Research Article

Fungal assemblage in the phyllosphere of *Grewia asiatica* and their role in plant development.

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ABSTRACT
Grewia asiatica locally known as Phalsa is an important fruit-bearing plant with diverse associated microbial communities that may influence plant health and physiological performance. Fungi are the eukaryotic microorganisms which are present almost in everywhere. The present study aimed to investigate the diversity of fungal microflora associated with different plant parts of *G. asiatica*. Fungal microflora were isolated from different parts of *G. asiatica* including stem, root, fruit, leaf and soil. Further, fungal characterization was carried out to confirm the fungal species. *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Alternaria alternata, Fusarium oxysporum and Colletotrichum falcatum.* Molecular identification was further performed using ITS sequencing and phylogenetic analysis which confirmed the evolutionary relationships of the isolated species with reference sequences. A total of eight fungal species belonging mainly to the phylum Ascomycota were isolated, with *Aspergillus* species showing predominance across most plant parts. Fungal diversity indices revealed higher diversity in soil and stem samples, while leaf samples exhibited the lowest diversity and similarity. Heat map analysis further supported variation in fungal diversity among plant compartments. The effect of the phyllosphere microbial community on photosynthetic pigments was assessed, revealing significantly higher photosynthetic pigment contents in plants treated with the phyllosphere microbial community compared to non-treated plants. Overall, the results indicate that fungal microflora associated with *G. asiatica* exhibit organ-specific distribution patterns and that the phyllosphere microbial community positively influences chlorophyll content and plant physiological performance.

Keywords: Fungal characterization, Fungal diversity, *Grewia asiatica* health, Phyllosphere microflora, Phylogenetic analysis.

INTRODUCTION
Grewia asiatica commonly known as Phalsa, associated with the family *Tiliaceae*. *G asiatica* is particularly cultivated in tropical and subtropical regions of the world (Kaur et al., 2024). Phalsa is a flowering plant with small fruits that normally bloom in tropical regions and have leaf fall during winter (Jalali et al., 2025). *G. asiatica* has two different varieties viz. tall and short that are found in India (khangarot et al., 2024). Over 150 species of *G. asiatica*, shrubs-originally from the Himalayas (Jariwala & Parmar, 2024). Phalsa is grown across the nation. Haryana, Punjab, Bihar, and Uttar Pradesh are the top states in northern India, It is also cultivated in Andhra Pradesh and Maharashtra in southern India. Worldwide, phalsa is found distributed and widely cultivated in Bangladesh, Pakistan, Sri Lanka, Nepal, Thailand, Vietnam, Laos, Philippines and some regions of the United States of America (Joshi et al., 2025). Almost 10 species are found in Pakistan, which are primarily grown in Punjab Province (Asghar et al., 2008). In Punjab, phalsa is cultivated on only 30 hectares (Kaur et al., 2024). *G. asiatica*, a plant species, has been traditionally utilized in Ayurveda, an ancient Indian medical book, for the treatment of several conditions such as ulcerative colitis, inflammation, fever wound healing, hypermenorrhea, and diabetes (Jariwala & Parmar, 2024). The fruit of the plant is a good source of different phytochemicals like alkaloids, phenolic compounds like flavonoids and isoflavonoids, and different volatile compounds with diverse properties like antidiabetic,

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hepatoprotective, anticancer, antifungal, analgesic, and antiviral. The fruit of the plant can be eaten raw or after processing in the form of different products like drinks and jams and is a good source of vitamins and proteins**.**(Sharma et al., 2023)**.** Microorganisms, including actinomycetes, bacteria and fungi, inhabit plants, where they can be either pathogenic or beneficial (Akwu et al., 2025). Climate change-driven changes in the composition and functions of plant microbiomes may impact host functions (warming and drought) on plant-microbiome interactions and their ecological functions from genome to ecosystem scales. The plant microbiota consists of different microorganism that inhabit different plant tissues that are accessible. The microbiome and the host plant develop intricate and dynamic relationships that inhabit the soil, rhizosphere, roots, and other plant parts. The comprehension of ecological principles that influence the construction and function of microbiomes in response to climate change will enhance our comprehension of the characteristics of microbiomes, such as resilience and resistance, that enhance plant fitness in new climatic settings. Microbes vary in their metabolism, physiology, and susceptibility to moisture and temperature (Thangjam et al., 2020). This study was designed to assess the total fungal microflora associated with different portions of *G asiatica* plants under field conditions and their impact on plant health, particularly chlorophyll contents in plant leaves. This study aims to develop a future step towards sustainable management of *G.asiatica* that how different combinations of microbial communities affect plant health.

