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Isolation and Characterisation of Plant Growth-Promoting Bacterial and Fungal Endophytes from Himalayan Yew (*Taxus wallichiana*) - An Economically Imperative Pant of Himalayas

(Pemencilan dan Pencirian Bakteria dan Kulat Endofit Penggalak-Pertumbuhan Tanaman daripada Himalayan Yew (*Taxus wallichiana*)-Tumbuhan Himalaya yang Penting daripada Segi Ekonomi)

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ABSTRACT

It is a known fact that the bacterial and fungal endophytes inhabit the plant tissues besides aiding in the better growth and health of the plants. The bark and leaves of *Taxus wallichiana* have drawn a lot of interest in recent years since they are the richest source of taxol, an anticancer drug. As it is a slow-growing tree that can only be regenerated via vegetative propagation, it has been classified as a critical rare species due to its extensive collection for medicinal and other purposes. Nonetheless, the use of endophytes as plant growth promoters is gaining much importance among environmentalists and agronomists because of their imperative role in crop production. Even then, there is hardly any information available regarding the growth-promoting endophytes isolated from bark and leaves associated with *T. wallichiana* commonly known as Himalayan Yew. Therefore, the present study was undertaken to isolate fungal and bacterial endophytes from *T. wallichiana* and to classify the growth-promoting properties of these endophytes. In total, seven fungal and ten bacterial endophytes were obtained from different parts of *T. wallichiana.* All of the isolated fungal and bacterial endophytes produced indole acetic acid while most of them also produced ammonia. Besides, the fungal and bacterial endophytes were also screened for antimicrobial and various enzymatic activities. Based on the above results, the two fungal endophytes were selected for their possible ability to promote seed growth. The results showed that the fungal endophytes isolated from *T. wallichiana* played an active role in increasing growth in other plant species and therefore, can be used as potential plant growth promoters.

Keywords: Antimicrobial activity; bacterial endophytes; fungal endophytes; plant growth-promoting endophytes (PGPE)

ABSTRAK

Adalah diketahui bahawa bakteria dan kulat endofit mendiami tisu tumbuhan serta membantu dalam tumbesaran dan kesihatan tumbuhan yang lebih baik. Kulit dan daun *Taxus wallichiana* telah menarik minat ramai sejak beberapa tahun kebelakangan ini kerana ia kaya dengan taxol, iaitu ubat antikanser. Memandangkan ia adalah pokok dengan tumbesaran yang perlahan dan hanya boleh dijana semula melalui pembiakan vegetatif, ia telah dikelaskan sebagai spesies langka yang kritikal kerana pengumpulannya dilakukan secara ekstensif untuk tujuan perubatan. Namun begitu, penggunaan endofit sebagai penggalak tumbesaran tumbuhan semakin penting dalam kalangan ahli alam sekitar dan ahli agronomi kerana peranan pentingnya dalam pengeluaran tanaman. Walaupun begitu, hampir tidak terdapat sebarang maklumat mengenai endofit penggalak tumbesaran yang diasingkan daripada kulit kayu dan daun yang dikaitkan dengan *T. wallichiana* yang juga dikenali sebagai Himalaya Yew. Oleh yang demikian, kajian ini telah dijalankan untuk mengasingkan endofit kulat dan bakteria daripada *T. wallichiana* dan pada masa yang sama, untuk mengelaskan sifat penggalak tumbesaran tanaman oleh endofit ini. Secara keseluruhan, tujuh kulat dan sepuluh bakteria endofit diperoleh daripada bahagian *T. wallichiana* yang berlainan. Kesemua kulat dan bakteria endofit yang dipencilkan menghasilkan asid asetik indol manakala kebanyakannya juga menghasilkan ammonia. Selain itu, kulat dan bakteria endofit juga

disaring untuk aktiviti antimikrob dan pelbagai enzim. Berdasarkan hasil di atas, dua endofit kulat telah dipilih kerana keupayaan mereka dalam menggalakkan tumbesaran benih. Hasil menunjukkan bahawa kulat endofit yang diasingkan daripada *T. wallichiana* memainkan peranan aktif dalam meningkatkan tumbesaran spesies tumbuhan lain dan berpotensi untuk digunakan sebagai penggalak tumbesaran tumbuhan.

Kata kunci: Aktiviti antimikrob; bakteria endofit; endofit penggalak tumbesaran tumbuhan (PGPE); kulat endofit

Introduction

Recently, novel microbial-inoculation approaches have gathered attention in mitigating the adverse effects of conventional farming techniques. Plants and microbes form a symbiotic relationship that benefits both parties equally. More importantly, plant-microbe symbiosis impacts plant health and growth, thereby improving agricultural characteristics and nutrient cycling leading to improved soil quality (Karthik et al. 2016; Khan et al. 2013; Puri et al. 2016). Plant tissues are inhabited by plant growth-promoting endophytes (PGPE), and the close association of endophytes within plant tissues enables nutrient exchange and enzyme activity (Khan et al. 2015; Murphy et al. 2014). PGPE produce numerous bioactive compounds with multiple biological activities that can be directly or indirectly identified as PGP (plant growth-promoting) agents. While most of the plants harbour endophytes within their tissues, the information available on PGPE and its biological activities is nowhere close to the high endophytic distribution (Hassan 2017). A better understanding of plant native endophytes, thus, can help to clarify their capacity and potential to enhance plant growth and create a sustainable crop production system.

Taxus wallichiana zucc., like most conifers, is an evergreen tree species that belongs to the Taxaceae family. The species is known as the Common Yew or the Himalayan Yew and is dioecious in nature, with female and male parts present on different trees (Bhuju & Gauchan 2018). It is a well-recognized evergreen tree of enormous medicinal importance and occurs beneath the Himalayan temperate locations (Adhikari & Pandey 2019). Several bioactive compounds like alkaloids, terpenes, steroids, and polyphenols have been isolated from this plant species and their pharmaceutical uses such as antimicrobial, anticancer, and many other medicinal applications was investigated (Adhikari & Pandey 2017; Fatima et al. 2016; Gauchan et al. 2021; Juyal et al. 2014). This species has attracted a lot of attention because of the existing exploitation of its bark for the extraction of the drug taxol. *T. wallichiana* regenerates only via seed germination, which is difficult as it is having a strong dormancy period of 1.5-2 years (Pandey et al. 2002).

