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Isolation, Morphological and Biochemical Characterization of Culturable Endophytic Microflora in *Paris polyphylla* Sm. from Nepal

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Abstract

Paris polyphylla Sm., also known as "Satuwa" in Nepal is a perennial medicinal herb, endemic to the Himalayan region. The plant is known to produce numerous bioactive compounds and provide medicinal use. Presently, it is in a state of vulnerability due to overexploitation, lower rate of seed germination, overharvesting, fragmentation of lands, and deforestation. Endophytic microbes of *P. polyphylla*. have been shown to produce numerous bioactive compounds in correspondence to the host plant. As there have been very few studies of *Paris* species in Nepal this study strives to explore the potential of the endophytic variety in the endemic species. The study investigated the endophytic microbiome in the rhizomes of the species from Dhunkharka, Nepal. A total of 18 endophytic bacterial isolates and 5 fungal isolates were obtained from the rhizome of *P. polyphylla*. Several isolates showed distinct morphological forms and were characterized using Gram staining, endospore staining, IMViC tests, catalase test, motility test, and triple sugar agar test. Among the endophytic bacterial isolates, 11 isolates showed gram-positive results and 5 were gram-negative, while endospore staining showed 10 negative and 6 positive isolates. Among the bacterial isolates, 6 were Methyl-Red (MR) positive, 2 were Voges-Proskauer (VP) positive, 15 isolates showed positive motility test, 10 were catalase-positive and 6 were citrate-positive. The fungal isolates could belong to the phylum Ascomycetes based on macroscopic and microscopic analysis. Further molecular characterizations are needed to accurately identify the endophytic isolates. The findings hint that these endophytic microorganisms could be a viable alternative for producing secondary metabolites, aiding in the conservation and medicinal use of the vulnerable *P. polyphylla*.

Keywords: Endophytes, Medicinal Plants, Microflora, *P. polyphylla*

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Introduction

Paris polyphylla Sm., also known as "Satuwa" in Nepal and India, Love apple in English, a member of the family *Melanthaceae*, is a perennial herb distributed in China, Taiwan, the Indian Subcontinent, and Indochina [1,2]. In Nepal, the species is populated in high-altitude forests between 1800-3500 m below tree-line elevations [3]. This medicinal plant plays an active role in treating burns, diarrhea, fever, stomachache, injuries, intestinal wounds, parasites, skin diseases, poisoning, snakebites, gastritis, typhoid, and hemostasis [4]. The significance of *Paris* species comes from its medicinal compounds which include steroidal saponins, triterpenoid saponins, flavonoids, flavonoid glycosides, sitosterol, pyrrolizidine alkaloids, B-ecdysone, fatty acid esters, and polysaccharides [5–7]. Because of the prevalence of such active compounds, the plant is in a state of constant overexploitation. The species has been listed as vulnerable by the International Union for Conservation of Nature (IUCN) and the Conservation Assessment and Management Plan (CAMP) 2001 workshop process. This drastic decline can be accounted for by the species' lower rate of seed germination, overharvesting, fragmentation of lands, and deforestation [8,9].

Endophytes are endosymbiotic microorganisms that reside within plant tissues and play a beneficial role for their host for most of their life cycle without affecting them negatively [7]. They offer different beneficial effects to the host plant, including; nutrient assimilation, fixation of atmospheric nitrogen, solubilization of minerals, stimulation of plant growth, and protection of host plants from pathogens and insects [10–12]. Additionally, some endophytes have been identified as sources of medicinally important compounds, such as Taxol from *Taxus wallachiana* [13–16]. Endophytes reside in the stems, roots, leaf segments, and inflorescence of weeds, petioles, and other parts of plants [17].

The rhizome of *P. polyphylla* var. *yunnanensis* was found to contain a great variety of endophytes, relatively more in their aerial organs [18,19]. This organ has been termed the "jack of all trades" because of its wide range of treating various ailments from diarrhea to cancer [20]. Ethnopharmacological study of the rhizome suggests

that its uses are similar in most countries. To elaborate, in Nepal, the rhizome of *P. polyphylla* is used against snake bites, insect bites, wounds, fever, and food poisoning, and it is also fed to cattle to reduce diarrhea and dysentery [21–23]. The same study concluded that the rhizome endophytic bacterial phyla mostly comprised of Cyanobacteria, Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria [18]. However, a similar study in *P. polyphylla* comprised Firmicutes, Gammaproteobacteria, Alphaproteobacteria, and Actinobacteria [24].

