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Research Article

Native fungal endophytes as potential bio-source for management of *Fusarium udum* in pigeonpea

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ABSTRACT: Fungal endophytes have gained a lot of importance as plant growth promoters and biological control agents against a variety of plant diseases. In the present investigation, *in vitro* studies were conducted to explore fungal endophytes native to the pigeonpea host system as potential biocontrol agents against the pigeonpea-infecting soil-borne pathogen, *Fusarium udum*. Fungal endophytes were isolated from healthy roots and leaves of pigeonpea seedlings. A total of 103 endophytes were isolated and categorized into 96 operational taxonomic units based on colony characteristics. Among the tested endophytes, one isolate identified as *Biscogniauxia petrensis* showed 61.9% inhibition against *F. udum* in the dual plate assay; further, the enrichment with *B. petrensis* also enhanced the growth of pigeonpea under greenhouse conditions. The findings pave the way for the application of native endophytic resources to biotic stress management in pigeonpea.

KEYWORDS: Biocontrol, fusarium wilt, native fungal endophytes, pigeonpea

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INTRODUCTION

Pigeonpea (*Cajanus cajan* L. Millsp.) is a climate-resilient legume crop grown in semi-arid and tropical sub-regions of Asia and Africa (Jorrin *et al*., 2021). Among the pulse crops grown in India, pigeonpea is the second most important pulse crop in the country, with a production of 3.89 million tonnes from an area of 4.53 million hectares (Anonymous, 2021), accounting for approximately 59 per cent of the total world production (FAO, 2018). It is primarily consumed in the form of split pulses, known as 'dal,' with a reported nutritional composition of approximately 20-22% protein, 1-2% fat, 65% carbohydrates, and 6.8% ash (Onweluzo & Nwabugwu, 2009), contributing to nutritional food security. Apart from its nutritional importance, pigeonpea is also used in soil improvement, agroforestry, intercropping, feed and fuel wood (Valenzuela & Smith, 2002).

Pigeonpea cultivation is affected by several factors, including biotic and abiotic stress. Among the numerous biotic and abiotic stress factors that impact pigeonpea, diseases are the most significant. Some of the important diseases affecting the pigeonpea in India are Fusarium wilt, Phytophthora stem blight, Cercospora leaf spot, Collar rot, Dry root rot, Alternaria leaf spot, Powdery mildew, Sterility mosaic and Phyllody (Kannaiyan *et al*., 1984). Among these diseases, vascular wilt disease caused by soil-borne pathogen *F. udum* Butler is a devastating and widespread fungal disease in all pigeonpea-growing areas and can cause significant grain yield loss with the range 30-100% (Nene *et al*., 1980, Kannaiyan & Nene, 1981). The incidence of the disease has been reported from 30% to 60% at flowering and crop maturity stages (Kannaiyan & Nene, 1981).

Fusarium udum was first reported in India (Butler, 1910) and later in Uganda (Small, 1922). Wilt disease can be diagnosed by symptoms such as loss of turgidity, slight inter-veinal chlorosis, internal browning of xylem vessels and a purple band on the stem extending upwards from the base. Due to the soil-borne nature of the pathogen and the existence of variants/races in *F. udum* (Subramanian, 1963), management of this pathogen has become a serious issue (Green *et al*., 1981).

Over the last few decades, management of wilt diseases has generally been achieved using resistant varieties, crop rotation, synthetic fungicides, and fumigation (Wang *et al*., 2018; Tang *et al*., 2020). However, generating resistant varieties has become a major issue due to the non-availability of the resistant dominant gene (Shanmugam & Kanoujia, 2011), and synthetic fungicides cause effects on human health and, the environment and pose risk to non-target beneficial

organisms, and develop pathogen resistance towards the fungicides (Dukare & Paul, 2021). The application of soil fungicides has been a limited success, and it is also not economical (Dukare & Paul, 2018). As a result, alternate strategies are necessitated for the eco-friendly and sustainable management of root-infecting soil pathogens.