Endophytes, obtained from plant species living in low-temperature mountain ecosystems, have attracted much interest in recent times for their role in plant growth and biocontrol (Pandey & Yarzábal 2019). *T. wallichiana*, which is listed on the IUCN Red List of Threatened Species (Thomas & Farjon 2011), is an important medicinal plant for research into the valuable endophytes that are significant for propagation, regeneration, conservation, and plant growth-related aspects (Das & Jha 2018). Understanding the beneficial microbiome, especially endophytes associated with the host species will be a prerequisite for utilizing the potential of endophytes for crop production. In this context, the main goal of this work was to isolate the possible fungal and bacterial endophytes from *T. wallichiana* and then using these isolates as PGPE inoculants so as to find an alternative method to chemical fertilizers for better crop productivity for sustainable growth and development. Since most chemical fertilizers, have negative effects on ecological components, along with soil fertility. As a result, bioinoculants and other organic agricultural practises have received a lot of attention as alternatives to chemical fertilizers.

Materials and Methods

Sample collection

Mature and healthy plants of *T. wallichaina* were picked for a sample collection from the Sanasar area of the Jammu Region, India. Sanasar is located at an altitude of 2,050 m is; 6730 ft above sea level, which is around 20 km distance west of Patnitop, in Ramban district on the national highway 1A. *T. wallichiana* is growing wildly in this area. Leaves and woody sections (bark and stem) were gathered at random for the study. The plant material was then brought to the laboratory in sterile bags and processed within 24 h after sampling.

Isolation of endophytes from *T. wallichiana*

Before processing, all the samples were properly rinsed in running tap water accompanied by double distilled

water. Samples of bark and stem were cut into 4.0 mm pieces. Initially, all the specimens were surface treated to remove epiphytic microorganisms (Petrini et al. 1993). The samples were then soaked in 70% ethanol for 1-3 min and thereby sterilized for 3-5 min with aqueous sodium hypochlorite. This was followed by rinsing in 70% ethanol for 2-5 s before final rinsing in sterilized double-distilled water. Each sample was then dried under aseptic conditions (Verma et al. 2007). Imprints of surface-sterilized samples were taken on potato dextrose agar (PDA) and Luria-Bertani agar plates to ensure surface sterilization effectiveness of the adopted method (Potshangbam et al. 2017). Sections of each sample were placed on potato dextrose agar (PDA) supplemented with streptomycin (100 mg/L) for fungal endophytes whereas for bacterial endophytes the section of each sample was placed on Luria-Bertani agar supplemented with cycloheximide (100 mg/L). The Petri plates were sealed with parafilm and incubated at 27 ± 2 °C for 4 weeks in case of fungal endophytes whereas in the case of bacterial endophytes plates were incubated at 35 ± 2 °C. Within 2 weeks of inoculation, much of the fungal growth had started. The fungi that was formed out of segments was periodically isolated and then shifted to fresh PDA plates. In the case of bacterial endophytes, colonies were selected on alternate days and purified on LB plates.

SCREENING OF ISOLATED ENDOPHYTES FOR PLANT GROWTH-PROMOTING PROPERTIES

The plant growth-promoting activities of fungal and bacterial endophytes that can indirectly or directly promote plant health and growth were investigated. The potential of the isolated endophytes to promote plant growth was tested *in vitro* as follows:

SCREENING FOR INDOLE ACETIC ACID PRODUCTION

IAA was calculated using the colorimetric method defined by Gordon and Weber (1951), albeit with few changes. Initially, the isolates were grown in 10% Tryptic soy broth (TSB), along with an addition of 5 mM of L-tryptophan at a temperature of 30 °C. After 24, 48 and 72 h of growth, 1 mL aliquots were centrifuged at 10,000 rpm for 12 min and treated with Salkowski's reagent (50 mL of perchloric acid (35%) and 1 mL of $FeCl_3$ solution (0.5 M)). The resulting solution was measured using a spectrophotometer at 530 nm and the concentration of IAA (0-100 µg mL^{-1}) was measured using the equation obtained from the standard commercial IAA curve.

SCREENING FOR AMMONIA PRODUCTION

Ammonia production was assessed using Cappuccino and Sherman method (Cappuccino & Sherman 1992). The isolates were grown for 96 h at 30 °C in 10 mL of peptone broth, 1 mL of grown culture was taken and centrifuged at 10,000 rpm, for 10 min. 0.5 mL of Nessler's reagent was added to the supernatant and consequently, the brownish colour appeared in the test tubes, which is considered as an indication of ammonia production. Non inoculated media was considered as control.

SCREENING FOR EXTRACELLULAR ENZYMES

The extracellular enzyme production by bacterial endophytes was done by adding the indicative substrates into Luria Bertani media and then inoculating the bacterial isolates in the media. The extracellular enzyme production by fungal endophytes was done by growing 3-4 mm plugs of fungal on the PDA plates supplemented with specific indicative substrates (Pansanit & Pripdeevech 2018). Amylase, cellulase, and protease enzymatic activities were evaluated by growing the endophytic isolates on media supplemented with 1% each of soluble starch, carboxy-methylcellulose (CMC), and skimmed milk, respectively. The appearance of the clear zone was measured after adding specific reagents (iodine, and 0.1% congo red solution to detect the amylolytic and cellulolytic, activities, respectively) which are used as indicators for extracellular enzymatic activities.

SCREENING FOR ANTIMICROBIAL ACTIVITY

All bacterial and fungal endophytes were checked for antimicrobial activity using Agar well diffusion method (Magaldi et al. 2004). In the case of bacterial endophytes, the isolates were grown in Lb broth for 6 days at 35 ± 2 °C at 180 rpm on a shaker. Where fungal isolates were grown in PD broth for 12 days at 28 °C at 180 rpm. The crude broth was blended thoroughly and centrifuged at 5000 rpm for 6 min. Liquid supernatant was extracted thrice with an equal volume of ethyl acetate. The organic extract was then evaporated and the crude extracts were dissolved in DMSO and then used for screening.