Scanty works have been reported on the endophytic microflora of *P. polyphylla* in the context of Nepal. Thus, this study aimed to isolate and identify the aforementioned microflora of the plant through morphological and biochemical means. The isolation of endophytic microbes from the plant and the production of antibacterial, antifungal, antagonistic, and antioxidant secondary metabolites from these endophytic strains may be used as a viable replacement for modern medicine.

Materials and Methods

Sample Collection

A healthy rhizome of *P. polyphylla* Sm. (PP_BET_180324) (**Figure 1A, 1B**) was collected from Bethanchowk-3 (BET), Dhunkharka, Bagmati, Nepal (27° 31' 18'' N, 85° 30' 8'' E) situated at an altitude of 2877 m above sea level (asl) at a local nursery in a sterile perforated plastic bag. The sample was brought to the Department of Biotechnology, Kathmandu University. It was visually inspected for any signs of disease and contamination, and stored at 4°C.

Figure 1A. A well-developed *Paris polyphylla* Sm. Plant in nursery. **Figure 1B**. Rhizome of *P. polyphylla* Sm. collected from the Sample Site. **Figure 1C**. Cutting of surface-sterilized rhizome pieces in LAF.

Surface Sterilization of Explant

The surface sterilization was carried out before the isolation of endophytes to remove any contamination of epiphytes. The rhizome explant was firstly thoroughly washed with running tap water for about 30 minutes to remove the adhered soil particles. It was then transferred to 1% Tween-20 solution, left undisturbed for 20 minutes as the surfactant is known to provide additional sterilization efficiency for isolation of endophytes [25]. Later, it was washed with distilled water until no froth was observed. The explant was then transferred into a Laminar Airflow hood and was dipped in 75% ethanol, followed by 1.3% Sodium hypochlorite (NaOCl), and 75% ethanol for 1 minute, 3 minutes, and 30 seconds respectively in 50 mL beakers each [26,27]. Finally, the explant was washed with distilled water thrice. The final wash solution was kept to determine the presence of contaminations (**Figure 1C**).

Isolation of Endophytic Bacteria

Two methods were implemented to isolate the endophytic bacteria from the rhizome of the plant, the transverse section method and the crushed root method. In the transverse section method, the outer layers of the rhizome piece were peeled off using a sterile scalpel and forceps and were cut into smaller pieces (V-shape) of 2-3 mm using a sterile blade (**Figure 1C**). Inoculation was done by directly inoculating the explant in the Nutrient Agar (NA) media (HiMedia, M001-500G) [28].

In the crushed root method, the rhizome sample was finely macerated into a paste using a sterile mortar and pestle. Then, the explant paste was serially diluted using distilled water to obtain 10^{-2} and 10^{-4} diluted solutions. The spread plate culture method was performed to isolate the endophytic bacteria by spreading 100 µl inoculum from undiluted, 10^{-2} and 10^{-4} diluted each in NA media. The culture media were incubated at 37±2°C for 24-48 hours [28].

Isolation of Endophytic Fungi

The transverse section method was followed for the isolation of endophytic fungi. Thin pieces of rhizomes were inoculated in Potato Dextrose Agar (PDA) media (HiMedia, GMH096-500G). The culture media was incubated at 28±2°C for about two weeks with regular monitoring [28]. 0.03 g l^{-1} of streptomycin was used in the PDA media to avoid bacterial contamination.

Selection and Purification of Bacterial and Fungal Endophytes

Morphologically variant bacterial colonies were selected: four colonies from the endophytic culture of the explant sample, two colonies from the undiluted homogenized sample, seven from the 10^{-2} diluted homogenized sample, and five from of 10^{-4} diluted homogenized sample. All fungal endophytes were chosen from the explant culture. Pure bacterial colonies were obtained by subculturing on NA media. Pure fungal colonies were obtained by subculture on PDA media.