Keeping environmental sustainability in view, the concept of "biological control" has made significant technological advancements in plant disease management, and the search for new and effective natural microbes against phytopathogens has become an emerging research area in agricultural sciences (Gao *et al*., 2017). Earlier, several fungal biocontrol agents, mainly *Trichoderma*, non-pathogenic *Fusarium*, and *Penicillium* strains, were evaluated to control Fusarium wilt, however, this lethal disease could not be controlled completely (Raza *et al*., 2017; Vos *et al*., 2014).

The plant-microbe interactions have emerged as a notable strategy for improving crop traits (Nataraja *et al.*, 2022). Among these interactions, the role of endophytes- the microorganisms living within the plant body- has gained prominence. Endophytes are organisms that establish mutualistic relationships with plants, residing within plant tissues and providing growth-promoting benefits (Durand *et al*., 2021). Endophytes can be either bacterial or fungal, but fungal endophytes have gained significant attention in various studies due to their widespread presence in plants, ranging from arctic to tropical regions.

Many endophytic species of fungi have been reported earlier, which have shown significant results in controlling fungal pathogens. Fo47 and CS-20 endophytic strains of *F. oxysporum* (*Fo*) strains have been shown to protect tomato against root pathogens and other crop species (De Lamo & Takken, 2020). The endophytic fungi can be successfully used to protect plants against pests and diseases as these fungi produce protective metabolites, inducing resistance to the host plants to biotic and abiotic stress by improving their growth and thus enhancing the yield (Kaul *et al*., 2016).

The current study was taken up to identify the potential endophytic species from the resistant pigeonpea plants which could be exploited against *F. udum,* to manage the wilt disease of pigeonpea in an eco-friendly way.

MATERIALS AND METHODS

Plant source

The fungal endophytes native to pigeonpea (*Cajanus cajan* L. Millsp.) were isolated at the seedling stage from leaves, stems and roots (variety ICP8863). The isolation was carried out at the plant pathology laboratory, Indian Council of Agricultural Research-All India Co-ordinated Research Project, University of Agricultural Sciences, Bangalore. The fresh plant samples were used for the isolation of the fungi through standard isolation protocol.

Isolation of endophytic fungi

The endophytic fungi from the leaves, stem, and roots of the healthy pigeonpea plant were isolated following the procedure by Shweta *et al*. (2010). Tissue samples were washed in running tap water to remove extraneous matter, and washed leaf, stem and root segments (0.5 cm^2/1 cm long) were excised using a sterile blade. Excised tissue segments were surface disinfected by immersion in 70% (v/v) ethanol for 3 min followed by 1% sodium hypochlorite for 4 min. After surface disinfection, tissue segments were rinsed 3-4 times in sterile distilled water and blotter paper dried. To check for the efficacy of surface sterilization, the disinfected tissue segments were imprinted onto Potato Dextrose Agar (PDA) in Petri plates and incubated (26°C under 12 h of light/ dark cycles) and observed for colony emergence. Surface-sterilized tissue segments were placed on PDA media amended with streptomycin sulphate (30 µg/mL). The plates were wrapped with cling film and incubated at 26°C under 12 h of light/dark cycles. Fungi emerging from the cut ends of the tissue segments were transferred using a fine-tipped needle onto fresh PDA media to obtain the pure cultures. Based on spore and colony morphology, the cultures were maintained as distinct isolates, assigned an isolate Operational Taxonomic Unit (OTU) identity number for future reference and deposited in the fungal culture collections.

Isolation of *F. udum*

The target soil-borne plant pathogenic fungus *F. udum* was isolated from the infected pigeonpea plant. The plant showing typical wilting symptoms was selected in the wilt-sick plot maintained. The root tissue showing the vascular discoloration was made into pieces with a sterile blade, and the pathogen was isolated using a standard isolation procedure. The pure culture of the pathogen was obtained through single spore isolation. The single spore culture of the pathogen was confirmed through morphological and molecular identification. After the confirmation, pure culture was maintained on PDA slants at 4°C until further use.

Dual culture assay

The endophytic fungal isolates from the stem, leaf and root were evaluated for their ability to inhibit the growth of *F. udum* by the dual culture technique described by Rahman *et al*. (2009). Briefly, the mycelial discs (5 mm) of the *F. udum* and the endophytes were excised from the margin of a freshly grown culture. Both the mycelial discs were placed in a Petri dish containing PDA medium at three centimetres from each other. The control plates contained the mycelial disc of the pathogen and the endophytic fungus alone. Plates

were incubated at 26°C under 12 h of light /dark treatment. The inhibition of the plant pathogenic fungi was calculated by the formula given by Gao *et al*. (2017).