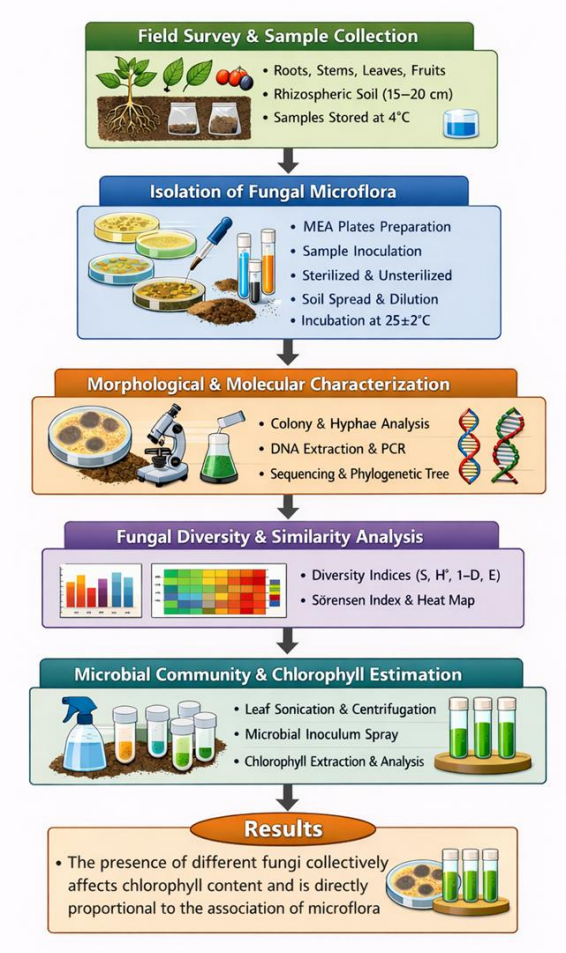


Figure 1. Flowchart of the methodology of this experiment

MATERIALS AND METHODS

Field Survey and Collection of Samples

Multiple surveys were conducted to obtain various samples such as rhizospheric soil, roots, stem, leaves, and fruits of the Phalsa plant from the field of Faculty of Agricultural Sciences (FAS), University of the Punjab, Lahore in 2025. The roots and rhizospheric soil surrounding them at approximately 15-20cm deep were collected for the study of fungal microflora. Similarly, the young to mature leaves emerging from the plant were collected. Mature fruits were obtained from the plants. These all samples were obtained,

brought to the laboratory in zip lock bag, and stored at 4ºC until fungal isolation.

Isolation of Fungal Microflora

To isolate fungal microflora, Malt Extract Agar (MEA) plates were prepared using the following composition (Cheng et al., 2023). For unsterilized samples, s mall portion of phalsa stem, root, leaf and fruit (approximately 3× 3mm) were aseptically inoculated on to MEA media plates and for sterilized samples, all these samples were surface sterilized for 30sec with 2 % sodium hypochloride. Soil sprinkle and serial dilution methods were done for isolation of fungal microflora from rhizospheric soil samples (Cheng et al., 2023, Sari et al., 2025). Purification of isolated fungal species was done when multiple fungi and incubated at 25±2ºC for 7 days and pure plates were stored at 4ºC for identification.

Characterization of Fungal Microflora

Macroscopic features such as color, margin, surface, colony shape were observed using a stereomicroscope. Microscopic features such as shape of hyphae, texture, and condial texture, and size were observed using a compound microscope (Gutiérrez-Sánchez et al., 2025). For molecular characterization, by using CTAB method genomic DNA were extracted from all isolated fungal microflora(Valiya Thodiyil et al., 2024). Polymerase chain reaction (PCR) was performed to amplify the internal tran-scribed spacer (ITS) region of ribosomal DNA (rDNA) using the universal primer pair ITS1 (5′ CTT GGT CAT TTA GAG GAA GTA A 3′) and ITS 4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (Nath et al., 2025). The PCR products were sequenced, phylogenetic tree were constructed by using MEGA X software (Gutiérrez-Sánchez et al., 2025).