The test organisms were spread on agar plates. The wells (6 mm) were bored in all plates and 100 µL of the extract was added into different wells based on the concentration. These plates were then incubated at 37 °C for 24 h. Antimicrobial activity was calculated by determining the diameter (mm) of the zone of inhibition. Strains that had antimicrobial activity against test

organisms formed a clear zone. All the experiments were carried out in triplicates. In the case of Gram-negative and Gram-positive test organisms, the control used was Ampicillin (10 µg/µL) whereas in the case of *Candida albicans* the control used was Fluconazole (20 µg/µL). The negative control used was dimethyl sulfoxide (DMSO).

Tested microorganisms are Gram-negative bacteria: *Escherichia coli* (MTCC-1652), *Klebsiella pneumoniae* (MTCC- 39), *Enterobacter aerogenes* (MTCC -111), *Mycobacterium smegmatis* (MTCC-994), *Proteus vulgaris* (MTCC-426), *Enterobacter species* (MTCC-7104), Gram-positive bacteria: *Bacillus subtilis* (MTCC-10619), *Staphylococcus aureus* (MTCC-3160), and Unicellular fungi: *Candida albicans* (MTCC-227). These tested microorganisms were brought from Microbial Type Culture Collection Chandigarh, India.

ENDOPHYTE IDENTIFICATION

DNA EXTRACTION AND PCR AMPLIFICATION OF THE INTERNAL TRANSCRIBED SPACER REGION

DNA isolation was done using the Liu et al. (1997) method with slight modifications. Analysis of the Polymerase Chain Reaction (PCR) was then carried out to amplify the ITS region from the isolated genomic DNA using the universal primers ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4R (5′-TCCTCCGCTTATTGATATGC-3′) (Chen et al. 2000) with the following thermocycler conditions: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 1min, 72 °C for 1 min 30 s; and a final extension at 72 °C for 10 min. The amplified sample size was verified by 1% agarose gel electrophoresis. The PCR products were purified using a PCR purification kit (Qiagen) and then sequenced. To identify the closest neighbour of selected endophytic fungal isolate, their nucleotide sequences were subjected to Blast search in the NCBI GenBank database. A phylogenetic analysis was carried out by obtaining related sequences from the NCBI and an evolutionary tree was created using the neighbor-joining analysis method and MEGA software, version 6.0, with 1000 bootstrap values.

EFFECT OF ENDOPHYTES ON PLANT ROOT GROWTH

Plant growth promotion was evaluated in fenugreek (*Trigonella foenum-graecum*) and mung bean (*Vigna radiate* L.), with the goal of investigating the response of endophytes obtained from *T. wallichiana* in other plant species. The seeds were surface disinfected by dipping in sodium hypochlorite (2.5%) for 5 min, then in 70% ethanol for 1 min, and then washed five times in sterile distilled water. Fungal isolates were inoculated at 28 °C in Potato Dextrose broth in an incubator shaker for 7 days at 180 rpm. Surface-disinfected seeds were incubated for a duration of 6 h at room temperature with bacterial or fungal suspensions, the broth without fungal and bacterial inoculation was used as control. Control and treated seeds were put in sterile Petri plates with wet sterilized filter papers and incubated in dark for 36 h at room temperature to measure the root length.

STATISTICAL ANALYSIS

The data was statistically analyzed by Statistical Package for the Social Sciences (SPSS Version 20). One-way analysis of variance (ANOVA) was used for multiple sample comparison, followed by Tukey HSD test at $P \leq 0.001$.

RESULTS

ISOLATION OF ENDOPHYTES

The standardized surface sterilization protocol for the isolation of endophytes was quite satisfactory as no growth was found on the control plate. The fungal isolates obtained were initially differentiated based on morphological characteristics. A total of seven fungal and ten bacterial endophytes were isolated from the leaves, bark and stem of *T. wallichiana* which suggests the presence of endophytic fungi and bacteria in plant tissue is not the same and are spread randomly.

SCREENING FOR INDOLE ACETIC ACID PRODUCTION

The results are shown in Figure1(A), 1(B) and Table 1 indicate that all the examined fungal and bacterial endophytes produced IAA with or without tryptophan as a precursor. Moreover, it was observed that the range of IAA production increased with an increasing concentration of tryptophan in the media. The highest IAA production was detected in two fungal endophytic strains FETW4 (85 µg mL^{-1}, 40.7 µg mL^{-1}) and FETW6 (89.7 µg mL^{-1}, 54.3 µg mL^{-1}) when supplemented with L-tryptophan or without tryptophan and $P \leq 0.001$. Whereas in case of bacterial endophytes the highest IAA production was detected in BETW5 (71 µg mL^{-1}, 35.5 µg mL^{-1}) and BETW9 (76 µg mL^{-1}, 38 µg mL^{-1}) when supplemented with L-tryptophan or without tryptophan and $P \leq 0.001$. In the case of comparison between bacterial and fungal endophytes, the highest IAA production was found in fungal endophytes.