Inhibition % = Cd-Td/Cd-5mm×100

where, Cd = radial growth of pathogen in pure culture and Td = radial growth of pathogen in dual culture.

Characterization of selected endophytic fungal isolates

The endophytic fungal isolates showing higher inhibition against *F. udum* were characterized based on the colony morphology and conidia/spores characters and by sequencing the Internal Transcribed Spacer (ITS) region of genomic DNA using universal primers ITS1 and ITS4 (White *et al*., 1990). The total genomic DNA of the best endophytic fungal isolates was extracted by the Cetyltrimethylammonium Bromide (CTAB) method (Vainio *et al*., 1998). The ITS region of genomic DNA of the fungus was amplified using universal primers ITS1 and ITS4 by PCR using an Eppendorf thermal cycler (Eppendorf, Germany) (White *et al*., 1990). The PCR conditions were set as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 15 s, 58°C for 1 min and 72°C for 45 s, and a final extension at 72°C for 7 min. The PCR mixture (50 µl) consisted of 1X PCR buffer, dNTP‟s (200 µM), 1.5 mM $MgCl_2$, and 5 pmol of each primer (Sigma Life Sciences), 10 ng of DNA and 1.5 units of Taq DNA polymerase. The amplified products were sequenced (Eurofins India Pvt. Ltd. Bengaluru, India). The nucleotide sequences were verified at the NCBI GenBank database using a basic local alignment algorithm for nucleotide sequences. The highest per cent identity, maximum query and score were used as a reference to assign the identity of the endophytic fungus species. Sequences of each of the fungal species and corresponding reference sequences from NCBI GenBank were subjected to ClustalW analysis. A maximum likelihood phylogenetic tree was constructed using MEGA 11 software. Obtained sequences are deposited in the NCBI database.

Scanning electron microscopy

To understand the biocontrol mechanism, the interaction of endophytic fungi and the *F. udum* was visualized under a Scanning Electron Microscope (SEM). Visualization of the sample in its native state without any treatment was done as per the protocol proposed by Venkatesh *et al.* (2018). The fungus was grown on a small piece of sterile Whatman No. 1 filter paper strips (4 × 4 mm; L × B) containing PDA medium. The PDA medium was poured into Petri dishes, and the Whatman No. 1 filter paper strips were placed on top of the semi-solid PDA medium and allowed to solidify, which eventually resulted in the formation of a thin layer on the surface of the filter paper. Later, mycelia discs were placed on both the side of the Petriplate to create an interaction zone, then Whatman No.1 filter paper strip containing the grown fungus at the interaction zone was immediately placed on the carbon adhesive tape stuck on to the aluminium stub (26 mm) and coated with gold in real time for 100 seconds using an ion sputter. The sputter-coated sample was taken for image analysis using the Scanning Electron Microscope (SEM). The processed samples were visualized under SEM (Zeiss – EVO 18, Germany) using a secondary electron detector (SE1) at a working distance (WD) of 7 to 9 mm at 10KV.

Double plate assay

The double plate assay is used to study the antifungal activity of non-diffusible compounds in inhibiting plant pathogenic fungi (Dennis & Webster, 1971). The endophytic fungal isolates which inhibited the growth of *F. udum* in dual culture assay were examined for their ability to inhibit the pathogens in double plate assay. A six-day-old culture of the endophytes and pathogens was used for the assay. 15 ml PDA was poured into two base plates (90 mm dia). The mycelial disc (5 mm dia) of an endophytic fungus and the pathogenic fungus was excised from the margin of a 5-day-old colony and placed on two different petri plates containing PDA medium. A petri plate having the inoculum of the pathogenic fungus was inverted over the petri plate inoculated with an endophytic fungus and incubated. Control treatment consisted of mycelia disc of pathogen or endophytic fungus alone. Plates were sealed with cling wrap and incubated at 26ºC under 12 h of light/dark cycles for 5 days. The inhibition percentage was calculated as described earlier.

