial structures (Fig. 2.b, c and d)) and subjected for molecular characterization. The sequence data of the PCR product confirmed the identity and *Fusarium sp.* as the closest match based on phylogeny (Fig. 3). This suggests that habitat-adapted fungal endophytes could successfully colonize the model system *Arabidopsis* symbiotically and promote early growth as observed in the case of *Serendipita indica* (Michal *et al*., 2011 and Vahabi *et al*., 2015).

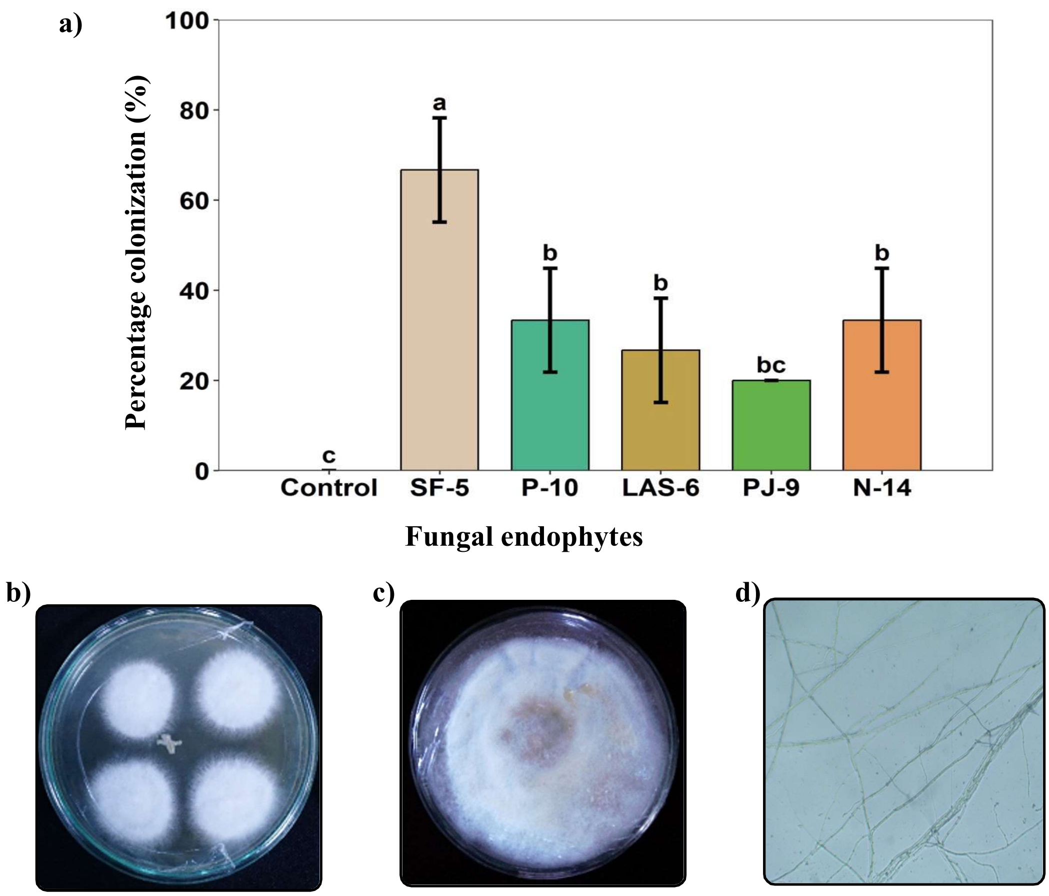

Fig. 2 : Assessment of fungal endophyte colonization in *A. thaliana.* a) Colonization percentage of the fungal endophytes in *A. thaliana*, b) confirmation of endophyte colonization (SF-5) in the tissue segments of *Arabidopsis* roots by re-isolation, c) comparison of colony morphology with mother culture and d) microscopic observation of the re-isolated fungus at 40x magnification