Assessment of Fungal Microflora Diversity and its Similarity

Fungal diversity was measured on the basis of total isolates recovered from different tissues of *Grewia asiatica*. Standard diversity metrics, including Species Richness (S), the Shannon–Wiener diversity index (H′), Simpson's diversity index (1−D), and Evenness (E), were calculated by using established formulas (Table 5). The SSI was calculated using the following formula:

SSI= 2C/ (A+B)

Heat Map Analysis

Heat map was created by using GraphPad Prism software to illustrate the distribution patterns of diversity indices across different plant parts. The dataset of fungal diversity and similarity index were included. The values were organized into a two-dimensional matrix, with plant organs represented along the rows and diversity parameters along the columns, enabling comparative visualization of fungal diversity among plant tissues (Figure 2).

Microbial Community and Detection of Total Chlorophyll Content

The healthy leaves of Grewia asiatica were cut into various pieces and put in a 50-ml centrifuge tube add about 25mL of MgCl2. The tube was sonicated in an ultrasonic bath for 20 min at 25°C and vortexed for 15s. All the leaflets were then removed and discarded, and both centrifuge tube was again centrifuged for 3 min at 6000 × g to pellet cells. Supernatant was added to a misting spray bottle with 50 ml of 10 mM MgCl2 with 0.1% tween 80. Two *Grewia asiatica* plants were sprayed with buffer for control and two sprayed with leaf inoculum in sterilized condition (Ehau-Taumaunu & Hockett, 2023). For check chlorophyll content the G. *asistica* leafs were collected. In the mortar, 0.5 g of the fresh *G. asiatica* leaf was added and macerated. In the mixture, 2 mL ethanol was added to 10 mL tubes and stirring for 1 mint. ensuring proper contact with the mixture. Then left for 30 min in the freezer in the dark light, after this mixture were centrifuged for 7 min at 3000 rpm. Readings were recorded at wavelengths of 663 nm and 645 nm(figure 3)

The obtained values were substituted in the following formulas, described in (Gu et al., 2016) for the estimation of photosynthetic pigments.

Chlorophyll a (mg/g) = 12.72 (OD A663) – 2.59 (OD A645)

Chlorophyll b (mg/g) = 22.88(OD A645) – 4.67 (OD A663)

Chlorophyll total (mg/g) = (OD A663) + (OD A645)

RESULTS

The isolated fungal microflora comprised eight species recovered from different plant parts, including stem, root, leaf, soil, and fruit (Table 1). Members of phylum Ascomycota such as *Aspergillus spp.* are found more in number on or around the G*rewia asiatica* plant parts as compared to other families of fungi. Ascomycota members are commonly known as the sac fungi or ascomycetes. It is the largest phylum of Fungi, with over 64,000 species. One feature that is present is most of the Ascomycota species is a reproductive structure known as ascus or asci. A total of 6 isolates belonging to 2 genera *Aspergillus and Alternaria.* Overall, the results indicate a predominance of *Aspergillus* species with variable distribution across different plant organs.

Table 1. Fungal microflora isolated from different *Grewia asiatica* parts.

Sr. No	Fungal microflora	Source

1	*Alternaria alternata*	Leaf
2	*Aspergillus fumigatus*	Root + soil
3	*Aspergillus terreus*	Root + soil+ fruit
4	*Aspergillus flavus*	Stem + root+ soil
5	*Aspergillus parasiticus*	Stem
6	*Aspergillus niger*	Stem +fruit+ soil
7	*Colletotrichum falcatum*	leaf
8	*Fusarium oxysporum*	Soil

Distribution Pattern of Isolated Fungal microflora (%) in Different *Grewia asiatica* parts

The isolated fungal flora comprised eight species recovered from different plant parts, including stem, root, leaf, soil, and fruit. *Aspergillus flavus* showed the highest frequency (80%)**,** with isolates mainly obtained from stem and root tissues. *Aspergillus terreus* and *Aspergillus niger* were the next most frequently isolated species, each showing a frequency of 60% and occurring in root, soil, and fruit samples. *Aspergillus fumigatus* exhibited a moderate frequency (40%) and was isolated from root and soil. In contrast, *Aspergillus parasiticus, Colletotrichum falcatum, Fusarium oxysporum* and *Alternaria alternata* were the least frequent species**,** each recorded with a frequency of 20%**,** and were restricted to stem and leaf tissues and rhizospheric soil respectively (Table 2).