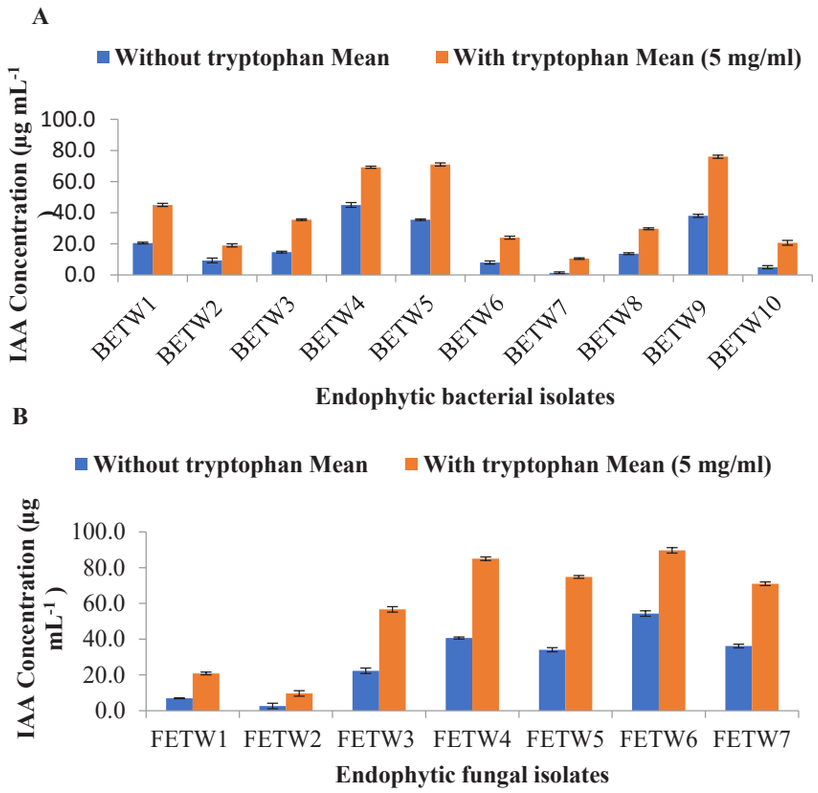

FIGURE 1. Quantitative production of IAA by bacterial and fungal endophytes isolated from *T. wallichiana* at 5 mg/mL concentration of tryptophan and without tryptophan; (A) endophytic bacterial isolates with and without tryptophan and (B) endophytic fungal isolates with and without tryptophan. Columns with error bars represent mean ± standard deviation from the pooled data of three repeated experiments

TABLE 1. Showing results of bacterial and fungal endophytes for IAA production

Sample	Type of microbes	IAA concentration (µg mL^{-1})	
		Without tryptophan	With tryptophan (5 mg/mL)
BETW1	Bacterial endophyte	20.5 ± 0.5^{c}	45 ± 1^{f}
BETW2		9.3 ± 1.5^{b}	19 ± 1^{b}
BETW3		14.7 ± 0.6^{b}	35.5 ± 0.5^{e}
BETW4		45± 28.6^{d}	69.2 ± 0.7^{g}
BETW5		35.5 ± 0.5^{c}	71 ± 1^{g}
BETW6		8 ± 1^{a}	24 ± 1^{c}
BETW7		1.3 ± 0.6^{a}	10.5 ± 0.5^{a}
BETW8		13.7 ± 0.6^{b}	29.7 ± 0.6^{d}
BETW9		38 ± 1^{d}	76 ± 1^{h}
BETW10		5 ± 1^{a}	20.7 ± 1.5^{b}
FETW1	Fungal endophyte	6.9 ± .2^{b}	20.8 ± 0.8^{b}
FETW2		2.7 ± 1.5^{a}	9.7 ± 1.5^{a}
FETW3		22.3 ± 1.5^{c}	56.7 ± 1.5^{c}
FETW4		40.7 ± .6^{e}	85 ± 1^{f}
FETW5		34.1 ± 1.2^{d}	74.8 ± 0.8^{e}
FETW6		54.3 ± 1.5^{f}	89.7 ± 1.5^{g}
FETW7		36.7 ± 1.0^{d}	71 ± 1^{d}

Values within the same column with different letters are significantly different (P≤0.001) by Tukey HSD test, values are means ± SD (n = 3). Means with the same letters in the parentheses are not significantly different according to Tukey's HSD test at P≤0.001

SCREENING FOR AMMONIA PRODUCTION

The results summarised in Table 2 shows that most of the isolated fungal and bacterial endophytes produced ammonia but their intensities varied as was clearly evident from the difference in the colours obtained in the experiment. However, one of the fungal strains (FETW2) and two bacterial endophytic strains (BETW2 & BETW7) were unable to produce ammonia (Figure 2).

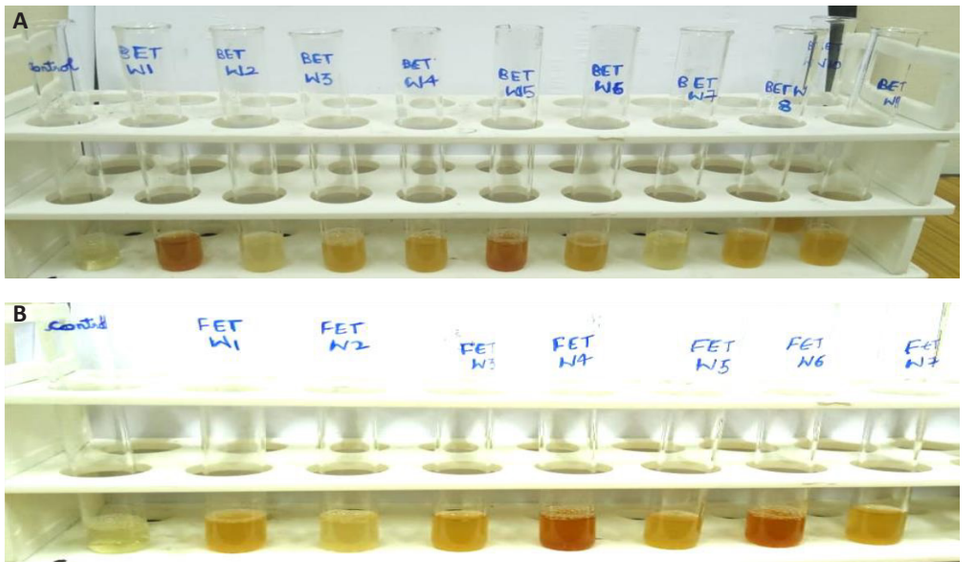

FIGURE 2. Photograph showing ammonia production by endophytes isolated from *T. wallichiana:* (A) Bacterial endophytes; (B) Fungal endophytes