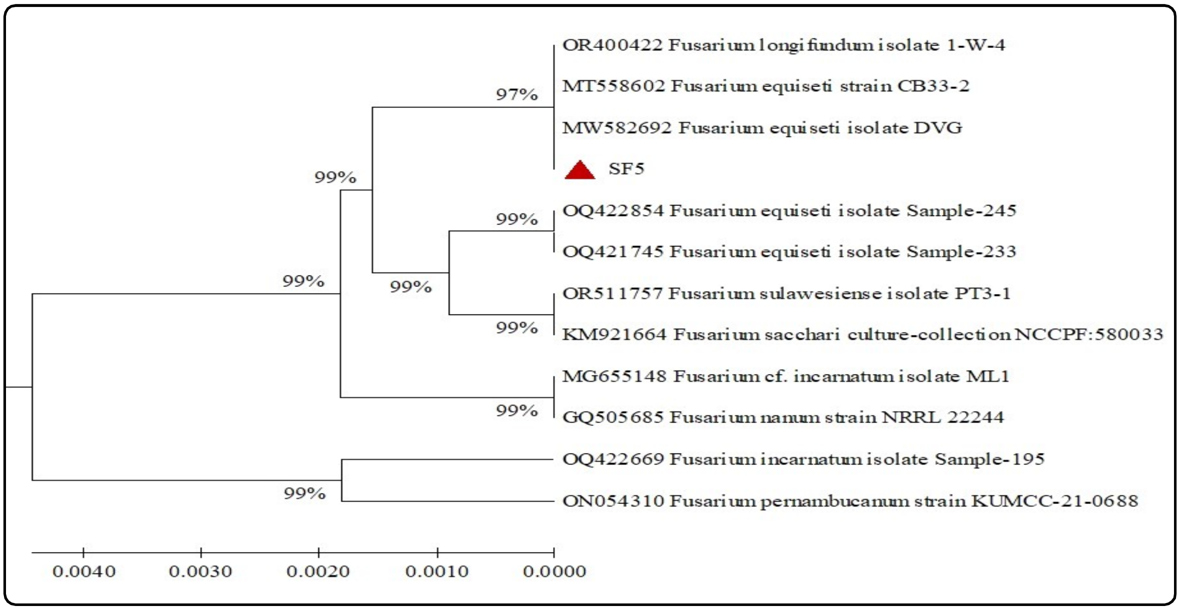

Fig. 3 : Phylogram generated from UPGMA (unweighted pair group method with arithmetic mean) analysis based on ITS sequence data

Endophyte-induced Activation of the Physiological Traits Associated with Growth

The study indicated that the five endophytes could efficiently colonize the new host (Fig. 2) and significantly increased rosette leaf area in the endophyte co-cultivated plants compared to the control, which did not show any growth response. The highest rosette leaf area was observed in the P-10 (122.43 mm^2) followed by SF-5 (98.13 mm^2), LAS-6 (87.10 mm^2), N-14 (85.06 mm^2) and control plants showed 31.10 mm^2 (Fig. 4 and 5a). This could be because, these endophytes are known to modulate