Table 1. Morphological Characteristics of Endophytic Bacterial Isolates

Name of Sample	Shape of Colony	Size of Colony	Color of Colony	Margin	Elevation	Texture	Opacity
PP_BET_020424_E1	Circular	Large	Pale yellow	Entire	Flat	Yellow	Opaque
PP_BET_020424_E2	Circular	Large	Pale yellow	Entire	Flat	Moist	Translucent
PP_BET_020424_E3	Circular	Large	Pale yellow	Entire	Convex	Dry	Opaque
PP_BET_020424_E4	Circular	Medium	Pale Yellow	Irregular	Convex	Dry	Opaque
PP_BET_020424_U1	Circular	Small	Pale yellow	Entire	Convex	Moist (oily)	Translucent
PP_BET_020424_U2	Irregular	Large	Pale yellow	Entire	Raised	Moist	Translucent
PP_BET_020424_X1	Irregular	Large	Pale yellow	Entire	Flat	Moist	Opaque
PP_BET_020424_X2	Circular	Large	Pale white yellow	Entire	Flat	Moist, shiny	Translucent
PP_BET_020424_X3	Circular	Small	Pale yellow	Entire	Raised	Moist, shiny	Translucent
PP_BET_020424_X4	Circular	Large	Pale yellow	Entire	Convex	Moist	Translucent
PP_BET_020424_X5	Circular	Medium	Pale yellow	Entire	Raised	Moist, Shiny	Translucent
PP_BET_020424_X6	Circular	Large	Pale yellow	Entire	Raised	Moist, Shiny	Opaque
PP_BET_020424_X7	Irregular	Large	Pale white yellow	Entire	Convex	Dry	Opaque
PP_BET_020424_Y1	Circular	Medium	Pale white yellow	Entire	Flat	Moist, shiny	Translucent
PP_BET_020424_Y2	Irregular	Large	Pale yellow	Irregular	Flat	Dry	Opaque
PP_BET_020424_Y3	Circular	Large	Pale yellow	Entire	Convex	Pink moist	Opaque
PP_BET_020424_Y4	Irregular	Large	Off-white	Entire	Flat	Dry	Opaque
PP_BET_020424_Y5	Circular	Large	Yellow/Neon	Entire	Flat	Moist	Translucent

Storage of Bacterial and Fungal Endophytes

The bacterial and fungal isolates were stored in NA and PDA slant culture at 4°C respectively. The slant cultures were sub-cultured once every two weeks during the project. Bacterial isolates were stored in cryovials with the addition of 70% glycerol at -80°C [29].

Morphological and Biochemical Characterization of Endophytes

The bacterial isolates were examined for shape, size, color, texture, opacity, and margin of the culture colonies. Gram staining and endospore staining of endophytic bacterial isolates were carried out following standard protocols and observed in 100x microscopic magnification [30,31]. All the bacterial isolates were tested for Indole, Methyl-red (MR), Voges-Proskauer (VP), Citrate (IMViC) test, catalase test, H_2S production test, and triple sugar iron (TSI) test following protocols from Bergey's manual of determinative bacteriology [32,33]. Similarly, the morphological analysis of fungal isolates was investigated including the isolates' colony pigmentation, type of hyphae, and their reproductive cells. Lactophenol cotton blue staining was performed, and the microscopy of fungal isolates was observed at 10x, 40x, and 100x magnification to visualize the distinct fungal morphologies [31].

Results

In this study, two methods were utilized to isolate the endophytic bacteria. The colonies were observed to grow from the edges of the explant and through the spread culture of the rhizome paste on NA media within 48 hours of incubation. Following the purification of colonies via subculture, a total of distinct 18 endophytic bacterial isolates and 5 endophytic fungal isolates were isolated from the rhizome tissue of *P. polyphylla*. 14 bacterial isolates (PP_BET_020424_U1, PP_BET_020424_U2, PP_BET_020424_X1, PP_BET_020424_X2, PP_BET_020424_X3, PP_BET_020424_X4, PP_BET_020424_X5, PP_BET_020424_X6, PP_BET_020424_X7, PP_BET_020424_Y1, PP_BET_020424_Y2, PP_BET_020424_Y3, PP_BET_020424_Y4, and PP_BET_020424_Y5) were isolated by the crushed rhizome method from the rhizome (weight of rhizome = 0.98 gm). Similarly, 4 isolates (PP_BET_020424_E1, PP_BET_020424_E2, PP_BET_020424_E3, and PP_BET_020424_E4) were isolated by the transverse section method from the rhizome (weight of rhizome = 1.239 gm) (**Figure 2**).