TABLE 2. Ammonia production of endophytic bacterial and fungal isolates from *T. wallichiana*

Sample	Type of microbes	Ammonia production
BETW1	Bacterial endophyte	++
BETW2		-
BETW3		+
BETW4		+
BETW5		++
BETW6		+
BETW7		-
BETW8		+
BETW9		+
BETW10		+
FETW1	Fungal endophyte	+
FETW2		-
FETW3		+
FETW4		++
FETW5		+
FETW6		++
FETW7		+

−, +, and ++ denote no, low, and high ammonia production, respectively

SCREENING FOR EXTRACELLULAR ENZYMES

Amongst the bacterial endophytes, isolates BETW4 and BETW9 exhibit the highest enzyme activity in the case of amylase, cellulase and protease enzyme ($P \leq 0.001$) whereas in the case of fungal endophytes, FETW6 was the isolate having the highest enzyme activity in case of all three enzymes, amylase, cellulase and protease enzyme ($P \leq 0.001$) (Table 3). In the case of comparison between bacterial and fungal endophytes, the highest enzyme activity was seen in fungal endophyte FETW6.

TABLE 3. Summarised results for the screening of bacterial and fungal endophytes for various extracellular enzymatic activities using solid media inoculated with different substrates

Sample	Type of microbes	Diameter of clear zone (mm)		
		Amylase	Cellulase	Protease
BETW1	Bacterial endophytes	10.3 ± 0.6^{b}	10.7 ± 0.6^{d}	9.3 ± 0.6^{b}
BETW2		6.7 ± 0.6^{a}	7.3 ± 0.6^{b}	6.3 ± 0.6^{a}
BETW3		9.7 ± 1.2^{b}	9.7 ± 0.6^{c}	11 ± 0^{c}
BETW4		15.33 ± 0.6^{c}	14.3 ± 0.6^{e}	15.7 ± 0.6^{d}
BETW5		9.7 ± 0.6^{b}	10.3 ± 0.6^{d}	10.7 ± 0.6^{c}
BETW6		6.33 ± 0.6^{a}	7.3 ± 0.6^{b}	7.7 ± 0.6^{b}
BETW7		-	-	-
BETW8		7.33 ± 0.6^{a}	8.3 ± 0.6^{b}	9.7 ± 1.5^{c}
BETW9		14.67 ± 0.6^{c}	14.3 ± 0.6e	15.7 ± 0.6^{d}
BETW10		6.33 ± 0.6^{a}	5.7 ± 0.3^{a}	7.3 ± 0.6^{a}
FETW1	Fungal endophytes	7.7 ± 1.5^{b}	7.3 ± 0.6a	7 ± 1a
FETW2		-	-	-
FETW3		6.3 ± 0.6^{a}	7.7 ± 0.6^{b}	8.7 ± 0.6^{b}
FETW4		12.7 ± 0.6^{c}	10.3 ± 0.6^{c}	18 ± 1^{c}
FETW5		9.3 ± 1.2^{b}	9.3 ± 0.6^{c}	10 ± 0.9^{b}
FETW6		16.3 ± 0.6^{d}	18.3 ± 0.6^{e}	20.3 ± 0.6^{d}
FETW7		14.7 ± 0.6^{c}	14 ± 1^{d}	16.7 ± 0.6^{c}

Values within the same column with different letters are significantly different ($P \leq 0.001$) by Tukey HSD test, values are means ± SD (n = 3). Means with the same letters in the parentheses are not significantly different according to Tukey's HSD test at $P \leq 0.001$

SCREENING FOR ANTIMICROBIAL ACTIVITY

Screening results of endophyte antimicrobial activities showed that growth of the five studied microorganisms (*B. subtilis* (MTCC-10619*), K. pneumoniae* (MTCC- 39*), M. smegmatis* (MTCC-994*), P. vulgaris* (MTCC-426*),* and *C. albicans* (MTCC-227)) out of nine was inhibited by bacterial endophyte BETW1, whereas BETW10 inhibited the growth of only *C. albicans*. Therefore, BETW1 and BETW10 positively impacted the pathogenic fungal development of *C. albicans*. The fungal endophytic strains FETW1, FETW4, and FETW6 showed inhibitory action against 5 tested microbial pathogens, and it was noticed that among all the tested strains, the highest antimicrobial activity was against *E. aerogenes* (Table 4). The zone of inhibition of the extracts is smaller than the control antibiotic.

IDENTIFICATION OF PLANT GROWTH-PROMOTING FUNGAL ENDOPHYTES

The amplification of the ITS fragment from the isolated fungal endophytic strains (FETW4 and FETW6) with plant growth-promoting properties was confirmed by agarose gel electrophoresis (Figure 3). The ITS gene sequences obtained in this study were deposited in the

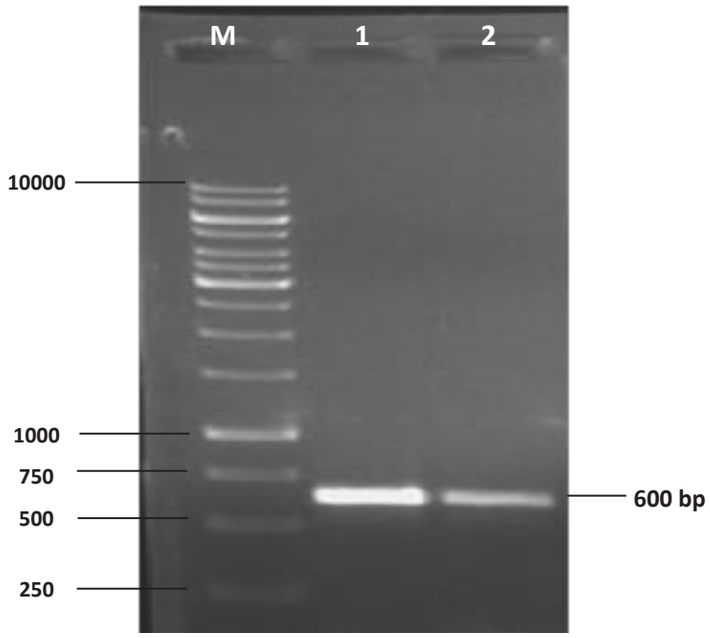

FIGURE 3. 1% agarose gel showing Purified PCR product of two fungal endophytes isolated from *T. wallichiana*: M-1 Kb DNA ladder; 1-FETW4; 2-FETW6