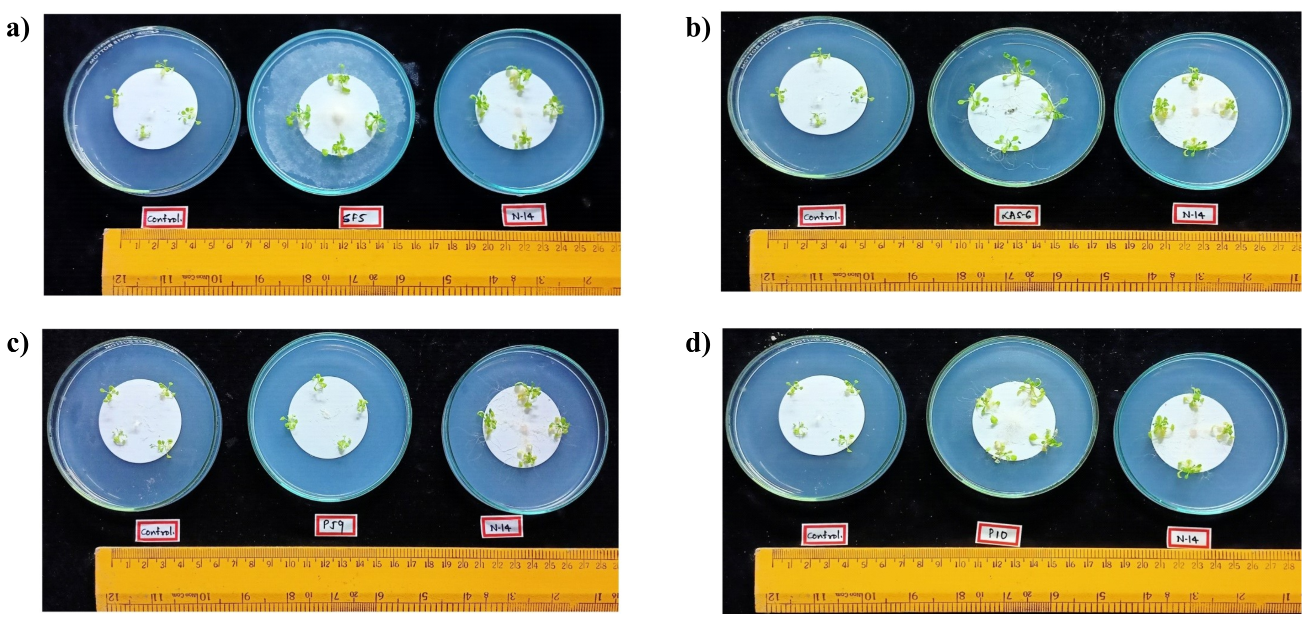

Fig. 4 : Co-cultivation of endophytic fungi with the young *Arabidopsis* seedlings. Photographs depicting the *in vitro* co-cultivation of *Arabidopsis* seedlings with endophytes, a) control, SF-5 and N-14 b) control, LAS-6 and N-14, c) control, PJ-9 and N-14 and d) control, P-10 and N-14