The isolates were characterized by using morphological and biochemical tests. The morphological characterization of 18 pure bacterial isolates is represented in **Table 1** and shown in **Figure 2**. Most of the colony shapes were found to be circular, with the colony size varying from small (less than 1 mm) to large (more than 1 mm), with larger sizes being the most common. The margin is commonly entire with flat, raised, and convex colony elevation. Out of 16 bacterial isolates, 11 were found to be gram-positive and 5 were gram-negative. 12 bacterial isolates were observed to be

bacilli (rod-shaped) and the rest were cocci (spherical-shaped). Similarly, 6 were found to be spore-forming bacteria, whereas, the rest were non-spore forming bacteria, as shown in the **Table 2** and **Figure 3**.

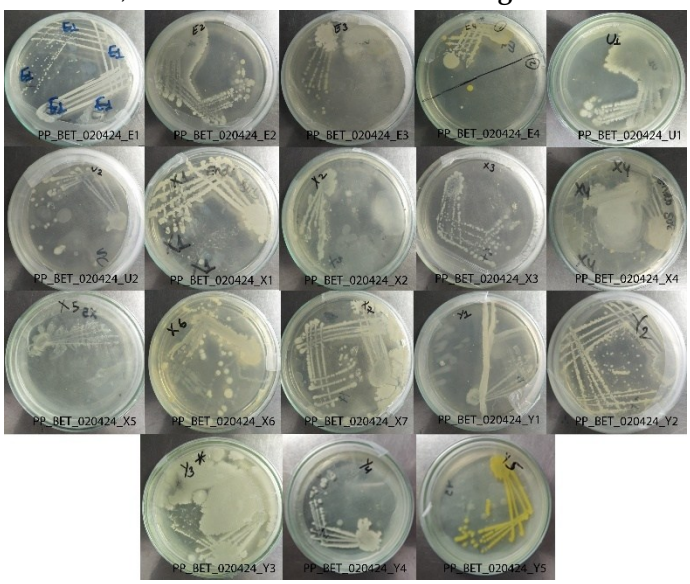

Figure 2. Cultures of Endophytic Bacterial Isolates in NA media incubated at 37±2°C for 24 hours (top-left-to-bottom-right: PP_BET_020424_E1 to PP_BET_020424_Y5)

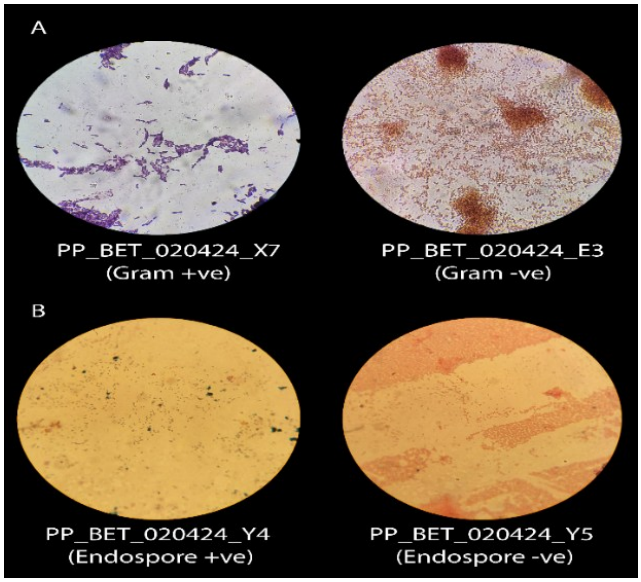

Figure 3A. Microscopy of Gram Staining of two Bacterial Isolates, rod-shaped gram-positive isolate PP_BET_020424_X7 (left), and rod-shaped gram-negative isolate, PP_BET_020424_E3 (right) observed at 100x magnification. The crystal-violet stain (violet) retains in gram-positive bacteria, whereas, the counterstain safranin (red) is retained in the gram-negative bacteria. **Figure 3B**. Microscopy of Endospore Staining of two Bacterial Isolates, endospore-positive isolate PP_BET_020424_Y4 (left), and endospore-negative isolate PP_BET_020424_Y5 (right) observed at 100x magnification. The malachite-green stain (green) retains in endospore-positive bacteria, whereas, the counterstain safranin (red) is retained in the endospore-negative bacteria.

