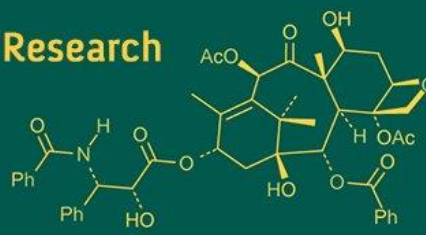
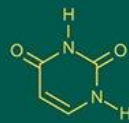
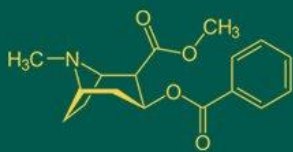


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Spatio-temporal distribution and diversity of bacterial endophytes in apple (*Malus domestica* Borkh.) and their antagonistic potential against *Alternaria mali*

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Abstract

Endophytic bacteria constitute an integral component of the plant microbiome and play a vital role in plant health, productivity, and disease management. Despite their importance, information on the spatio-temporal distribution, tissue specificity, and functional potential of bacterial endophytes in perennial fruit crops such as apple remains limited. The present investigation was undertaken to study the spatial and seasonal dynamics, diversity, and biocontrol potential of bacterial endophytes associated with two apple cultivars, Gala Red Lum and Oregon Spur, grown under temperate conditions of the Kashmir Valley at two locations, SKUAST-K, Shalimar and ICAR-CITH, Budgam. A total of 111 bacterial endophytes were isolated from different apple tissues, including leaves (47), twigs (36), flowers (15), and fruits (13). Season-wise analysis revealed the highest recovery of endophytes during summer (44 isolates), followed by springs (39) and autumn (28). Among the tissues examined, leaves exhibited the highest endophytic colonization, followed by twigs, flowers, and fruits. Diversity analysis based on morphotype grouping revealed distinct spatial and temporal variations in endophytic bacterial communities, with higher alpha diversity recorded at SKUAST-K, particularly during the spring season. Beta diversity analysis further indicated pronounced community turnover across locations and seasons. The results demonstrate that tissue type, season, and location significantly influence the structure and diversity of bacterial endophyte communities in apple.

In vitro evaluation of the isolated endophytes against *Alternaria mali* using dual culture and volatile assays identified 2 promising bacterial isolates (I-38, I-50) exhibiting strong antagonistic activity. Among these, isolate I-38 showed minimum mycelial growth (11.3mm) with the highest mycelial growth inhibition (87.3%), followed by I-50 with mycelial growth of (12.5mm) and (87.2%) mycelial growth inhibition. Volatile-mediated inhibition assays further confirmed the biocontrol potential of these isolates, with I-38 and I-50 exhibiting 80.2% and 69.6% inhibition, respectively. These two endophytes were characterized by molecular means and were characterized as *Bacillus cereus* group, I-38 as *Bacillus cereus* and I-50 as *Bacillus wildmanni*. The study highlights the potential of native apple endophytic bacteria as effective biocontrol agents against *Alternaria mali* and provides valuable insights for the targeted isolation and utilization of beneficial endophytes in sustainable apple disease management.

Keywords: Spatio-temporal dynamics; Tissue specificity; Microbial diversity; Biocontrol potential; Seasonal variation

Introduction

The term endophyte (Greek: endon = within and phyton = plant) was first introduced by Anton de Bary in 1866 to describe microorganisms that inhabit internal plant tissues without causing apparent harm to their host. Since then, the concept has expanded to encompass phylogenetically diverse bacterial and fungal communities that colonize healthy plant tissues for part or all of their life cycle (Hallmann *et al.*, 1997; Rosenblueth and Martinez-Romero, 2006) [18, 36]. These endophytic microorganisms establish intricate associations with their host plants, ranging from commensalistic to mutualistic interactions, and play a pivotal role in enhancing plant growth, stress tolerance, and resistance to biotic challenges. Plant endophyte interactions are governed by a complex interplay of factors, including host genotype, tissue specificity developmental stage, environmental conditions, and geographical location

(Arnold and Lutzoni, 2007; Qawasmeh *et al.*, 2012) [5, 33]. Seasonal fluctuations in temperature, humidity, and host physiological activity further influence endophytic population dynamics, resulting in highly diverse and temporally dynamic microbial communities (Ek-Ramos *et al.*, 2013) [15].

Consequently, endophytic assemblages exhibit pronounced spatial and temporal variation, emphasizing the need for systematic studies to understand their distribution patterns and functional attributes in perennial crops.

Apple (*Malus domestica* Borkh.) is one of the most economically important temperate fruit crops worldwide and represents a complex perennial agroecosystem that harbours diverse microbial communities. Apple production is severely constrained by several diseases, among which *Alternaria* leaf blotch, caused by *Alternaria mali*, is a major concern due to its destructive impact on foliage, fruit quality, and yield. Currently, disease management relies heavily on chemical fungicides, which remain the most commonly adopted strategy for protecting apple orchards (Karacic *et al.*, 2024) [21]. However, prolonged fungicide use has led to the development of pathogen resistance, accumulation of pesticide residues on fruits, environmental pollution, and disruption of beneficial microbial communities, thereby raising serious concerns regarding sustainability and food safety (Chowdappa *et al.*, 2013) [13]. These limitations necessitate the exploration of eco-friendly and biologically sustainable alternatives.

In this context, bacterial antagonists particularly endophytic bacteria functioning as biological control agents (BCAs) have gained considerable attention as promising tools for disease management in perennial fruit crops, including apple (Pane and Zaccardelli, 2015; Shoaib *et al.*, 2019) [31, 41]. Endophytic bacteria inhabit internal plant tissues, share ecological niches with pathogens, and effectively suppress disease through mechanisms such as nutrient competition, antibiosis, volatile production, and induction of systemic resistance. Among the diverse bacterial BCAs, *Bacillus* spp. are regarded as exceptionally promising due to their Gram-positive nature, ability to form endospores, and tolerance to adverse environmental conditions, making them suitable for formulation, storage, and field application (Kukreti *et al.*, 2023; Ankitha *et al.*, 2023) [3, 23].

Several endophytic bacterial taxa, including *Bacillus velezensis*, *Paenibacillus* spp., and *Bacillus pseudomycoides*, have demonstrated strong antagonistic activity against a wide range of phytopathogens. *Bacillus velezensis*, described by (Ruiz-Garcia *et al.* 2005) [38], promotes plant growth through enhanced nutrient acquisition and phytohormone production while suppressing fungal pathogens via bioactive secondary metabolites such as lipopeptides, polyketides, and extracellular enzymes (Kim *et al.*, 2017) [22]. Similarly, *Paenibacillus* spp. are known to produce hydrolytic enzymes, form protective biofilms, and emit volatile organic compounds that inhibit fungal growth (Timmusk and Wagner, 1999; El-Deeb *et al.*, 2013) [16, 43]. *Bacillus pseudomycoides* has also exhibited notable biocontrol efficacy against both soil- and foliar-borne pathogens (Yanti *et al.*, 2018) [47].