RESULTS

Isolation and screening of endophytic fungi

A total of 24 endophytic fungi were isolated from the root, stem and leaf segments of pigeonpea wilt resistant variety ICP8863 at the seedling stage. Of the total 24 entophytic fungi isolated, 13 endophytes emerged from the leaf region, 6 endophytes emerged from the stem region, and 4 endophytes from the root portion of the plant. Further, the isolated endophytes were categorized into different OTUs based on the morphology of the fungal colony and spore characteristics. All 24 endophytes were further subjected to antagonistic assay using dual culture plates for the preliminary screening of the endophytes against *F. udum*.

Dual culture assay

All the isolates were subjected to dual culture assay for their antagonistic activity against *F. udum*. Of the 24 OTUs, 6 OTUs effectively inhibited the mycelial growth of the pathogenic fungus viz; ROP1 IV, G yellow, G brown, ROP V, III PRR3, II PRR 1 with per cent inhibition of 62.91, 60.88, 61.90, 57.08, 55.33 and 60.76 per cent, respectively. Among the six effective endophytes, maximum mean per

cent inhibition was observed in ROP IV (62.91 %), (Figure 1) followed by G brown (61.90%), G yellow (60.88%), II PRR 1 (60.76%), ROP V (57.08%) and III PRR3 (55.33%).

Inhibition of the pathogenic fungus could be due to mycoparasitism, production of diffusible or production of volatile organic compounds.

Molecular characterization of the selected endophytes

The native endophytes that performed well under the dual culture assay were further identified through molecular characterization. Fungal endophytes were identified through their ITS rDNA sequences obtained from the Sanger sequencing. The region searched for homology using the Basic Local Alignment Search Tool (BLASTn) of the National Centre for Biotechnology Information indicated that the ITS rDNA sequences of the isolates G yellow and G brown closely related to *B. petrensis with* 98.68 per cent homology. Whereas ROP IV, ROP V, III PRR 3 and II PRR 1 were closely related to *Sordaria fimicola, Fusarium oxysporium,* and *Emmia lacerate* of 99.80, 99.74 (Figure 3) and 99.64 per cent, respectively.

Mechanism of inhibition of pathogen by endophytes

The mechanisms of inhibition of pathogens by endophytes involve a complex interplay of various factors. Several mechanisms have been reported to be adopted by the biocontrol agents, particularly fungi, *viz*; mycoparasitism, production of antimicrobial compounds, competition for resources by endophytes, and production of non-diffusible compounds.

Mycoparasitism: The selected endophytes were studied through scanning electron microscopy to see the active interaction between the fungal endophytes and the *F. udum*. Out of six endophytes under this study, only one endophyte, *i.e*., *B. petrensis,* showed a more competitive nature with the pathogenic mycelia, resulting in the modification of *F. udum* mycelial characteristics. In the dual culture assay plate at the interaction zone, *F. udum* showed bulged mycelia and reduced or no sporulation, and it is evident under the scanning electron microscopic picture (Figures 4C and E), further, the dense coiling of *B. petrensis* mycelia around the *F. udum* was also observed.

Antifungal activity of non-diffusible compounds

The selected six endophytes were further tested by double plate assay for their ability to produce non-diffusible compounds with the assumption that selected endophytes might produce some important non-diffusible compounds which may act on the *F. udum*. Interestingly, one endophyte significantly reduced the growth of *F. udum* with a per cent inhibition of 43.53 percent (Figures 5a and b). The nature of inhibition by this endophyte was stable, with pathogens inhibited even after 20 days of incubation. Further, the ethyl acetate culture filtrate extract of the selected endophytes