10 isolates showed positive results for the catalase test, whereas, the remaining were found to be negative. All of the bacteria showed positive results for the indole test with most of them being motile. Only 3 (PP_BET_020424_X3, PP_BET_020424_Y1, and PP_BET_020424_Y4) were found to be non-motile (**Figure 4B**). The MR-VP test resulted in 6 isolates with positive results for MR and only 2 positives for the VP test. Here, *Escherichia coli* was used as positive control for the MR test, and *Staphylococcus aureus* for the VP test (**Figure 4B, 4C**). The citrate utilization test revealed 6 bacteria with positive (blue coloration) (**Figure 4D**).

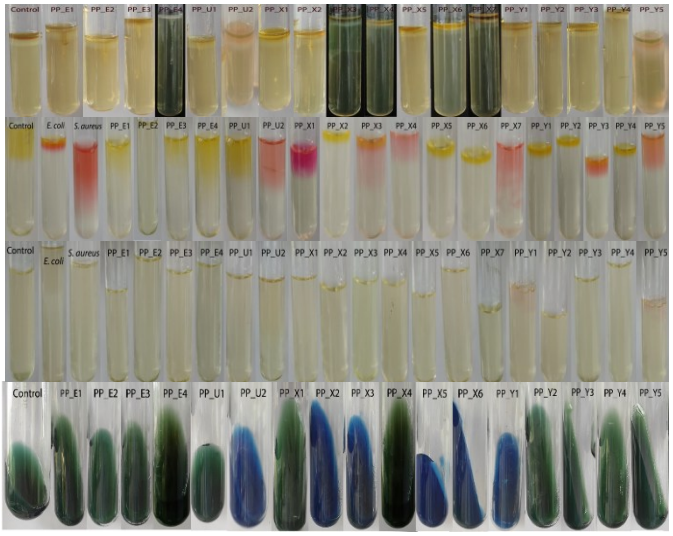

Figure 4A. Sulphur-Indole-Motility (SIM) Test of bacterial isolates in SIM media (HiMedia) (left-to-right: control to PP_Y5) after 24 hours of incubation at 37±2°C, and upon the addition of 2-3 drops of Kovac's reagent. Red coloration denotes +ve result, whereas, yellow coloration denotes -ve result for indole test. **Figure 4B**: Methyl Red (MR) test of bacterial isolates in MR-VP broth medium (HiMedia) (left-to-right: control to PP_Y5) after about 48 hours of incubation at 37±2°C. Red coloration indicates +ve result for MR test, and yellow color indicates -ve result for VP test. **Figure 4C**: Voges-Proskauer (VP) Test of bacterial isolates in MR-VP broth medium (HiMedia) (left-to-right: control to PP_Y5) after 24 hours of incubation at 37±2°C. Red coloration indicates +ve result for MR test, and yellow color indicates -ve result for VP test. **Figure 4D**: Citrate Utilization Test of bacterial isolates in Simmon's citrate media (left-to-right: control to PP_Y5) incubated at 37±2°C for 24 hours. Growth with color change from green to intense blue along the slant indicates +ve result, whereas, no growth and no change of color (green slant) indicates -ve for citrate utilization test.

The macroscopic and microscopic morphology of 5 fungal isolates on PDA media were characterized, as shown in **Table 3** and **Figure 5**. All of the colonies were comprised of septate hyphae. 3 isolates, PP_BET_240424_F1, PP_BET_240424_F3, and PP_BET_240424_F6 were observed to consist of asexual conidia, whereas, a distinctly clear spiky conidiophore was found in PP_BET_240424_F5 isolate.