Previous studies have extensively characterized *B. velezensis* strains A6 and P42, originally isolated from pomegranate and rice, respectively, revealing their ability to produce a wide array of antimicrobial secondary metabolites, including fengycin, iturin, bacillomycin, and surfactin (Amruta *et al.*,

2018; Prasanna *et al.*, 2021) [2, 32]. These strains exert antagonistic effects through siderophore production, hydrogen cyanide, volatile and non-volatile compounds, and lytic enzymes, while also inducing systemic resistance via activation of key defense-related enzymes in host plants (Shabanamol *et al.*, 2017; Kukreti *et al.*, 2023) [23, 40]. In addition to disease suppression, such endophytes enhance plant growth by improving nutrient uptake and modulating phytohormone levels (Rana *et al.*, 2011; Manivannan *et al.*, 2012) [28, 34].

Although host specificity of endophytes remains debated, with some studies suggesting host-restricted benefits (Long *et al.*, 2008) [26] and others demonstrating cross-host colonization (Sessitsch *et al.*, 2005; Ma *et al.*, 2011) [27, 39], non-host endophytes offer significant advantages due to their ecological adaptability and broad-spectrum disease suppression (Byregowda *et al.*, 2022) [11]. Such traits are particularly valuable in perennial orchard systems, where environmental variability and long-term sustainability are critical considerations (Choudhary *et al.*, 2023) [12].

Despite growing interest in plant-associated microbiomes, comprehensive investigations on the spatio-temporal distribution and tissue-specific diversity of bacterial endophytes in apple remain limited, particularly under the temperate Himalayan conditions of the Kashmir Valley. Elucidating these patterns is essential for identifying ecologically adapted and functionally efficient endophytes that can be harnessed for sustainable orchard management. Therefore, the present study was undertaken to investigate the seasonal and tissue-specific distribution and diversity of bacterial endophytes associated with apple cultivars grown at two distinct locations in the Kashmir Valley and to evaluate the antagonistic potential of selected endophytic bacteria, including *Bacillus cereus* and *Bacillus Wildmannii* against *Alternaria mali* using *in vitro* dual culture, volatilome assays.

2. Materials and Methods

2.1 Collection of Plant Samples

Healthy and asymptomatic plant samples of apple (*Malus domestica* Borkh.) were collected from two high-density cultivars, Gala Red Lum and Oregon Spur, cultivated at two geographically distinct experimental sites in the Kashmir Valley, India: the Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K), Shalimar, Srinagar, and the ICAR-Central Institute of Temperate Horticulture (CITH), Budgam. These locations were selected to represent contrasting agro-climatic conditions and orchard management regimes prevailing in the region. Sampling was conducted during three distinct phenological seasons—spring, summer, and autumn—to capture seasonal variability in bacterial endophytic communities. Tissue sampling was performed in accordance with seasonal physiological availability. During the spring season (April), flowers, leaves, and twigs were collected; in the summer season (late June to early July), fruits, leaves, and twigs were sampled; while during autumn (September), sampling was confined to leaves and twigs. All samples were collected exclusively from apparently healthy, disease-free apple trees to minimize the likelihood of pathogen contamination. Immediately following collection, samples were placed in sterile polyethylene bags, appropriately labelled with cultivar, location, season, and tissue type, and transported to the laboratory under cooled and aseptic conditions. Samples were processed at the earliest possible

time for the isolation of bacterial endophytes using standard microbiological protocols.

2.2 Surface Sterilization of Plant Tissues

Plant samples were thoroughly washed under running tap water to remove adhering debris, and tissues exhibiting visible surface injuries were discarded. Surface sterilization was carried out following the protocol of Araujo *et al.* (2001)^[4] with minor modifications. Briefly, tissues were sequentially immersed in 70% (v/v) ethanol for 1 min, followed by sodium hypochlorite solution (2.5% available chlorine) for 4 min, then again in 70% ethanol for 30 s, and finally rinsed three times with sterile distilled water. The efficacy of surface sterilization was confirmed by plating 100 µL aliquots of the final rinse water onto nutrient agar (NA) plates. Plates were incubated at 25 ± 1°C for 24 h and monitored for microbial growth to ensure the absence of epiphytic contamination.

2.3 Isolation of Bacterial Endophytes

Surface-sterilized plant tissues were aseptically sectioned into small fragments, airdried under sterile conditions, and placed onto nutrient agar plates. The plates were incubated at 37°C for 48 h and monitored periodically for the emergence of bacterial colonies. Colonies originating from within the plant tissues were considered endophytic in nature and selected for further purification.

2.4 Purification and Maintenance of Bacterial Isolates

Emerging bacterial colonies displaying distinct morphological features were purified through repeated streaking on fresh nutrient agar plates until homogeneous and morphologically consistent colonies were obtained. Pure cultures were maintained on nutrient agar slants at 4°C for short-term preservation and periodically sub-cultured to maintain viability. Each isolate was assigned a unique identification code based on its location, season, cultivar, and tissue of origin.

2.5 Morphological and Microscopic Characterization

Morphological characterization of purified isolates was performed based on colony characteristics observed on nutrient agar plates after 24-48 h of incubation, including colony shape, colour, margin, elevation, and surface texture. Microscopic characterization was carried out using Gram staining to determine Gram reaction and cellular morphology, following standard procedures (Boone *et al.*, 2005; Barik, 2015)^[6, 10].

2.6 Grouping of Bacterial Endophytes into Morphotypes

Based on combined morphological and microscopic characteristics, bacterial isolates were grouped into distinct morphotypes. Isolates exhibiting similar colony morphology, Gram reaction, and cellular features were assigned to the same morphotype, whereas those with distinct traits were categorized separately. These morphotypes were treated as operational taxonomic units (OTUs) for subsequent diversity analysis. Morphotyping minimized redundancy among phenotypically similar isolates and facilitated robust estimation of endophytic bacterial diversity.

2.7 Assessment of Spatial and Temporal Distribution

The spatial and temporal distribution of bacterial endophytes was assessed by categorizing isolates according to location (SKUAST-K and CITH), season (spring, summer, autumn), cultivar, and tissue type. The frequency and distribution of

isolates and morphotypes within each category were documented to evaluate patterns of endophytic colonization.

2.8. Diversity Analysis of Bacterial Endophytes

2.8.1 Shannon-Wiener Diversity Index

Bacterial endophyte diversity was quantified using the Shannon-Wiener diversity index (H'), which integrates both morphotype richness and evenness, and was calculated using the formula:

$$H' = - \sum (p_i \times \ln p_i)$$

where p_i represents the relative abundance of the i th morphotype, calculated as the proportion of isolates belonging to that morphotype within a given location-season combination.

2.8.2 Spatial and Temporal Variation Analysis

Spatial variation in endophytic diversity was evaluated by comparing Shannon index values between locations, while temporal variation was assessed by comparing diversity indices across seasons within each location.