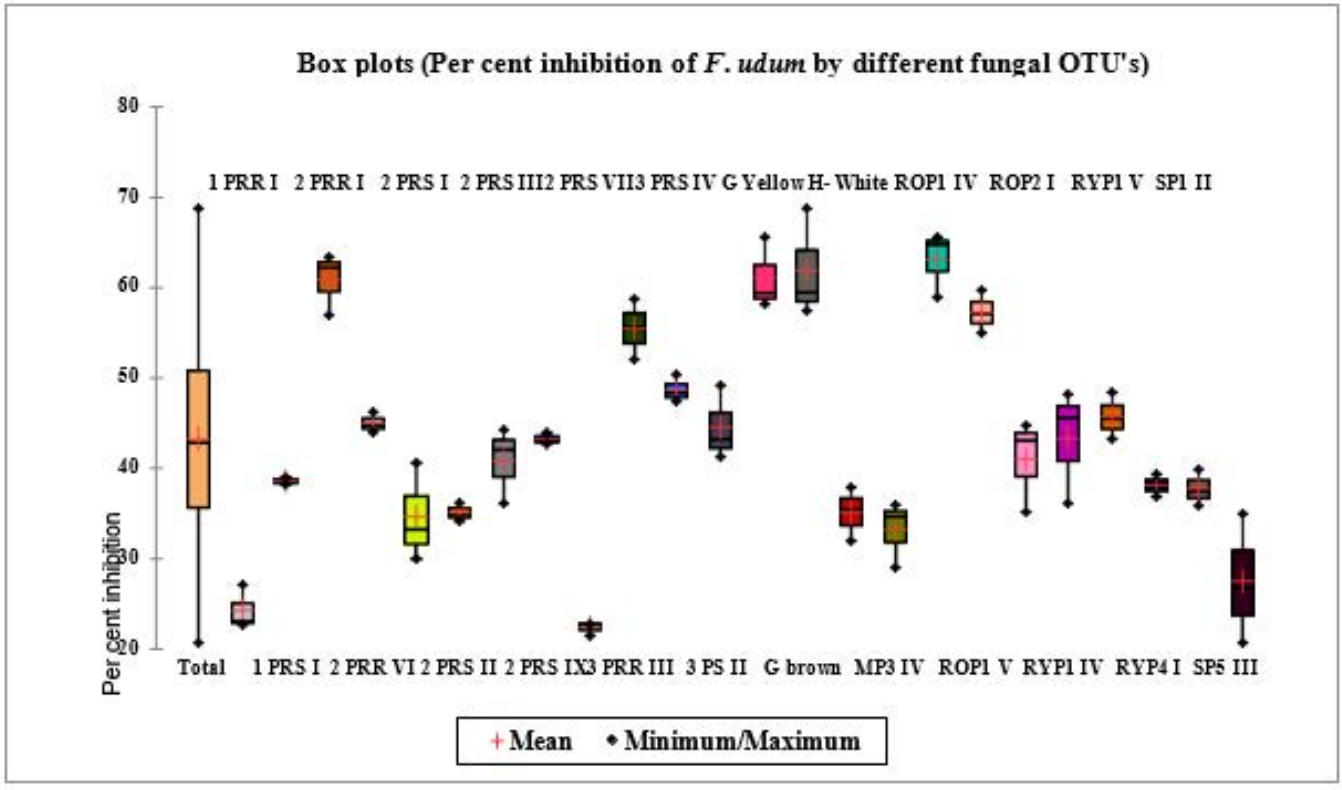


Figure 1. Inhibition of *F. udum* by different endophytes in dual culture assay.



Figure 2. Inhibition of *F. udum* by native fungal endophytes associated with pigeonpea.