Discussion

P. polyphylla rhizome was collected from the Hilly region of Nepal at an altitude of 2877 m above sea level in April. The plant from which the rhizome was collected was

Table 2. Biochemical Characteristics of Endophytic Bacterial Isolates.

Name of Sample	Gram Staining	Endospore Staining	Shape	Catalase	Sulfur	Indole	Motility	MR	VP	Citrate Utilization Test
PP_BET_020424_E1	+	+	Cocci	+	-	-	+	-	-	-
PP_BET_020424_E2	-	+	Rod	+	-	-	+	-	-	-
PP_BET_020424_E3	-	-	Rod	+	-	-	+	-	-	-
PP_BET_020424_E4	+	-	Rod		-	-	+	-	-	-
PP_BET_020424_U1	+	-	Cocci	-	-	-	+	+	-	-
PP_BET_020424_U2	+	-	Rod	-	-	-	+	-	-	-
PP_BET_020424_X1	+	-	Rod	+	-	-	+	+	-	-
PP_BET_020424_X2	+	-	Rod	+	-	-	+	+	+	+
PP_BET_020424_X3	-	-	Cocci	+	-	-	-	+	-	+
PP_BET_020424_X4	+	+	Rod	+	-	-	+	-	+	-
PP_BET_020424_X5	+	-	Rod	+	-	-	+	+	-	+
PP_BET_020424_X6	-	+	Rod	-	-	-	+	-	-	+
PP_BET_020424_X7				+	-	-	+	+	-	-
PP_BET_020424_Y1	-	-	Rod	-	-	-	-	-	-	+
PP_BET_020424_Y2	+	+	Rod	+	-	-	+	-	-	-
PP_BET_020424_Y3				-	-	-	+	-	-	-
PP_BET_020424_Y4	+	+	Rod	-	-	-	-	-	-	-
PP_BET_020424_Y5	+	-	Cocci		-	-	+	-	-	-

+ ve: Positive response, - ve: negative response.

Table 3. Macroscopic and Microscopic Observation of 5 Endophytic Fungal Isolates

Fungal Isolate	Macroscopic Observation on PDA Media	Microscopic Observation at 100x magnification			
	Pigmentation	Mycelia	Hyphae	Reproductive cells	Potential class of Fungi
PP_BET_240424_F1	Greenish center, white periphery	White mycelia	Septate	Conidia (asexual)	Ascomycetes
PP_BET_240424_F3	White center, cream periphery	White mycelia	Septate	Conidia (asexual)	Ascomycetes (Eurotiomycetes)
PP_BET_240424_F4	Brown in the periphery, white in the center	White and brown mycelia	Septate	-	-
PP_BET_240424_F5	Dark greyish center, white periphery	Yellowish pink mycelia	Septate	Conidiophores (asexual)	Ascomycetes (Sordariomycetes)
PP_BET_240424_F6	White center, white periphery	White mycelia	Septate	Conidia (asexual)	Ascomycetes

approximately three years old and grown in a shaded region in the greenhouse. As endophytic communities vary based on geographic location, seasonal changes, plant species, age, and specific plant organs [34–38], these environmental and growth parameters could have influenced the isolation of endophytes in our study. A study in *P. polyphylla* var. *yunnanensis* mentioned that endophytic diversity varies with the age of the plant [27]. Because the plant's rhizome was collected from an early plant, it should nurture plenty of endophytes.

In our study, the isolation of the endophytic microbiome was done from the rhizomes, because the Chao and Shannon diversity indices for root endophytes were found to be significantly higher in the roots (Chao index = 614.36 ± 44.24, Shannon index = 2.66 ± 0.72) than in other plant organs [39].

This is most likely because the roots are closer to the rhizosphere, which harbors a plethora of microorganisms and is more in contact with the soil than any other parts of the plant [40–42]. It has been also reported that the continuous and progressive filtering of microbes by the plant's immune system also plays a significant role in the life of endophytes owing to the lesser endophytes in aerial parts of the plant [41].

Similarly, the rhizomes of *P. polyphylla* with a large repertoire of medicinal compounds, especially the Paris saponins have been reported to affect the diversity of endophytes in the organ [5,20,43,44].