2.9 Multivariate Analysis of Bacterial Community Composition

Differences in bacterial endophyte community composition were assessed using the Jaccard dissimilarity index based on presence-absence data of morphotypes. Pairwise dissimilarity matrices were generated and visualized through heat map analysis. Principal Coordinates Analysis (PCoA) was employed to ordinate samples and visualize clustering patterns across locations and seasons. Multivariate analyses were conducted using PAST software (version 4.0) and the R statistical environment (vegan package).

2.10 Statistical Analysis and Data Visualization

All diversity indices and multivariate analyses were computed using PAST and R software. Graphical outputs were generated to facilitate interpretation of spatial and temporal patterns in bacterial endophyte diversity.

2.11 In Vitro Evaluation of Antagonistic Activity

2.11.1 Dual Culture Assay

The antagonistic activity of bacterial endophytes against *Alternaria mali* was assessed using the dual culture technique. A 5 mm mycelial disc from the actively growing margin of a 7-day-old *A. mali* culture grown on potato dextrose agar (PDA) was placed on one side of a PDA plate, while 48 h-old bacterial cultures grown on nutrient agar were streaked on the opposite sides of the plate. Each treatment was replicated three times (Latha *et al.*, 2009)^[24]. Plates were incubated at 28 ± 1 °C. Control plates contained the pathogen alone. Mycelial growth was measured after complete colonization in control plates, and percent inhibition was calculated using the formula described by Vincent (1947)^[45]:

$$\text{Percent inhibition} = [(C - T) / C] \times 100$$

Where C = growth in control, T = growth in treatment.

2.11.2 Volatile Organic Compound (VOC) Assay

The inhibitory effect of volatile organic compounds (VOCs) produced by bacterial endophytes against *A. mali* was evaluated using a sealed plate assay. A 5 mm agar plug from

an actively growing 7-day-old *A. mali* culture was placed on PDA in the base plate, while the corresponding lid plate containing nutrient agar was streaked with the bacterial isolate. The two plates were immediately sealed together using a double layer of parafilm to create a closed system and incubated at $28 \pm 1^\circ\text{C}$ until maximum growth was observed in control plates (Rouissi *et al.*, 2013)^[37]. Percent inhibition was calculated using the Vincent (1947)^[45] formula as described above.

3. Results and Discussion

3.1 Isolation of Endophytic Bacteria from Apple Cultivars

The bacterial endophytic micro-organisms were isolated from two cultivars of apple Gala Red Lum and Oregon Spur, collected from two locations, namely SKUAST-K, Shalimar and ICAR-CITH, Budgam, during three different seasons. Plant tissues such as leaves, flowers, fruits and twigs were used for the isolation of bacterial endophytes. (Table 1). Based on colony morphology, a total of 111 bacterial endophytic isolates were obtained during the course of the study.

Table 1: Season-wise sampling schedule and plant tissues collected for isolation of bacterial endophytes from two cultivars of apple

S. No.	Season	Month	Collected samples
1	Spring	April	Flowers, twigs, leaves
2	Summer	Last week of June to first week of July	Fruits, twigs, leaves
3	Autumn	September	Twigs, leaves

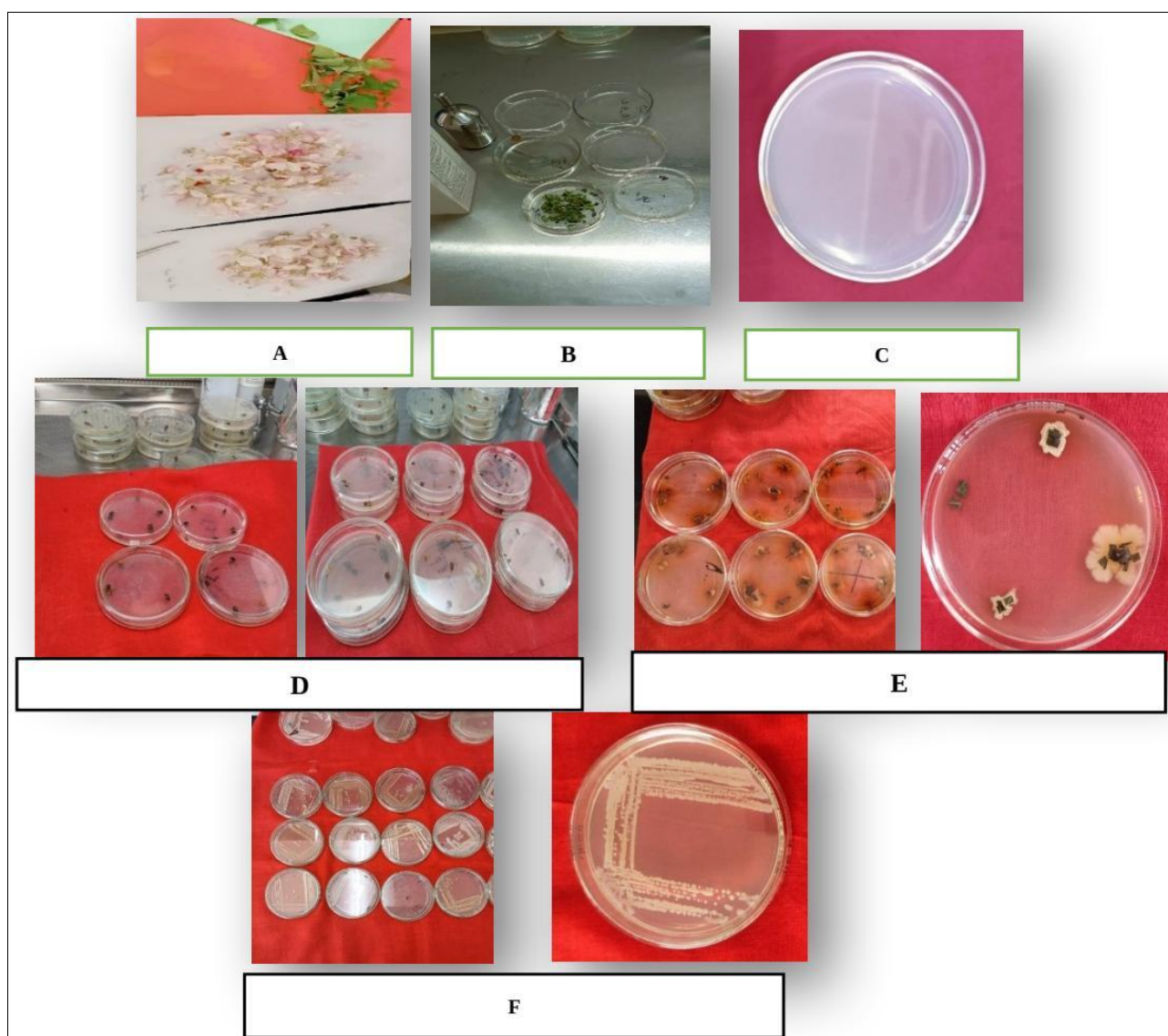


Plate 1: Isolation of bacterial endophytes from different apple tissue

- **A:** Sample collection
- **B:** Surface sterilization
- **C:** Sterilization confirmation
- **D:** Inoculation
- **E:** Bacterial endophyte growth after incubation
- **F:** Pure culture of endophytes

3.2 Morphological characterization of endophytic bacterial isolates

The bacterial isolates had varying morphological characters. Colony colour varied between cream, creamy white, yellow,

white and light yellow. Colony margin was of entire and erose. Whereas the elevation was observed to be of convex,

raised and flat. And based on the opacity the cultures were of opaque, transparent and translucent in their nature.