TABLE 4. Summarized results of antimicrobial activity of bacterial and fungal endophytes against different organisms

Sample	Diameter of the clear zone (mm)								
	B. subtilis	*S. aureus*	*E.coli*	*K. pneumoniae*	*E. aerogenes*	*M. smegmatis*	*P. vulgaris*	*Enterobacter species*	*C. albicans*
BETW1	12.3 ± 0.6[a]	-	-	17.7 ± 0.6[b]	-	-	-	-	10.6 ± 0.5[a]
BETW2	-	-	-	16.3 ± 0.5[a]	-	-	-	-	-
BETW3	-	-	-	19.5 ± 0.5[c]	-	-	-	-	-
BETW4	-	-	-	-	-	-	-	-	-
BETW5	-	-	-	-	-	-	-	-	-
BETW6	13 ± 1[a]	-	-	16.3 ± 0.6[a]	-	-	-	-	-
BETW7	-	-	-	-	-	-	-	-	-
BETW8	12.4 ± 0.4[a]	-	-	-	-	-	-	-	-
BETW9	-	-	-	-	-	-	-	-	-
BETW10	19.1 ± 0.2[b]	-	-	-	-	11.9 ± 0.9[a]	-	-	12.4 ± 0.4[b]
Control	30.5 ± 0.5[c]	31.7 ± 0.6[a]	28.9 ± 0.2[a]	30.4 ± 0.4[d]	33.2 ± 0.3[b]	29.6 ± 0.2[b]	28.3± 0.3[a]	30.2 ± 0.3[a]	31 ± 0.4[c]
FETW1	11.7 ± 0.6[c]	-	-	17.1 ± 0.2[b]	-	19.3 ± 0.6[a]	20.2 ± 0.6[c]	-	11.3 ± 0.6[a]
FETW2	-	-	19.4 ± 0.4[b]	-	-	-	-	-	-
FETW3	-	-	16.7 ± 0.6[a]	-	-	-	-	-	-
FETW4	12.0 ± 0.2[c]	-	-	12.2 ± 1.3[a]	-	-	18.4 ± 0.4[a]	11.3 ± 0.6[a]	13.3 ± 0.6[b]
FETW5	-	-	-	-	-	-	-	-	-
FETW6	10.9 ± 0.2[b]	12.6 ± 0.5[a]	-	-	20.5 ± 0.5[a]	-	19.3 ± 0.4[b]	-	11.3 ± 0.6[a]
FETW7	10.5 ± 0.5[a]	-	-	-	-	-	-	-	-
Control	30.5 ± 0.5[d]	31.7 ± 0.6[b]	28.9 ± 0.2[c]	30.4 ± 0.4[c]	33.2 ± 0.3[b]	29.6 ± 0.2[b]	28.3± 0.3[d]	30.2 ± 0.3[b]	31 ± 0.4[c]

Values within the same column with different letters are significantly different (P≤0.001) by Tukey HSD test, values are means ± SD (n = 3). Means with the same letters in the parentheses are not significantly different according to Tukey's HSD test at P≤0.001

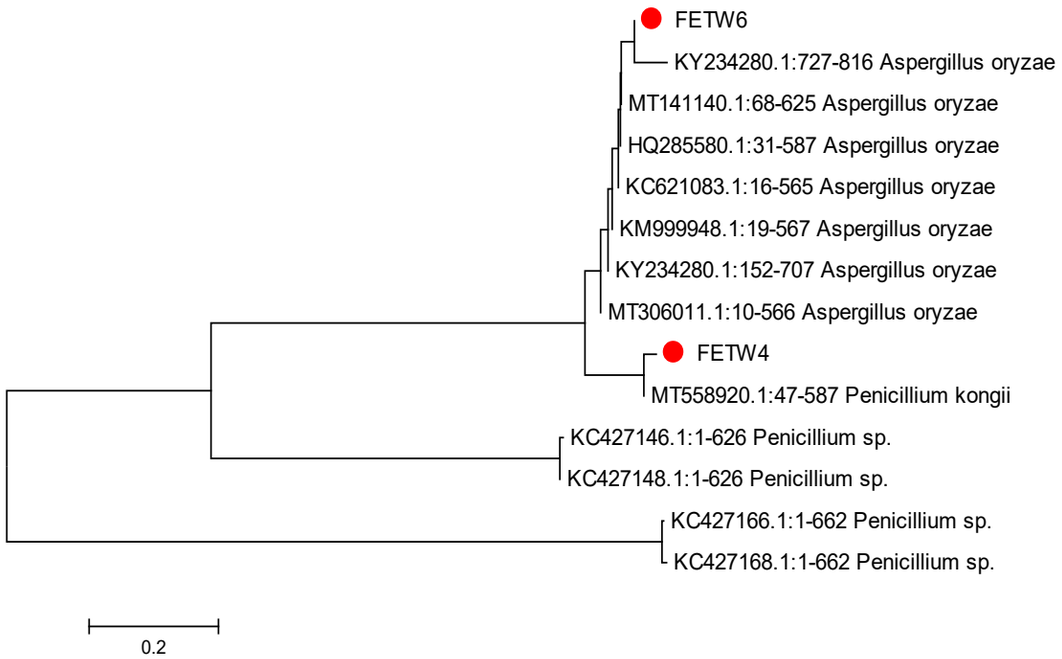

FIGURE 4. Phylogenetic analysis of FETW4 and FETW6 isolated from *T. wallichiana* based on ITS gene sequences and closely related sequences by the neighbor-joining algorithm

GenBank under the accession numbers MT844019 and MT845819, respectively. A phylogenetic tree was constructed using the neighbour-joining method based on ITS gene sequences of the endophytic fungal isolates and selected fungal strains of related taxa. The phylogenetic results show that the ITS sequence of fungal endophyte FETW4 was more related to *Penicillium kongii* (MT558920) with 98.16% similarity and FETW6 to *Aspergillus oryzae* (KY234280) with 97.14% homology (Figure 4).