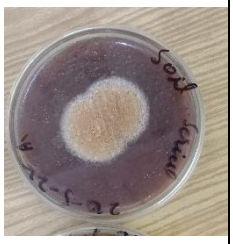
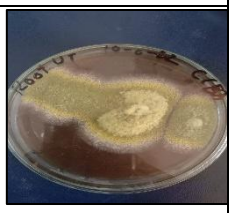
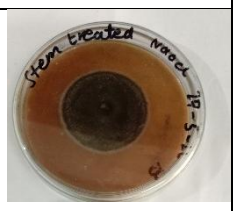
Table 2. Distribution of isolated fungal species from different parts of *Grewia asiatica* and their relative frequency of occurrence.

Sr.no	Isolated fungal microflora	No of isolated species					Total isolates	f	$\chi 2$
		Stem	Root	Leaf	Soil	Fruit			
1	*Aspergillus flavus*	1	2	0	1	0	4	80%	1
2	*Aspergillus terreus*	0	1	0	1	1	3	60%	2
3	*Aspergillus fumigatus*	0	1	0	1	0	2	40%	3
4	*Aspergillus parasiticus*	1	0	0	0	0	1	20%	4
5	*Aspergillus niger*	1	0	0	1	1	3	60%	2
6	*Alternaria alternata*	0	0	1	0	0	1	20%	4
7	*Colletotrichum falcatum*	0	0	1	0	0	1	20%	1
8	*Fusarium oxysporum*	0	0	0	1	0	1	20%	1
Total							16		
	P= 0.82					**df=7**			

Morphological Characterization of Isolated Fungal microflora

Morphological characterization of isolated fungal flora involves the visible features such as colony color, texture, shape, and growth pattern show in table 3, as well as microscopic features like hyphal structure, philides, vesicle, conidial shape and reproductive structures were observed as shown in table 4.

Table 3. Macroscopic characteristics of isolated fungal Microflora

Sr.no	Species names	Colony color	Surface	Colony shape	Picture
1	*A. fumigatus*	Dark greenish to gray	Velvety to powdery	Circular (round) margin are slightly irregular	
2	***A.*** *terreus*	Pale yellow to orange	Velvety to powdery	Circular	
3	*A. flavus*	Yellow to green	Velvety	Circular to slightly irregular**.** Margin Irregular. Rapid and spreading with uneven.	
4	*A. niger*	black	Powdery to granular	Circular (round)	
5	*A. alternata*	Initially white than turning to brown	Velvety or cottony	Circular	
6	*A. parasiticus*	Olive green to dark green	Floccose	Circular	

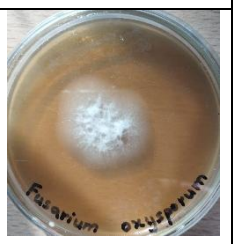
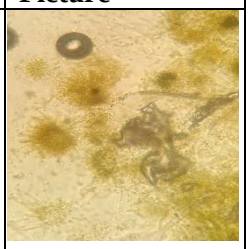
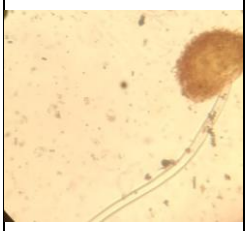
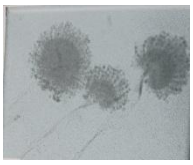
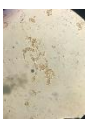
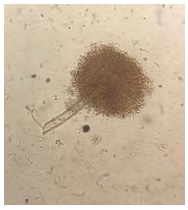
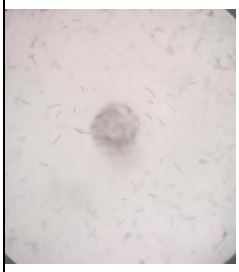
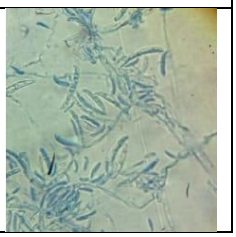
7	*Colletotrichum falcatum*	Initially white to greyish	Cottony to velvety	Circular, with entire to slightly irregular margins	
8	*Fusarium oxysporum*	Initially white, later becomes pale pink	Cottony to floccose**,**	Circular	