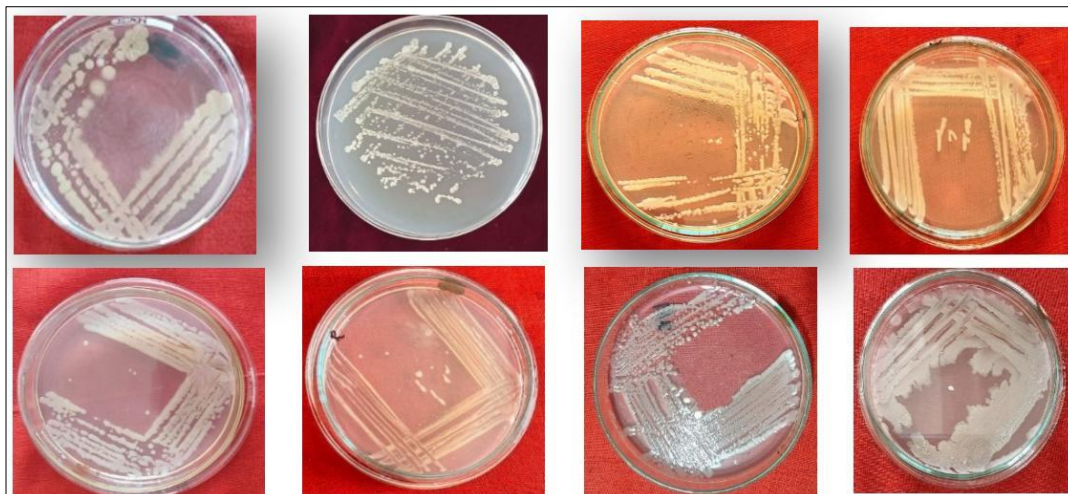


Plate 2: Colony morphology of bacterial endophytic isolates

Representative bacterial endophytes

3.3 Microscopic observation of endophytic bacterial isolates

Microscopic observation was carried out through Gram staining under 100 X oil immersion to study the differences

among bacterial isolates. The isolates were found to be of both Gram positive and Gram negative with shape varied between cocci, rod and coccobacilli.

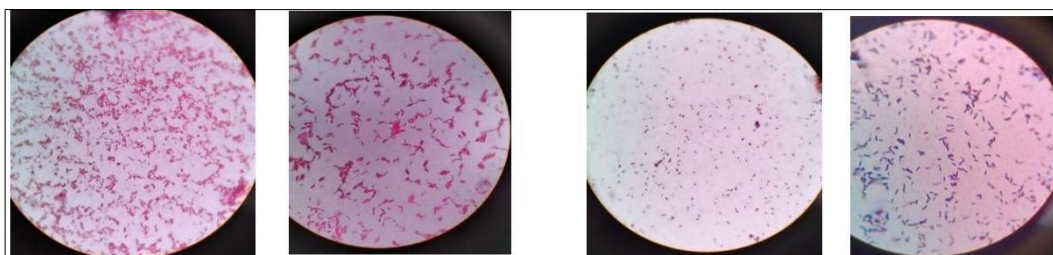


Plate 3: Microscopic characteristics of bacterial endophytic isolates

Gram negative

3.4 Season-wise, Location-wise, Cultivar-wise and Tissue-wise Distribution of Endophytic Bacteria

The season-wise, location-wise, cultivar-wise and tissue-wise distribution of bacterial endophytes isolated from apple is presented in Table 2.

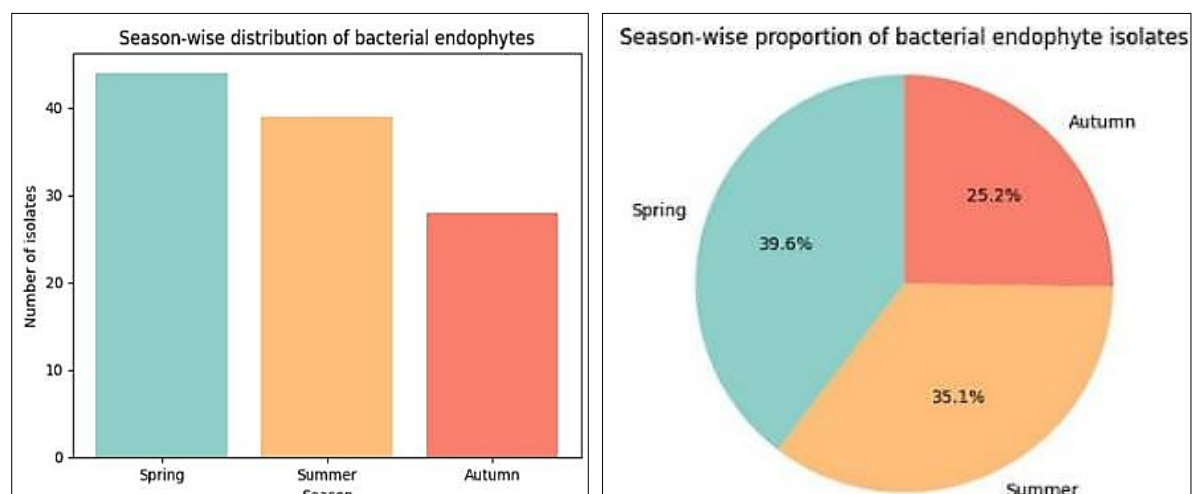
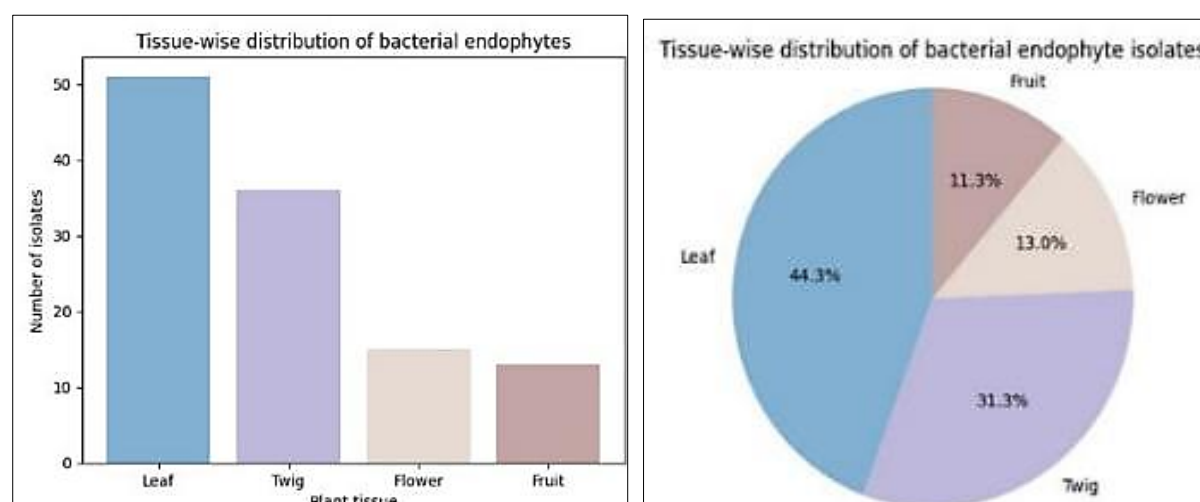
Endophytes diversity across seasons: The distribution pattern of bacterial endophytic microorganisms showed a considerable variation in their population across different seasons. The highest population of bacterial endophytic microflora (44) was observed during spring season followed by summer (39) and autumn (28). Location-wise comparison revealed that SKUAST-K yielded a higher number of isolates (62) compared to ICAR-CITH (49).