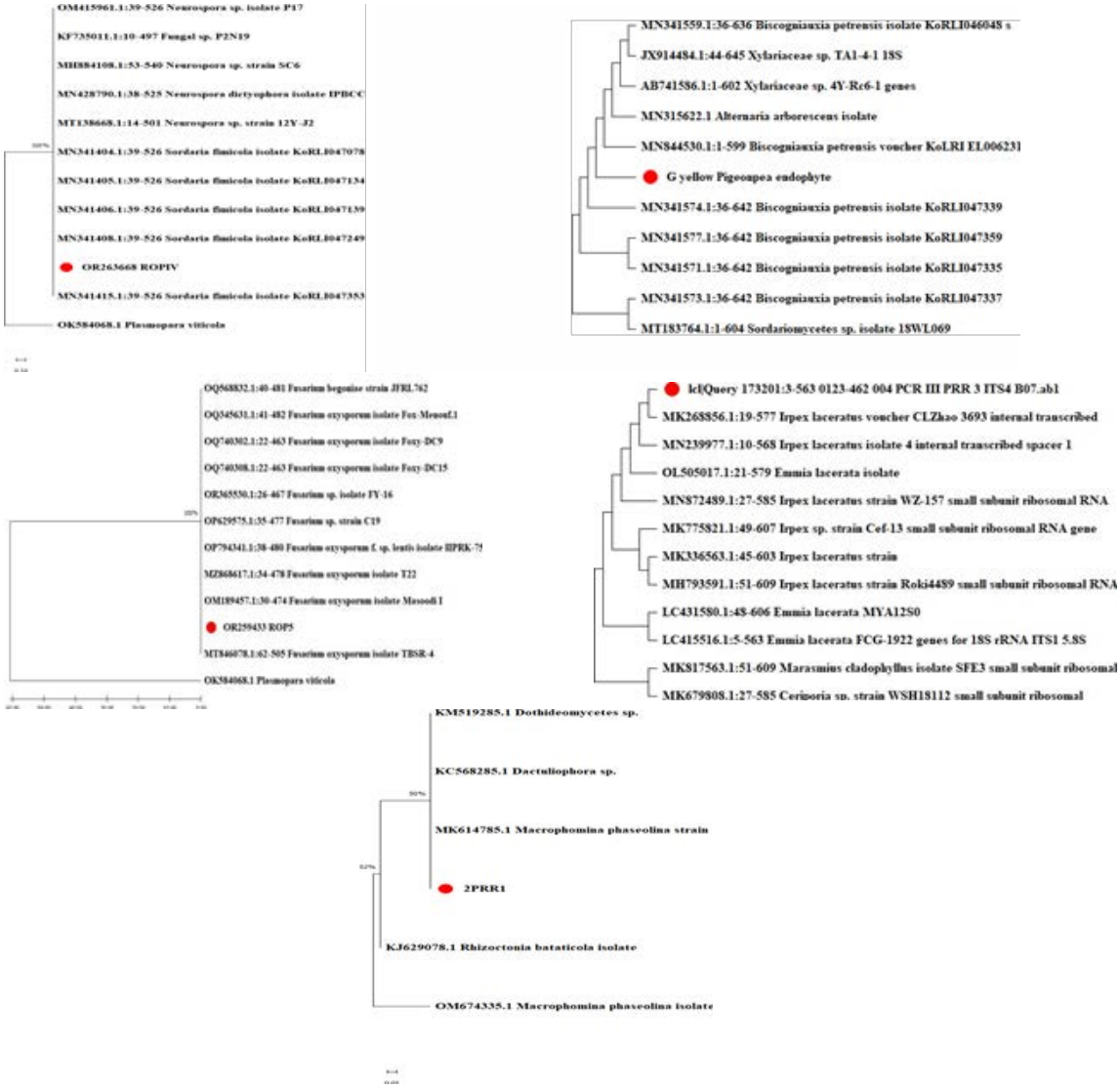


Figure 3. Maximum likelihood phylogenetic tree based on ITS rDNA sequence of different endophytic species and its closest ITS rDNA matches from Gen Bank. The phylogenetic tree was constructed with a bootstrap value of 1000 replicates. Species marked with the solid bullet are from the current study.

Table 1. Details of the selected OTUs and their percent identity with NCBI accession number

OTU Name	Name of the organism	Percent identity	NCBI Accession number
G brown	*Biscogniauxia petrensis*	98.68	OR236255
G yellow	*Biscogniauxia petrensis*	98.68	OR263955
ROP IV	*Sordaria fimicola*	99.80	OR263668
ROP V	*Fusarium oxysporium*	99.74	OR259433
IIIPRR3	*Emmia lacerate*	99.64	OR259100