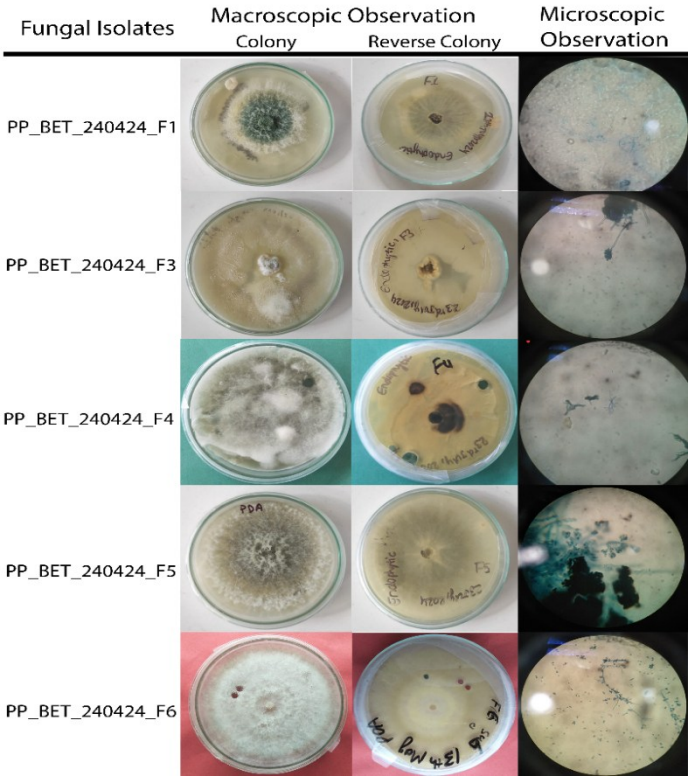

Figure 5.Macroscopic and microscopic observation of 5 fungal isolates at 40x (PP_BET_240424_F1, PP_BET_240424_F3, PP_BET_240424_F4, PP_BET_240424_F5, and PP_BET_240424_F6)

This highlights that the microphyla might have a potential role in the assimilation of such compounds. There have been several endo-microbiomes in varying medicinal plants that are known to produce such compounds [6,7,13,14,45,46]. One such example is of the isolate *Bacillus cereus* LgD2 and *Fusarium oxysporum* TPB isolated from *P. fargesii* Franch. have been reported to significantly increase Polyphyllin content in the rhizomes by 90 dpi (31). Therefore, the possibility of the endophytic microbiomes assisting in such processes cannot be completely diminished.

Previous studies in the endophytes of *P. polyphylla* suggest that most of the bacterial microbiomes within this plant belong to the family of Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria [18]. In our research, we found that 8 (50%) of the gram-positive isolates are rod-shaped, which suggests that they belong to genera such as *Bacillus*, *Clostridium, Corynebacterium, Clostridiodes,* or *Listeria. Bacillus* and *Corynebacterium* are endospore+ bacteria, whereas, *Clostridiodes* and *Listeria* do not produce endospores [47–50]. Our study identified three gram-positive and endospore-positive rods, and 5 gram-positive endospore-negative rods which could belong to the following genera. Gram-positive endophytes play integral role in biocontrol, bioremediation, plant growth, control of soil-borne pathogens, and support of host plant defense against environmental stresses [49]. Similarly, the identification of endospores is important for understanding the environmental adaptability, its ability to confer resistance to environmental stresses, and the pathogenic potential of these isolates as conducted in these studies [51–53]. In contrast, the isolates, PP_BET_020424_E1, PP_BET_020424_U1, PP_BET_020424_Y3, and PP_BET_020424_Y5 being gram-positive and cocci-shaped (25%) might belong to the *Staphylococcus* or *Streptococcus* genera. One of them (PP_BET_020424_E1) is an endospore+ cocci-shaped bacterium, whereas, the rest of the bacteria do not have such protective mechanisms. Four rod-shaped gram-negative isolates (25%) (PP_BET_020424_E2, PP_BET_020424_E3, Y6, and PP_BET_020424_Y1) are suspected to be *Escherichia coli*, *Pseudomonas*, *Proteus*, or *Klebsiella* species [30,54]. This correlates with the findings that endophytes can be gram+ or gram- with varying shapes [7].