Endophytes diversity across plant parts: The diversity in endophytes, regardless of plant tissues was astonishingly very high, evident from (Table 2), showing highest (47) numbers of endophytic bacteria confined to apple leaf samples followed by twigs (36). Whereas apple flowers (15) and fruits (13) samples showed a comparatively lower degree of diversity out of total bacterial endophytes.

Overall, the results clearly demonstrate that the recovery of bacterial endophytes in apple cultivars is strongly influenced by season, tissue type, location, and cultivar, with spring season and leaf tissues being the most favourable sources for endophyte isolation.

Table 2: Season-wise, location-wise and tissue-wise distribution of bacterial endophytes isolated from two apple cultivars (Gala red-lum and oregon spur).

Season	Location	Cultivar	Leaves	Flowers	Fruits	Twigs	Total IsolatesS
Spring	SKUAST-K Shalimar	Gala red lum	5	4	-	3	12
		Oregon spur	3	5	-	3	9
	ICAR-CITH Rangreth	Gala red lum	3	3	-	3	9
		Oregon spur	3	3	-	3	9
Total spring							39
Summer	SKUAST-K Shalimar	Gala red lum	6	-	4	3	13
		Oregon spur	5	-	5	3	13
	ICAR-CITH Rangreth	Gala red lum	3	-	3	3	9
		Oregon spur	3	-	3	3	9
Total Summer							44
Autumn	SKUAST-K Shalimar	Gala red lum	6	-	-	3	9
		Oregon spur	4			3	6
	ICAR-CITH Rangreth	Gala red lum	3			3	7
		Oregon spur	3			3	6
Total Autumn							28
Grand total	47 15 13 36						111

**Fig 1:** Season-wise distribution of bacterial endophyte isolates recovered from apple cultivars**Fig 2:** Tissue-wise distribution of bacterial endophyte isolates in apple cultivars

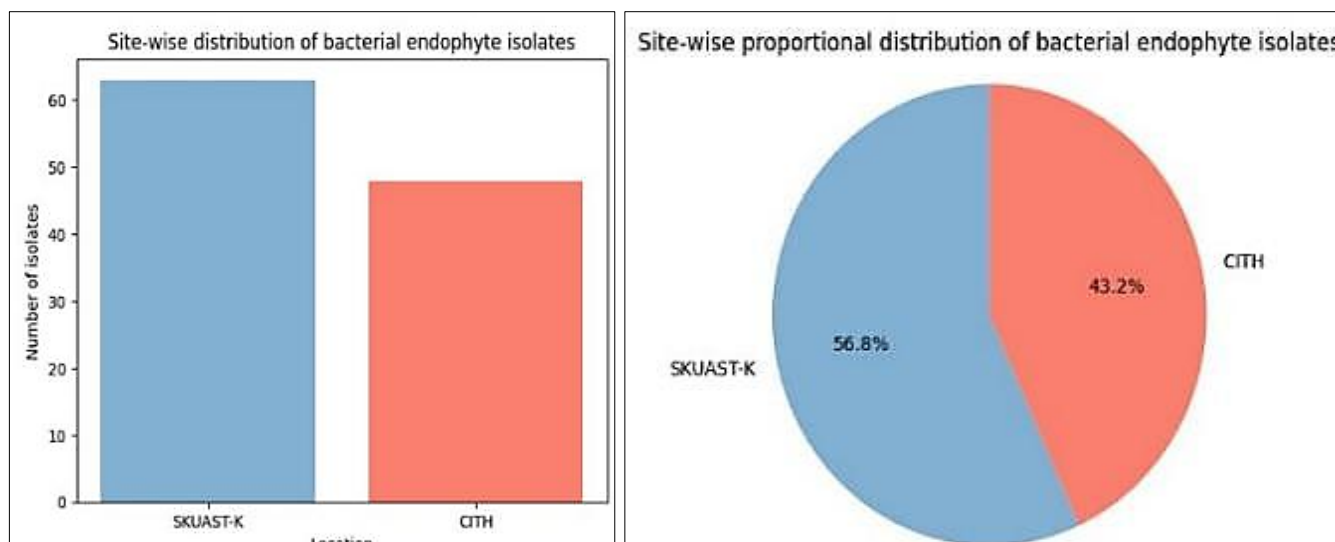


Fig 3: Site-wise distribution of bacterial endophyte isolates in apple cultivars

3.5 Grouping of Endophytic Bacterial Isolates into Morphotypes

Based on colony morphology, pigmentation, margin, elevation, opacity, Gram reaction and cell shape, the bacterial endophytic isolates were grouped into five distinct morphotypes (M1-M5). Isolates showing similar cultural and microscopic characteristics were grouped under a single morphotype to reduce redundancy and facilitate diversity analysis. Each morphotype was considered as an Operational Taxonomic Unit (OTU) for the calculation of diversity indices.

3.6 Alpha Diversity of Bacterial Endophytes across Locations and Seasons

Alpha diversity of bacterial endophytes was assessed using morphotypes as Operational Taxonomic Units (OTUs) to evaluate within-sample diversity. The Shannon diversity index (H') revealed noticeable variation across locations and seasons (Table 3).

At ICAR-CITH, the Shannon diversity index remained 0.000 during spring, summer and autumn, indicating a highly

uniform bacterial endophyte community with no detectable variation across seasons. In contrast, SKUAST-K consistently exhibited measurable bacterial endophyte diversity during all three seasons. The highest Shannon diversity index at SKUAST-K was recorded during summer (0.308), followed by autumn (0.231), while the lowest value was observed during spring (0.116).

Overall, Shannon diversity index values were consistently higher at SKUAST-K compared to ICAR-CITH, suggesting a greater richness and evenness of bacterial endophytes at SKUAST-K. These results clearly indicate that spatial location and seasonal variation significantly influenced the alpha diversity of bacterial endophytes associated with apple.