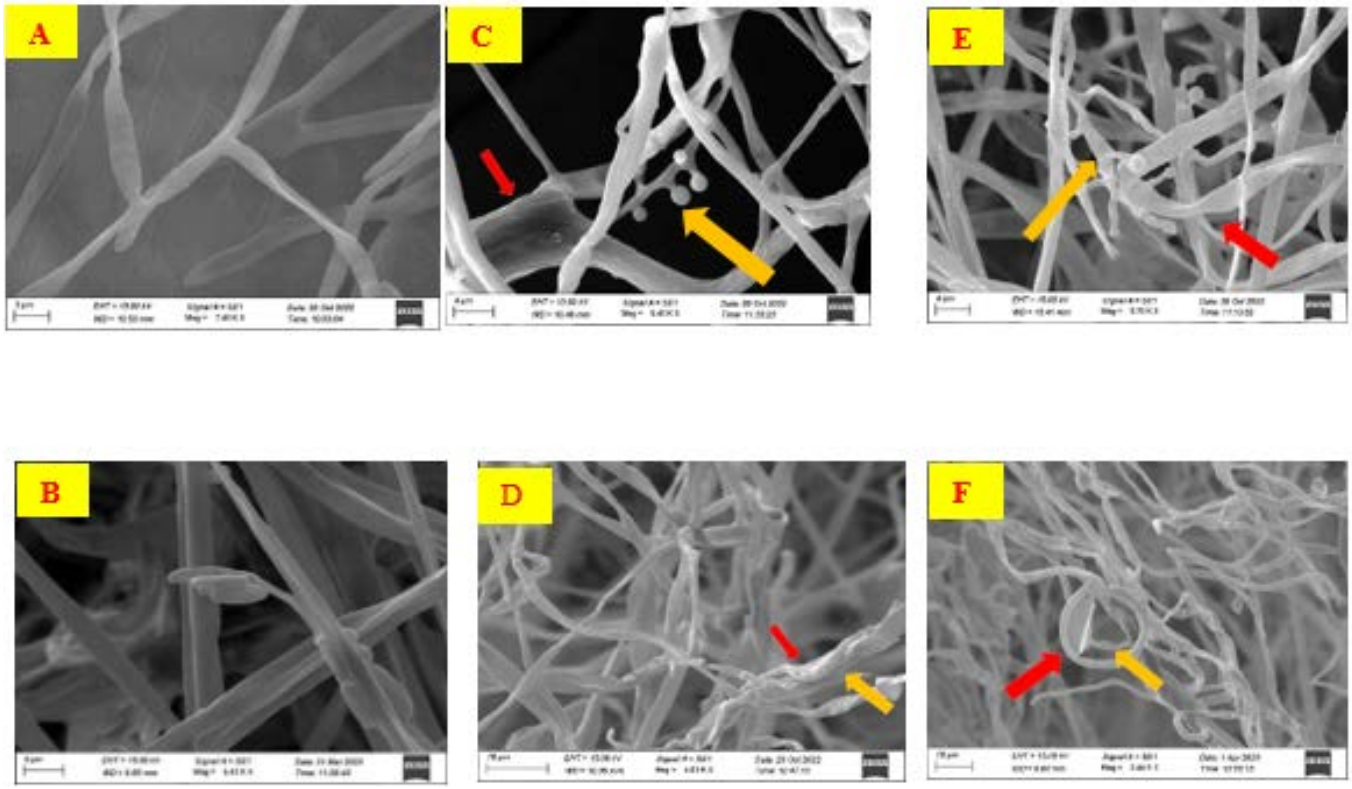


Figure 4. Scanning Electron Microscopy image showing mycoparasitism of *F. udum* by *B. petrensis*. **(A)** Mycelial characteristic of the endophytic fungus **(B)** Spore and mycelial characterstics of *F. udum* **(C)** Changes in mycelial characteristics of *F. udum* (Endophyte: arrow in red, Pathogen: arrow in yellow) **(D)** Parasitizing of *B. petrensis* mycelia over *F. udum* **(E)** Similar mycelia modification like bulged mycelia **(F)** *B. petrensis* coiling over *F. udum*.

(a)



(b)

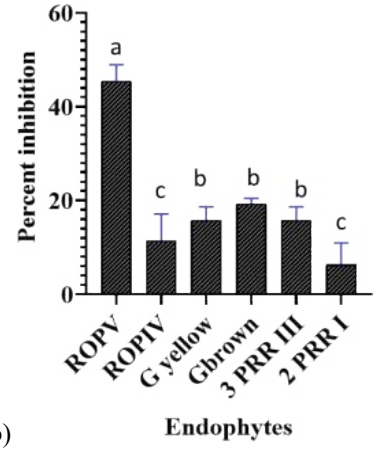


Figure 5. (a) Inhibition of *F. udum* by *Sordaria fimicola* (OR263668) (ROP IV) in double plate assay. **(b)** Percent inhibition of *F. udum* by different endophytes.

was tested against *F. udum* by disc diffusion assay. Among all the tested endophytes, none of them showed inhibition of *F. udum*, indicating that these endophytes use the other mechanism of inhibition of the pathogen.