EFFECT OF FUNGAL ENDOPHYTES ON SEED GROWTH

The plant growth-promoting activities of fungal endophytes that could indirectly or directly promote seed growth were examined. The findings showed that all of the isolated fungal endophytes produced IAA in varying amounts and most of them produced ammonia as well. Since fungal isolates FETW4 and FETW6 produced the highest concentration of both IAA and ammonia, these two were taken for further investigation of their effect on seed growth of *Trigonella foenum-graecum* and *Vigna radiate* L. These findings showed that both the isolated fungal endophytes *P. kongii* FETW4 and *A. oryzae* FETW6 showed growth-promoting properties in fenugreek and mung bean seeds as compared to the control as shown in Figure 5 and Table 5.

TABLE 5. Effect of endophytic fungal isolates on the root length (cm) of *Trigonella foenum-graecum* and *Vigna radiate* L. seedlings

Fungal treatments	Root length (cm) *Trigonella foenum-graecum*	Root length (cm) *Vigna radiate* L.
Control	1.5 ± 0.5^{a}	1.3 ± 1.4^{a}
FETW4	4.5 ± 0.3^{b}	4.7 ± 0.6^{b}
FETW6	3.7 ± 0.2^{b}	3.1 ± 0.4^{b}

Values within the same column with different letters are significantly different ($P \leq 0.001$) by Tukey HSD test, values are means ± SD (n = 3). Means with the same letters in the parentheses are not significantly different according to Tukey's HSD test at $P \leq 0.001$

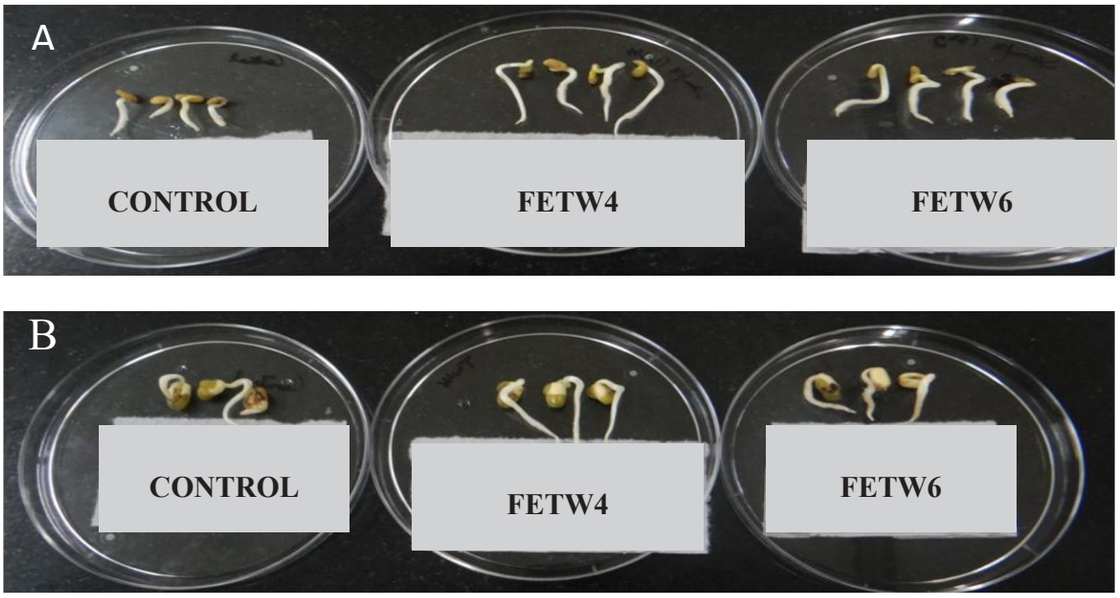

FIGURE 5. Effect of endophytic inoculants on the growth of (A) *Trigonella foenum-graecum* seedlings and (B) *Vigna radiate* L. seedlings with two endophytic fungal strains isolated from *T. wallichiana*

DISCUSSION

Endophytes are considered to be the most abundant class of micro-organisms that can enable host plants to counter the negative impacts of biotic and abiotic stress conditions (Bokhari et al. 2019; Chadha et al. 2015; Halo et al. 2015; Hamayun et al. 2018). Within plant tissues, bacterial and fungal endophytes are common residents and have been shown to assist in plant health and growth. Nevertheless, very little is known about medicinal plant endophytes that promote plant growth (PGPE) (Hassan 2017). Endophytes promote plant growth by producing phytohormones, among several other ways of stimulating growth (Ali et al. 2017; Khan et al. 2017). The endophytes have the ability to prevent or reduce the deleterious effects of certain pathogenic organisms. The positive effects of endophytes on their host plant become visible through various mechanisms (Ryan et al. 2008) including antibiosis (antibiotic production), growth promotion, inducing host defences (induced systemic resistance, ISR), parasitism, competition and signal interference (quorum sensing) (Amer & Utkhede 2000; Jayaraj et al. 2005; Jorjani et al. 2012). Increased use of synthetic fertilizers negatively affects the production of agricultural crops and the health of plant linked ecosystems. Endophytes can serve as a substitute to chemical fertilizers because of their role as biofertilizers (Audipudi et al. 2017; Celador-Lera et al. 2018; Kumar et al. 2017; Pappas et al. 2018; Tumangger et al. 2018).