Table 4. Microscopic characteristics of isolated fungal microflora

Sr no	Species names	Nature of hyphae	Vesicle	Conidia shape	Philides	Microscopic Picture
1	*A. fumigatus*	Septate	globose to sub-globose	Small, greenish, smooth	Uniseriate	
2	*A. flavus*	Septate	Globose to sub-globose	Rough-walled	Uniseriate and biseriate	
3	*A.terreus*	Septate	Globose	Columnar conidial heads	Biseriate	
4	*A.niger*	Septate, hyaline	Globose to subglobose	Radiate	Biseriate, Cover entire vesicle	
5	*A. alternata*	Absent	Instead of vesicle it produces chains of conidia	Ellipsoidal, ovoid, obclavate,	Absent	

			directly from conidiophores.			
6	*A.parasiticus*	Septate	spherical	Surface rough	Uniseriate	
7	*Colletotrichum falcatum*	Septate, hyaline	Absent	unicellular, falcate	Short,simplehyaline.	
8	*Fusarium oxysporum*	septate	Absent	Macroconidia Microconidia	Short, simple phialides	

Phylogenetic analysis of isolated fungal microflora

The dendrogram illustrates clustering of fungal isolates using accession numbers and species names. Species such as *Aspergillus fumigatus* and *Colletotrichum falcatum* form distinct clades indicating genetic divergence. The branching pattern reflects similarity in DNA sequences used for phylogenetic analysis.

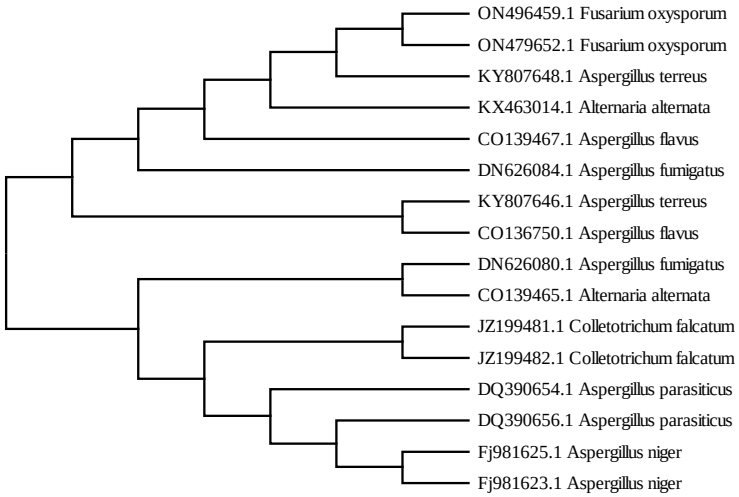


Figure 2. Phylogenetic relationships among fungal isolates based on nucleotide sequence data. The tree was constructed using the Maximum Likelihood (ML) method with 1000 bootstrap replicates to assess branch support. Bootstrap values ≥50% are shown at the nodes. The scale bar represents the number of nucleotide substitutions per site**.**

Fungal Diversity across *G. asiatica* Parts and rhizospheric Soil

A total of 16 fungal isolates representing eight species were recovered from different plant parts (stem, root, leaf, fruit) and soil. The stem samples yielded three isolates belonging to three species, showing a Shannon–Wiener diversity index (H′) of 1.099, a Simpson index (1–D) of 0.667, and complete evenness

(E = 1.0). The average Sørensen similarity index (SSI) for stem-associated fungi was 0.267. The root samples recorded the highest number of isolates (N = 4) with three species, resulting in an H′ value of 1.040, Simpson index of 0.625, and high evenness (E = 0.947). The average SSI for root-associated fungal communities was 0.350. The leaf samples showed the lowest diversity, with two isolates representing two species, giving an H′ value of 0.693 and a Simpson index of 0.500, while evenness remained 1.0, indicating equal distribution of species. No similarity with other plant parts was observed (SSI = 0.000). The soil samples yielded three isolates belonging to three species, exhibiting a Shannon–Wiener index of 1.099, a Simpson index of 0.500, and complete evenness (E = 1.0). The soil fungal community showed the highest average Sørensen similarity index (0.450) among all sample types. The fruit samples yielded two isolates comprising two species, with H′ and Simpson index values of 0.693 and 0.667, respectively, and complete evenness (E = 1.0). The average SSI for fruit-associated fungi was 0.400.