DISCUSSION

The increasing severity of plant diseases worldwide due to climatic abnormalities has urged the urgent and essential requirement for the deployment of a secure and efficient method to control plant diseases. The research on endophytic microorganisms has demonstrated their significance for the capacity to enhance plant growth and various beneficial activities, including antimicrobial properties (Attia *et al*., 2024). In the present study, 24 endophytes have been isolated from resistant genotype pigeonpea plants with the hypothesis that the resistance is gene mediated along with the activity of beneficial microbes as "hologenome". Another idea of selecting the resistant genotype of pigeonpea, which is the host of the *F. udum,* is that the endophytes native to the target host could easily be colonized than the endophytes from different hosts and cross inoculation. Interestingly, the endophytic fungi isolated from pigeonpea could easily be colonized, and six of them showed the potential to inhibit the growth of *F. udum* under dual culture assay with inhibition ranging from 55.33 to 62.91 per cent. A similar result on endophytes has also been reported where an *Alternaria* sp. isolated from *Nicotiana nimmoniana* was able to effectively inhibit the growth of *Sclerotium rolfsii* both under *in vitro* conditions by 46.62 per cent. In the present study, an endophyte identified and characterised as *B. petrensis,* apart from inhibiting the pathogen species *F. udum* by 61.90 per cent, also modified the mycelial characters of the test fungus, which was evidenced by the SEM examination. This is the first study reporting the potential of this fungus against the *F. udum*. The endophytic fungi have evolved different modes of action on the plant pathogens, using coiling around hyphae and, after penetration, competition for food and space, the degradation of hyphal cytoplasm (Cao *et al*., 2009). In the present study, strong competition between the endophytic fungi and the *F. udum* was noticed.

Another endophytic fungus identified as *Sordaria fimicola* inhibited the pathogenic fungus both in dual plate and double plate assay. Results from the study indicated that under double plate conditions, an *S. fimicola* would have released the unknown non-diffusible volatile organic compounds that strongly inhibited *F. udum.* Though the study did not reveal the exact compound acting against the *F. udum,* it paved the way for identifying the endophyte effective against the wilt-causing pathogen. The researchers have reported *S. fimicola* as an endophyte which was isolated from soil (Dewan *et al*., 1994) and several plants (Newcombe *et al*., 2016). Further, the biocontrol effect of *S. fimicola* has been reported with multiple modes of action as competition for nutrients and space due to the rapid growth of the fungus and production of certain volatile compounds that may strongly suppress fungal growth (Abdallah *et al*., 2018). The biocontrol nature of *S. fimicola* has also been reported in several crops, where *S. fimicola* was able to control soil-borne plant diseases in cucumber, spinach, komatsuna, and Japanese black pine (Watanabe *et al*., 1991).

Apart from the abovementioned endophytes, another three endophytes, *viz*., *Fusarium oxysporium* and *Emmia lacerate* showed the significant inhibition under dual culture assay but did not show any inhibition or competition in double plate assay, disc diffusion assay and microscopic examinations. The ability of these endophytes to inhibit the *F. udum* may be attributed to the competition for resources.

CONCLUSION

Native endophytes represent an invaluable component of plant ecosystems, contributing to plant health, biodiversity conservation, and ecosystem functioning. Exploring their diversity and understanding their ecological roles can lead to innovative strategies for sustainable agriculture, ecosystem management, and conservation efforts. The present work demonstrated that *B. petrensis* and *S. fimicola* isolated from a resistant pigeonpea genotype have a potential role in the biocontrol of *F. udum*. Further, evaluating them at the plant level and deciphering molecular mechanisms of endophyte-induced resistance against *F. udum* could help better exploitation of the endophyte in protecting the pigeonpea system from wilt disease. Identified endophytes can be further used to evaluate the performance under field conditions to control the *Fusarium* wilt in pigeonpea, and they may be used as an alternative to the traditional biocontrol agents.

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