Several researchers have isolated endophytic fungi and bacteria from various parts of the *T. wallichiana*, such as the leaves, bark, roots, and stem (Adhikari & Pandey 2019, 2020; Ashkezari & Fotouhifar 2017; Collins & Jacobsen 2003; Kumar et al. 2019; Zaiyou et al. 2017). As far as the Jammu region particularly Sanasar is concerned there are no reports related to bacterial and fungal endophytes of *T. wallichiana.* Therefore, in the present work, ten bacterial and seven fungal endophytes were isolated from the medicinal plant *T. wallichiana*. These results are inconsistent with Ashkezari research in that the presence of the type and number of endophytic fungi on each part of the plant is not the same (Ashkezari & Fotouhifar 2017).

Microbial endophytes promote plant growth directly through the development of plant hormones, particularly IAA. The IAA aids in plant growth by increasing the number of root hairs and lateral roots (Husen 2016). In the current study, all fungal and bacterial endophytes which were tested for IAA production gave positive results and similar results have been reported previously by Ismail et al. (2021). However, our study is focused only on the growth of the seedlings rather than taking into account the growth attributes like biomass,

shoot and root length, and chlorophyll content. Fungal endophytes displayed higher IAA biosynthesis than those synthesized by bacterial endophytes and our results are in close agreement with the studies of Hassan (2017). Ammonia production can help satisfy the nitrogen demand of the host plant and when in excess reduces the colonization of plants by pathogens (Mbai et al. 2013). The production of ammonia by endophytes is a desirable feature of plant growth and soil fertility (Li et al. 2016) and our results show that most of the isolated fungal and bacterial endophytes produced ammonia in different intensities as evident from the difference in the colours obtained in the experiment.

Antimicrobial and enzymatic activities are indirect mechanisms that endophytes demonstrate for the enhancement of plant growth. The enzymatic activities of endophytes give their host plants defence against pathogenic microorganisms via the hydrolysis of the pathogens cell-wall (Glick 2012). Our results show that cellulase, protease, and amylase enzymes are produced by all isolated endophytes. Similar studies have been reported from bacterial and fungal endophytes screened for enzymatic activity (Borah et al. 2019; Ntabo et al. 2018; Patil et al. 2015). Antimicrobial activity results showed that all isolated bacterial and fungal endophytes showed a significant zone of inhibition against at least one of the microbial pathogens studied. Hence, endophytes exhibiting antimicrobial activity along with PGP characters can be potentially beneficial for crop production. These results are inconsistent with Hassan (2017) research according to which all bacterial and fungal endophytes exhibit a significant zone of inhibition against at least one of the tested microbial pathogens.

In the present study, since fungal endophytes displayed good results as compared to bacterial endophytes, so fungal endophytes; FETW4 and FETW6 were selected for further analysis. The two selected endophytic fungi were identified as *Penicillium kongii* and *Aspergillus oryzae* based on sequences of the ITS region which is a commonly used tool for identifying fungi. Phylogenetic analysis showed the taxonomic positions of the fungal endophytes to the genus *Penicillium* and *Aspergillus* and both belong to the phylum Ascomycota. Our results are in line with other studies, which reported that the endophytic fungi are mainly from the phylum Ascomycota (Ababutain et al. 2021; Adhikari & Pandey 2019; Gashgari et al. 2016). In order to better understand the unique connection among endophytes and plants, various screening methods based on culture-dependent or independent methods of determining the endophyte-plant association are needed. Therefore, the identification of plant-associated endophytes could be beneficial for biotechnological applications of endophytes as plant growth-promoting agents or biocontrol.

It has been reported that fungal endophytes produce phytohormones which enhance plant growth in a number of crops (Ansari et al. 2013; Bilal et al. 2018; Chand et al. 2020; Xia et al. 2019). There are various studies in which *Aspergillus* and *Penicillium species* are reported as plant growth-promoting endophytes (Adhikari & Pandey 2019; Nath et al. 2015; Wani et al. 2016). From the present study, it is also concluded that the isolated fungal endophytes *Penicillium kongii* FETW4 and *Aspergillus oryzae* FETW6 possess great potential and have a wide range of applications in increasing crop production. Both the fungal strains can be used to promote plant growth to harness their potential as bio inoculants in agriculture for sustainable crop production.

Taxus wallichiana has been best known for various medicinal uses like anticancerous, and antimicrobial (Adhikari & Pandey 2017; Juyal et al. 2014). *T. wallichiana* has been studied for plant growth-promoting endophytes but not to a satisfactory level. To the best of our knowledge, the previous studies on plant growth-promoting endophytes from *T. wallichiana* are related only to roots endophytes (Adhikari & Pandey 2020, 2019). In this background, the present work focuses on the plant growth-promoting endophytes of *T. wallichiana* from the leaves and bark region.

Seed germination is one of the most crucial stages in plant growth and development. The germination test was used to assess the plant growth-promoting effect of FETW4 and FETW6 on the fenugreek and mung bean seeds. Previous studies have shown a positive response to inoculation with fungal endophytes (Bi et al. 2018; Bilal et al. 2018; Khan et al. 2021). Endophytes and their host plants form a unique bond that is likely to have a significant impact on the formation of metabolic products in plants (Jia et al. 2016). In the present work seeds of fenugreek and mung bean were selected as both these plants grow well in warmer climates so we need to check whether there is any effect of endophytes isolated from a plant growing in a colder region on these plants. The fenugreek and mung bean seeds which were inoculated with isolated fungal endophytes (FETW4 & FETW6) showed more growth as compared to control. Hence, these isolates can be utilised and explored in detail for the growth-promoting effect in fenugreek and mung bean seeds.

Conclusions

Being a valuable source of natural products, endophytes have gained prominence in various fields such as agriculture and biotechnology over the past few decades. The present study showed that *T. wallichiana* is an ecological niche for diverse fungal and bacterial endophytes. These endophytes exhibit various indirect and direct mechanisms for plant growth promotion without symptomatic injury. Therefore, inoculation of fenugreek and mung bean with fungal endophyte isolates stimulates plant growth compared to the uninoculated. This study indicates that the possible application of these endophytes in agriculture could result in ameliorating plant production and health, and, may lead to increased soil quality as well as fertility. Nevertheless, continued isolation of the endophytes and repeated field studies are needed to support the present findings.

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