Table 3: Shannon diversity index (H') of bacterial endophytes across locations and seasons

Location	Spring	Summer	Autumn
CITH	0.000	0.000	0.000
SKUAST-K	0.116	0.308	0.231

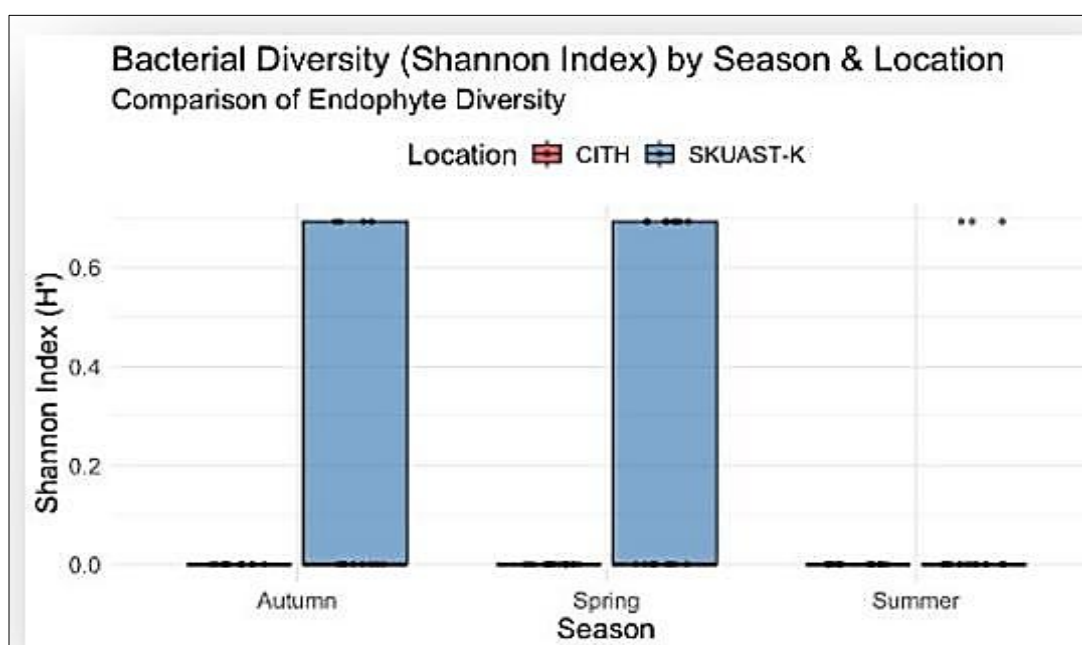


Fig 4: Season-wise comparison of Shannon diversity index (H') of bacterial endophytes across locations

3.7 Beta Diversity Analysis: Spatial and Temporal Variation in Bacterial Endophyte Communities

Beta diversity analysis was carried out using the Jaccard dissimilarity index based on presence-absence data of bacterial morphotypes to evaluate differences in community composition across locations and seasons.

The heat map (Figure 5) illustrates the degree of similarity and dissimilarity among bacterial endophyte communities.

Variations in colour intensity represent differences in community composition, with similar colour patterns indicating greater similarity and contrasting patterns indicating higher dissimilarity. The observed variation in colour distribution among samples clearly suggests that bacterial endophyte community composition varied across both spatial (location) and temporal (seasonal) scales, demonstrating pronounced heterogeneity.

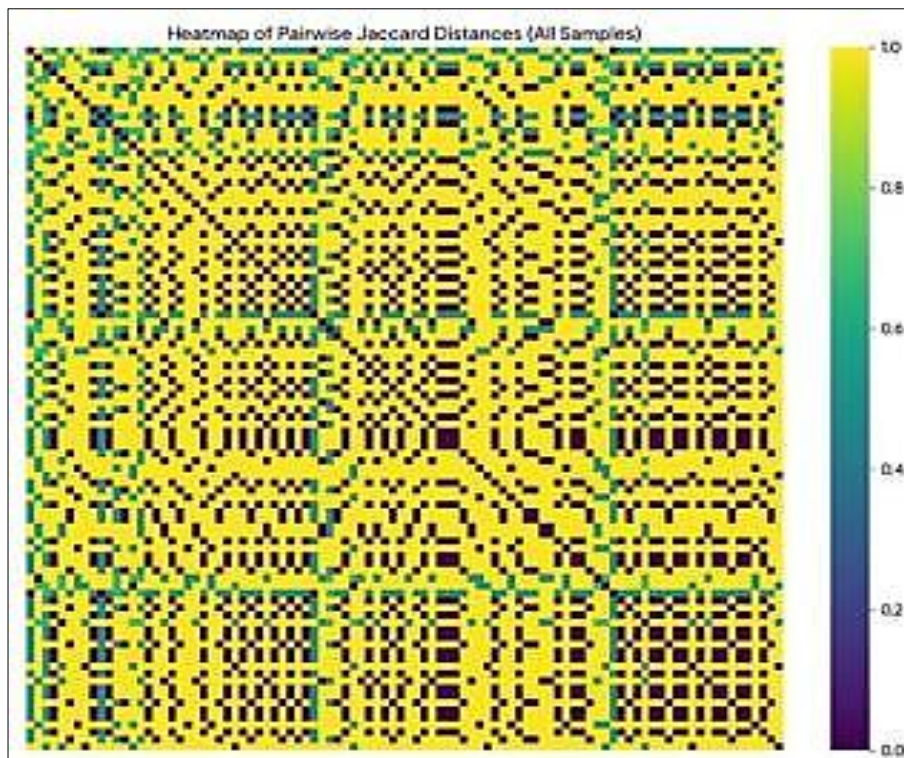


Fig 5: Heat map showing pairwise Jaccard dissimilarity among bacterial endophyte communities across seasons and locations

3.8 Principal Coordinate Analysis (PCoA) Based on Jaccard Dissimilarity

To further examine spatial and seasonal variation in bacterial endophyte community structure, Principal Coordinate Analysis (PCoA) based on Jaccard dissimilarity was performed (Figure 6).

The PCoA plot revealed distinct clustering patterns of bacterial endophyte communities, indicating differences in

community composition among samples collected from different locations and seasons. The separation of clusters along the principal coordinate axes suggests that both location and season exerted a significant influence on the structure of bacterial endophyte communities associated with apple.