Table 5. Diversity indices and Sørensen similarity of fungal microflora associated with different *G. asiatica* parts and rhizospheric soil

Plant part	Total isolates (N)	Species richness (S)	Shannon-wiener index (H)	Simpson index (1-D)	Evenness(E)	Avg. Sørensen similarity index (SSI)
Stem	3	3	1.099	0.667	1	0.267
Root	4	3	1.040	0.625	0.947	0.350
Leaf	2	2	0.693	0.500	1	0
Soil	4	3	1.099	0.500	1	0.45
Fruit	2	2	0.693	0.667	1	0.4

Heat map showing fungal diversity indices in rhizospheric soil and plant parts

Fungal diversity, as indicated by Shannon-Wiener (H′) and Simpson (1-D) indices, varied among plant parts and rhizospheric soil. Diversity was lowest in leaves, suggesting a more selective fungal community, whereas stems and soil exhibited the highest diversity, reflecting richer and more complex fungal assemblages. Roots and fruits showed intermediate diversity. Sørensen similarity index (SSI) further indicated that leaf fungal communities were the most distinct, while soil and fruit communities shared more species in common with other plant parts. Overall, these patterns highlight organ-specific differences in fungal composition, with soil and stems supporting the most varied communities and leaves harboring a more restricted set of fungi.

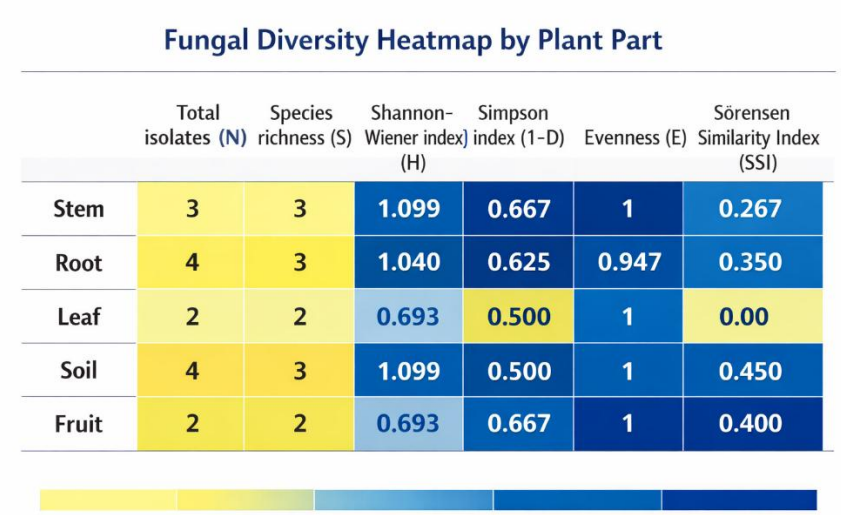


Figure 3. Heat map of fungal diversity indices and Sørensen similarity across plant parts and rhizospheric soil**.**

Effect of Phyllospheric Microbial Community on Chlorophyll Content

The photosynthetic pigments in the samples differed considerably among treatments. FOR Phyllosphere microbial community (PMC) treatment photosynthetic pigments was in higher amounts with chlorophyll a = 4.1mg/g, chlorophyll b = 7.48mg/g, and total chlorophyll = 9.5mg/g, than the Non Phyllosphere microbial community (NPMC) treatment, which was lower amounts (chlorophyll a = 3.3mg/g, chlorophyll b = 3.3mg/g, total chlorophyll = 7mg/g,). The optical density (OD) was at wavelength of at 645 nm (OD645) and 663 nm (OD663) were also higher in the PMC treatment (0.35 and 0.41, respectively) than in the NPMC treatment (0.21 and 0.31, respectively), showed that PMC promoted a higher amount of photosynthetic pigments than NPMC. In general, the results show that LAM has a positive effect on chlorophyll content and photosynthetic pigment accumulation.