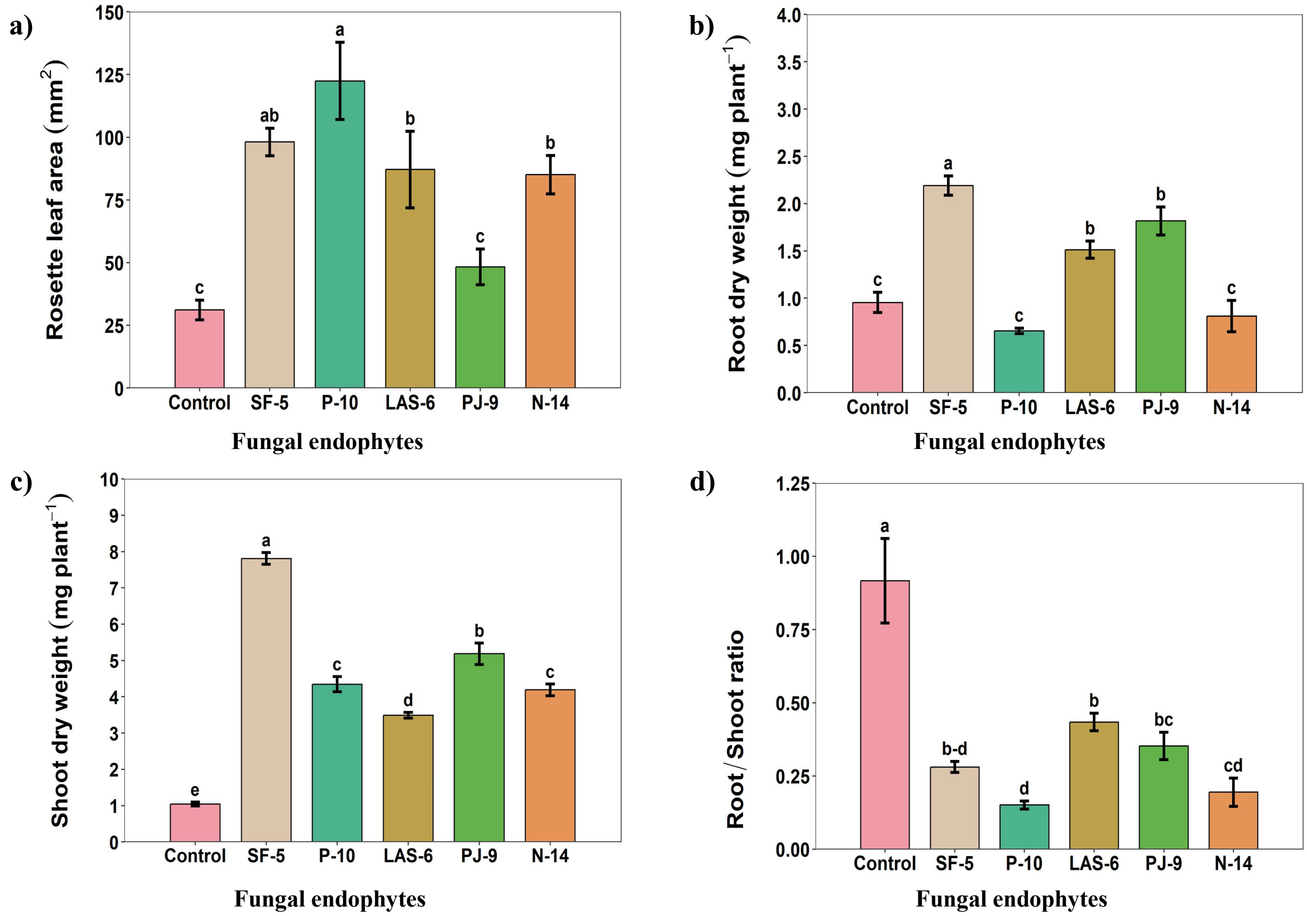

Fig. 5 : Growth response of the endophyte-enriched plants in the young *Arabidopsis* seedlings. a) Effect of fungal endophyte colonisation on the photosynthetic leaf area of *Arabidopsis* seedlings, b) Root dry weight (mg plant^{-1}), c) shoot dry weight (mg plant^{-1}) and d) root/shoot ratio

phytohormone levels in the host to induce growth promotion and impart stress resilience (Xu *et al.*, 2018 ; Suebrasri *et al.*, 2020). Additionally, N-14 was taken as a negative control, however, there was also a significant increase in the leaf area of plants treated with N-14 compared to control plants. Fungal endophytes form essential constituents of the leaf intercellular spaces and have a huge impact on photosynthesis (Suryanarayanan *et al.*, 2022 and Bangari & Nataraja, 2023).

Interestingly, while comparing SF-5, P-10, LAS-6, PJ-9 and N-14 treated seedlings to the control group, the difference in leaf area was significant and was approximately two-fold and higher, indicating a substantial and noteworthy impact of these fungal endophytes in increasing the photosynthetic leaf area (Fan *et al.*, 2020 and Rozpądek *et al.,* 2018).

Plant root and shoot biomass are crucial parameters for assessing the plant response to carbon, nutrient cycling and biomass partitioning. The root-to-shoot biomass ratio is a key indicator of plant resource allocation (Qi *et al.,* 2019). The present study showed an increased root dry weight (mg plant^{-1}) and shoot dry weight (mg plant^{-1}) in endophyte colonised *Arabidopsis* seedlings compared to control plants (Fig. 5b and c). Further, the root-to-shoot ratio was highest in the control group (0.91), followed by LAS-6 (0.43), PJ-9 (0.35), SF-5 (0.28), N-14 (0.19) and P-10 (0.15) (Fig. 5d). This suggests that endophytes balance the growth of the plant and improve water and nutrient absorption. Increased biomass accumulation in shoots suggests an increase in growth with improved resource acquisition facilitated by the endophytes, plants can allocate more energy to shoot growth, leading to a lower root-to-shoot ratio. This results in more significant above-ground biomass (Fig. 5c), which is often advantageous for photosynthesis and overall plant productivity. This observation shows the impact of endophyte colonization on plant physiological aspects, shedding light on its ability to optimize resource utilization for sustained growth and development. Endophytes play a crucial role in enhancing plant vitality by improving the uptake of macro and micronutrients from the soil organic substances and increasing the availability of these nutrients to the host (Rana *et al*., 2020; Mei *et al*., 2024 and Xue *et al*., 2024).

Identification of trait-specific endophytes, capable of activating inducible traits will have a huge impact on improving plant's water mining and uptake of nutrients. This kind of physiological adaptation aims to enhance the plant's ability to extract water and nutrients under water-limited conditions. This study highlights the potential of selected endophytes to activate the physiological traits, offering valuable insights into plant growth promotion and sustained yield. This approach not only signifies a potential strategy for mitigating the adverse impacts of climate change but also underscores its pivotal role in advancing crop improvement efforts.

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