The bacterial isolates were differentiated based on their oxygen tolerability using the catalase test [32,55]. Ten isolates showing positive catalase test indicate aerobic or facultatively anaerobic bacteria, potentially belonging to genera such as *Staphylococcus, Bacillus,* or *Pseudomonas.* The remaining eight resulted catalase negative likely belonging to anaerobic or microaerophilic genera such as *Streptococcus, Lactobacillus,* or *Clostridium* [55–58].

Our isolates demonstrated negative results for the indole test in Sulphur-Indole-Motility (SIM) media suggesting that they are not capable of producing the enzyme tryptophanase in contrast to previous findings in other plants [59,60]. Similarly, 6 MR+ isolates indicate their ability to ferment glucose and produce a stable acid end-product [61]. Two isolates were positive for the VP test indicating acetoin production in MR-VP media. The positive citrate utilization test confirmed that six isolates could metabolize citrate as their sole carbon source showing metabolic diversity among the isolates [33].

15 isolates were motile indicating the presence of flagella or another locomotory organ. This is significant because endophytes are known to have a special locomotory organ. These organs help them to colonize and offer a symbiotic relationship to the plant [41,62–64]. None of the isolates were found to produce H_2S gas reflecting that these bacteria are unlikely to play roles in sulfur metabolism [57,65,66].

The macroscopic and microscopic observations of the isolated fungal species revealed distinct morphological characteristics that aided in their preliminary identification. Most of the isolates were found to be of the

phylum Ascomycota, as they were found to be septate and formed sexual spores called ascospores, and conidia in some members [68]. Two genera, *Aspergillus* and *Trichoderma* were identified for the isolates PP_BET_240424_F3 and PP_BET_240424_F5. In the PP_BET_240424_F3 isolate its creamy or light brown coloration with some wrinkling appears characteristic of *Aspergillus terreus* or *Aspergillus flavus* [69]. The PP_BET_240424_F5 isolate's dense wooly dark grey-green-white appearance with a radial pattern, branched structure, and conidiophore arrangement aligns with the features of *Trichoderma*, suspected to be *Trichoderma harzianum* or *Trichoderma viride* [71]. Gauchan et al. demonstrated that two isolates of *Trichoderma* spp., MUSH and BIOC, potentially belonging to *T. harzianum* and *T. viride*, exhibited in-vitro inhibition of several plant pathogens, with inhibition ranges of 47–77% and 41–72%, respectively. These fungal species could, therefore, be used as a potent biopesticide and biofertilizers [71]. Further molecular analysis, such as sequencing of the Internal Transcribed Spacer (ITS) region or other barcoding genes, is required to confirm the exact species. The possibility of finding novel microorganisms cannot be entirely diminished either. Studies showed the isolation of novel gram-negative, facultative anaerobic identified to be *Azospirillum endophyticum* as catalase+ having flagella in the root of *P. polyphylla* Sm. var. *yunnanensis* [72]. Similarly, another novel bacterium by the name of *Oceanobacillus endoradicis* sp. nov. was found to be aerobic, gram-positive, spore-forming, rod-shaped, and having peritrichous flagella isolated from the same plant [73]. The isolation of such novel bacteria shows how little is known about the endophytic microbiome in this plant.

Conclusion

This study illustrates that *P. polyphylla* collected from Nepal's Hilly region can be an authentic resource for the isolation of the endophytic microflora. The findings broaden the understanding of endophytic communities and build the foundation for further research. Our isolated microorganisms may also play vital role for the growth and development of the plant in environmental stress.

Future research work should focus on identifying the molecular interactions between *P. polyphylla* and its endophytes, as well as investigating their potential applications in improving plant growth and resistance to stress factors. Understanding seasonal variations and their impact on the endophytic community could also provide insights on optimizing the use of these microorganisms in plant-based biotechnological innovations for conservation, sustainable agriculture, medicinal plant production, and environmental resilience.

Author's contribution

RS, SB, SR, RP, and AG conducted the project. DPG developed the project concept and assisted with sample collection. DPG, BMK and TS provided supervision and guidance throughout the study.

Competing Interests

No competing interests were disclosed.

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Ethical Approval

Not applicable.

Data availability

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