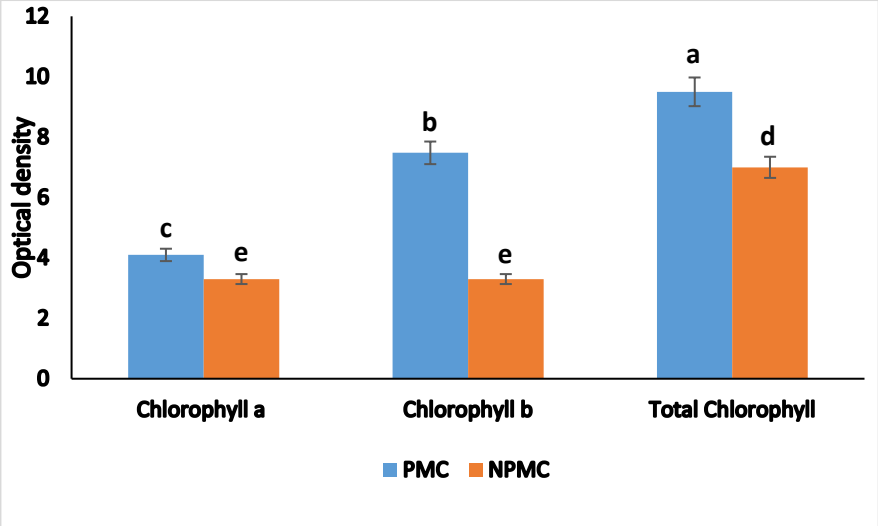


Figure 4. Comparison of chlorophyll a, chlorophyll b, and total chlorophyll content in *Grewia asiatica* under Phyllosphere microbial community (PMC) and Non Phyllosphere microbial community (NPMC) treatments. Statistical significance was determined using Tukey's Test at $p \leq 0.05$, and all values represent the mean ± standard deviation of three biological replicates (n = 3).

DISCUSSION

The current study clearly shows that Grewia asiatica has a varied fungal microflora in different plant parts and rhizosphere soil, thus establishing fungal microflora may vary among plant organs and could be effected by micro-environmental factors (Thangjam et al., 2020; Kaur et al., 2024). Ascomycota, especially Aspergillus spp., dominated in most plant parts, which is in line with international literature that states Ascomycota is the most dominant fungal group in plant-associated environments (Egidi et al., 2019). The dominance of *Aspergillus flavus, A. niger, and A. terreus* in stem, root, soil, and fruit tissues indicates their adaptability, saprophytic character, and high colonization ability in both epiphytic and endophytic environments. The limited distribution of Alternaria alternata and Colletotrichum falcatum to leaf tissues primarily suggests tissue specificity, which can be explained by the surface chemistry of the host, leaf microclimate, and plant defense systems (Gutiérrez-Sánchez et al., 2025). The diversity indices of the fungi showed that the rhizospheric soil and stem had higher diversity, while the lowest diversity and similarity were found in the leaf tissues, thus confirming the idea that soil is the primary reservoir of plant-associated fungi (Egidi et al., 2019; Jalali et al., 2025). The low Sørensen similarity index value in leaf tissues emphasizes the selective nature of the phyllosphere habitat, which is characterized by UV radiation, lack of nutrients, and humidity fluctuations, thus making it unfavorable for fungal colonization (Ehau-Taumaunu & Hockett, 2023). The combination of morphological and

molecular characterization based on ITS ensured the accurate identification of the fungal microflora, which is in accordance with the recommendations for fungal taxonomy and phylogenetics (Valiya Thodiyil et al., 2024). The phyllosphere microbial community (PMC) showed a significant increase in chlorophyll a, chlorophyll b, and total chlorophyll content compared to the non-treated plants, which indicates the positive effect of microbial association on improving photosynthetic efficiency, which can be attributed to the microbial synthesis of growth-promoting substances and enhanced availability of nutrients (Ghosh & Roy, 2025).The data supports the hypothesis that the collective presence of diverse fungal microflora has a direct influence on chlorophyll content, which is proportional to the degree of microbial association and not the presence of a single fungal species. In general, the organ-specific fungal distribution and positive influence of the phyllosphere microbial community on chlorophyll content indicate that native fungal microflora have a crucial role in the physiological health of plants and can be harnessed for sustainable management of *G.asiatica*.

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