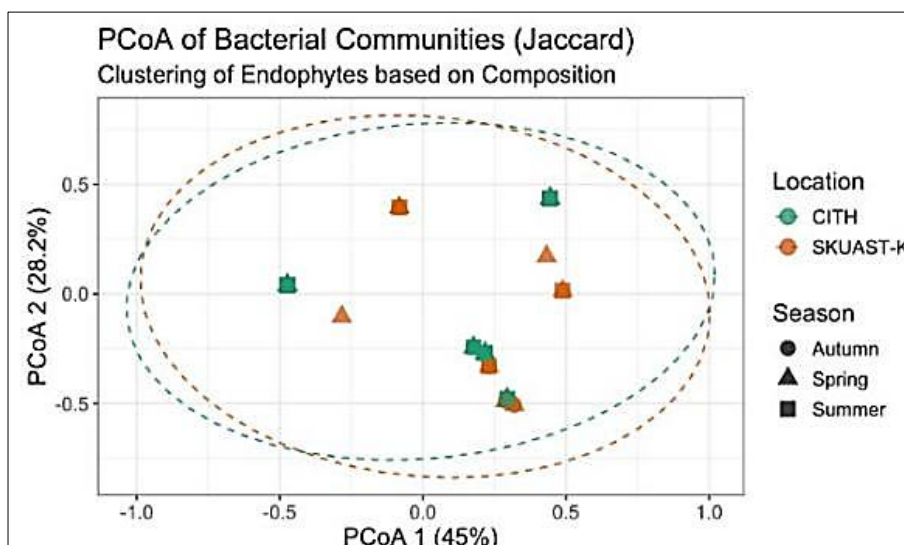


Fig 6: Principal Coordinate Analysis (PCoA) based on Jaccard dissimilarity showing clustering of bacterial endophyte communities

PCoA Showing Explicit Location- and Season-wise Separation of Bacterial Endophyte Communities

A detailed PCoA plot illustrating explicit separation of bacterial endophyte communities based on location and season is presented in Figure 7.

The plot demonstrates clear grouping of samples according to their respective locations and seasons, indicating that

bacterial endophyte communities differed significantly across spatial and temporal gradients. The spatial separation of sample points along the principal coordinate axes reflects variation in community composition, thereby confirming the role of environmental conditions and seasonal dynamics in shaping bacterial endophyte diversity in apple.

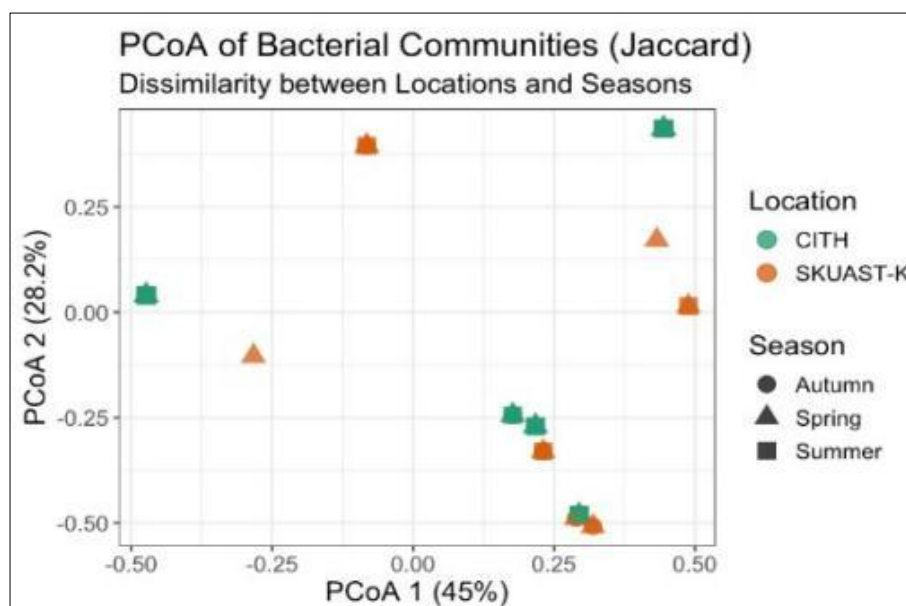


Fig 7: Principal Coordinate Analysis (PCoA) based on Jaccard dissimilarity showing separation of bacterial endophyte communities across locations and seasons

3.10 *In vitro* evaluation of bacterial endophytes against *Alternaria mali*

The antagonistic potential of bacterial endophytes isolated from apple was evaluated against *Alternaria mali*, the causal agent of Alternaria leaf blotch disease, under *in vitro* conditions using the dual culture technique.

3.10.1 Antagonistic effect of bacterial endophytes on mycelial growth of *Alternaria mali*

A total of 111 bacterial endophytes were screened for their ability to suppress the mycelial growth of *A. mali* by the dual culture assay. The mycelial growth of the pathogen varied significantly in the presence of different bacterial isolates, indicating variability in their antagonistic potential.

Among all the tested endophytes, isolate I-38 exhibited the maximum suppression of fungal growth, recording the lowest mean mycelial growth of 11.3 mm. This was followed by isolates I-50 (12.5 mm). The remaining isolates were comparatively less effective in restricting the growth of *A. mali*. Statistical analysis revealed significant differences among treatments at the 5% level of significance (Table 4 Plate 4).

3.10.2 Percent Inhibition of Mycelial Growth of *A. mali*

The antagonistic efficacy of bacterial endophytes was further expressed in terms of percent inhibition of mycelial growth of *A. mali*. The percent inhibition varied significantly among the 111 isolates tested.

Consistent with the mycelial growth results, isolate I-38 (87.3%) recorded the highest inhibition of the pathogen, followed by isolates I-50 (86.0%) (Table 4 Plate 4). These isolates demonstrated strong antagonistic activity against *A. mali* under *in vitro* conditions. The observed inhibition may

be attributed to the production of antifungal metabolites, competition for nutrients and space, or the secretion of cell wall degrading enzymes by the bacterial endophytes.

Table 4: Effect of bacterial endophytes on mycelial growth of *Alternaria mali* under *in vitro* conditions (dual culture assay)

Treatment	Mycelial Growth of pathogen (mm)	Mycelial growth inhibition of pathogen
I-38	36.833 (6.110 ^{uu})	87.367 (69.181 ^a)
I-50	33.833(5.859 ^{yz})	86.067 (68.085 ^b)
Control	90.00	0.00

Values having different superscripts differ significantly ($p < 0.05$) (Data depicted in parenthesis is square root transformed values)

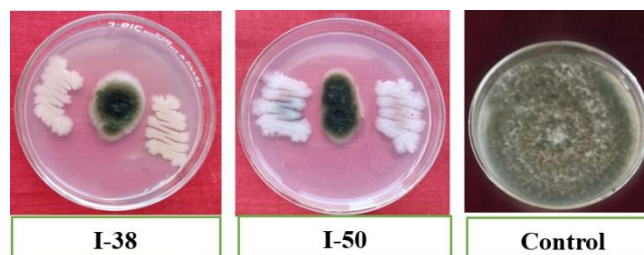


Plate 4: Antagonistic potential of bacterial endophytes against *Alternaria mali*

3.11 Effect of Volatile Metabolites Produced by Bacterial Endophytes

3.11.1 Effect of volatile metabolites produced by bacterial endophytes on mycelial growth of *Alternaria mali*

The volatile metabolite assay demonstrated considerable variation among the selected bacterial endophytes in their ability to suppress the mycelial growth of *Alternaria mali* under *in vitro* conditions. The extent of pathogen growth

differed significantly in response to volatile compounds released by the bacterial isolates (Table 5, Plate 5). Among the tested endophytes, isolate I-38 resulted in the lowest mycelial growth of *A. mali*, indicating strong antifungal activity mediated through volatile metabolites.

This was followed by isolate I-50 which also caused substantial reduction in fungal growth. The observed differences among treatments were statistically significant at the 5% level.

Table 5: Effect of volatile metabolites of selected bacterial endophytes on mycelial growth of *Alternaria mali*

Treatment	Mycelial growth (mm)	Mycelial growth inhibition (%)
I-38	17.667 (4.260 ^b)	80.267 (63.639 ^a)
I-50	27.333 (5.272 ^a)	69.600 (56.555 ^b)
Control	90.00	0.00

3.11.2 *In vitro* Inhibition of *Alternaria mali* by Volatile Metabolites

The inhibitory effect of volatile metabolites produced by bacterial endophytes on *A. mali* was further expressed in terms of percent inhibition of mycelial growth. Significant variation in volatile-mediated inhibition was observed among the bacterial isolates evaluated (Table 5 Plate 5).

Isolate I-38 exhibited the highest percent inhibition of *A. mali* due to volatile metabolite production, followed by isolate I-50. These results suggest that volatile metabolite production plays an important role in the antagonistic interaction between certain bacterial endophytes and *A. mali*.

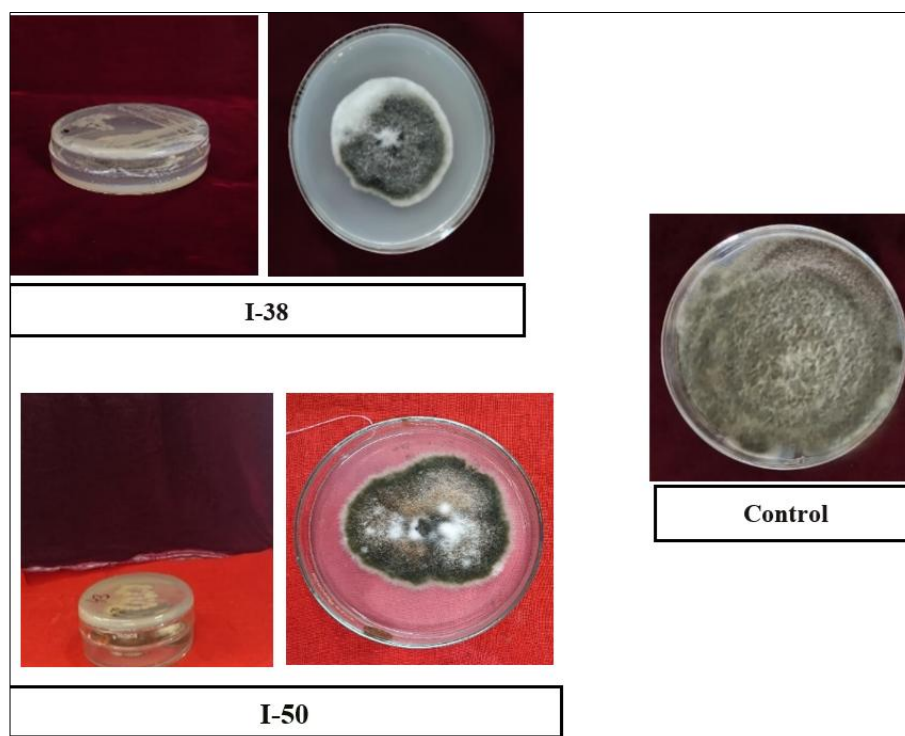


Plate 5: Volatile test of bacterial endophytes against *Alternaria mali*

Discussion

The present investigation revealed a rich and ecologically structured assemblage of bacterial endophytes associated with apple cultivars, as evidenced by the recovery of 111 isolates across tissues, seasons, and locations, confirming apple as a stable reservoir of endophytic bacterial diversity. The perennial woody nature of apple and the presence of longlived internal tissues likely provide favourable niches for sustained endophytic colonization, as also reported in earlier studies (Miliute *et al.*, 2015; Liu *et al.*, 2017) [25, 30]. The pronounced seasonal variation observed, with maximum endophyte recovery during spring followed by summer and autumn, underscores the strong influence of host phenology and environmental conditions on endophytic colonization, where active vegetative growth, enhanced sap flow, and increased nutrient availability during spring promote microbial establishment and proliferation (Miliute *et al.*,

2015; Liu *et al.*, 2017; Yin *et al.*, 2013; Martins *et al.*, 2019) [29, 48]. Tissue-specific distribution patterns revealed leaves as the primary niche for endophytic bacteria, followed by twigs, while fruits and flowers harboured comparatively lower diversity, likely due to their shorter developmental periods and stronger physical and biochemical defense barriers, a trend consistent with previous reports in apple and other woody perennials (Liu *et al.*, 2017; Berg *et al.*, 2014; Abdelfattah *et al.*, 2021) [1, 7, 8]. Spatial analysis indicated significantly higher endophytic diversity at SKUAST-K compared to ICAR-CITH, suggesting that local agro-climatic conditions, altitude, temperature regime, and orchard management practices play a decisive role in shaping endophytic community composition, as similarly demonstrated in landscape-scale studies on perennial crops (Bodenhausen *et al.*, 2013; Coleman-Derr *et al.*, 2016; Abdelfattah *et al.*, 2021) [9, 14]. Morphotype-based

classification revealed substantial phenotypic heterogeneity among isolates, supporting its ecological relevance as a practical proxy for diversity assessment under culture-dependent conditions (Hughes *et al.*, 2001; Martins *et al.*, 2019) ^[20]. Higher alpha diversity recorded at SKUAST-K, particularly during spring, reflects favourable microclimatic conditions that support coexistence of multiple endophytic taxa, whereas reduced alpha diversity at ICAR-CITH indicates strong environmental filtering and site-specific constraints, as reported in orchard ecosystems under restrictive conditions (Martins *et al.*, 2019; Wassermann *et al.*, 2019; Abdelfattah *et al.*, 2021) ^[46]. Furthermore, beta diversity analysis based on Jaccard dissimilarity, supported by PCA ordination and heatmap visualization, revealed pronounced community turnover across locations, seasons, and tissues, highlighting the dynamic and context-dependent nature of apple-associated endophytic bacterial communities (Martins *et al.*, 2019; Trivedi *et al.*, 2020; Berg *et al.*, 2021) ^[44]. The fungal pathogen isolated from diseased apple leaves was morphologically and pathologically confirmed as *Alternaria mali*, exhibiting diagnostic cultural and microscopic characteristics and fulfilling Koch's postulates, consistent with classical and contemporary descriptions of the pathogen and its role as the causal agent of *Alternaria* leaf blotch in apple (Roberts, 1924; Simmons, 2007; Filajdic and Sutton, 1995; Hartevelde *et al.*, 2014) ^[17, 19, 35, 42]. Collectively, these findings demonstrate that bacterial endophyte diversity and ecological distribution in apple are governed by a complex interplay of host traits, environmental factors, and temporal dynamics, providing a strong ecological foundation for the targeted exploitation of endophytic bacteria in sustainable apple disease management.

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