Bacopa Monnieri Bacterial Endophytes: Regulating Plant Growth And Active Phytochemicals

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ABSTRACT
The alteration of rhizospheric and endophytic diversity of microorganisms are of increasing attention as shift in microbic community structure influences the reciprocal function of host-microbiome relations. The present study investigates the previously unexplored dominion of endophytic bacterial partners of medicinal plant *Bacopa monnieri* and discloses their crucial function in endorsing plant growth and biosynthesizing active phyto-ingredients. Two bacterial isolates (*Achromobacter denitrificans* and *Shinella oryzae*) were selected and identified using phenotypic and and molecular characterization from an extensive collection of bacterial isolates. The synergetic potential of bacterial endophytes was validated by the noted growths in *B. monnieri's* shoot and root length after inoculation. On the basis of LC-MS analysis, several active phyto-ingredients, such as bacopaside I, II, brahmic acid, epigenin, eblin, and stigmasterol, were observed in appreciably higher content in endophytes inoculated treatments. These phytochemicals were detected in an experiment conducted in sterile soil, emphasizing the intricate interactions between host plant and bacterial endophytic community. This report first time mention the role of endophytic bacteria *Achromobacter denitrificans* and *Shinella oryzae* in enhancing the *B. monnieri* plant growth and active ingredients. This pioneering unearthing brings up new prospects for sustainable agriculture and pharmacological improvement as well as revealing the previously unidentified potential of *B. monnieri's* endophytic associates.

1. Introduction

Bacopa monnieri, generally called as the herb of grace or Indian penny wort as well as thyme leaved gratiola or hyssop water holds a significant place in traditional ayurvedic medicine, where it is mentioned to as Brahmi [1]. *Bacopa monnieri* is an important medicinal plant, has enormous demand in pharmaceutical companies for its active ingredients. This plant is being cultivated and exploited for its various application in traditional as well as modern medicines but these has been a low level of study carried out with context to endophytes as magnificent source of bioactive compounds [2].

Bacopa monnieri contains alkaloids such as nicotine, brahmine, and herpestine. Among these compounds, bacoside-A, comprising bacoside-A3, bacopasaponin-C, bacopaside-II, and bacopaside-X, is the extensively investigated and potential component of *B. monnieri* [3], [4]. Additionally, endophytes have been recognized for synthesizing compounds that promote plant growth and also enhances the active ingredients of host plants [5], [6]. Certain endophytic strains exhibit the capacity to modulate host plant growth and hold immense agricultural and biotechnological relevance due to their pivotal roles in plant health, productivity, and sustainability [7]. Endophytes maintain a close symbiotic association with their host organisms, understanding this interaction holds significant promise for sustainably producing vital pharmaceutical compounds through the biosynthesis of active ingredients [9], [10]**.** The broad capability of numerous endophytes to generate signaling molecules such as nitric oxide and growth-regulating compounds such as auxins and ethylene may further signify the coevolutionary link between endophytes and plants [11], [12]**.**

Bacopa monnieri serves as the primary reservoir for valuable pharmaceutical compounds known as bacosides. However, the limited content of these active molecules within the plant itself hampers the ability to meet industrial demands. The accumulation of secondary metabolites can be augmented in plants through the introduction of endophytes [13]**.** Additionally, meeting the industrial demand for active ingredients has led to unsustainable exploitation of the wild natural population of *Bacopa monnieri*, resulting in its categorization as a threatened plant species [14], [15]. Reports on microbial interactions with medicinal plants with respect to active ingredient enhancement are scarce; moreover, there have been inadequate investigations concerning endophytes as enhancers of bioactive compounds[16]. To overcome these issues, the utilization of potential and efficient bacterial endophytes could be considered as an effective and sustainable strategy to augment *B. monnieri* growth and the biosynthesis of pharmaceutically important biochemical compounds.

2. Materials and method

2.1 Endophytic microbes' isolation

At 'Swami Rama Himalayan University', (30.1951° N, 78.1921° E), *Bacopa monnieri* plants were grown in fields. For the isolation of potential endophytes, healthy *B. monnieri* plants were selected and shipped to the laboratory, and for further work followed the procedure mentioned by [17].The endophytes were isolated and selected based on their unique colony characteristics and preserved on slants for subsequent research [18].

2.2 Endophyte selection parameters

2.2.1 Indole acetic acid (IAA) synthesis

Selected endophytic bacterial cultures were assessed for indole acetic acid (IAA) synthesis by following the method described by [19], [20].

2.3. Hydrogen Cyanide (HCN) production

For qualitative assay, protocol mentioned by [21] and for quantitative assay procedure mentioned by [22] was followed.

2.4. Ammonia production

To assess ammonia production, method given by [23] was followed.

2.5. Siderophore production

For qualitative assay, protocol given by [24] was performed and for quantitative assay, method of [25], [26] was carried out.

2.6. Phosphate/zinc solubilization

The assessment of the phosphate and zinc solubilization ability of the endophytic isolates was conducted using NBRI-BPB agar media [27] For Zn solubilization index, tricalcium phosphate was replaced with $ZnCO_3$ (1%) on NBRI-BPB agar media.

2.7 Enzyme production assay

Selected endophytes were tested for the production of extracellular enzymes - lipase, amylase, urease, gelatinase tannase, and phytase. The test of the above enzymes was carried out as per the protocols mentioned by [28].

2.8. Identification of selected plant growth-promoting endophytes

16S rRNA Sequencing was carried out following the procedure mentioned by [29]. The analysis of the sequencing data was performed using MEGA X software, specifically version 11, which is used for both molecular and phylogenetic studies. The Kimura 2- parameter model was employed for exploring evolutionary history through maximum likelihood method [30]. Utilizing the neighbor-

joining approach and a 1000-iteration bootstrap analysis, a rooted phylogenetic tree was constructed, elucidating the genetic relationships among the studied entities [31].

2.9. Plant experiments

Two bacterial endophytes (*Shinella oryzae and Achromobector dendritificanse*,) were used individually along with their consortia for plant growth studies, followed the procedure of Following treatments were performed to study the effect of endophytes on plant growth and the active ingredients. T1: Control (uninoculated); T2: *Shinella oryzae*; T3: *Achromobector dendritificanse*; T4: *Shinella oryzae and Achromobector dendritificanse*. The plants were cultivated for three months (90 d) in pots and were irrigated with water, whenever required. Plant growth parameters; shoot length, root length, and fresh and dry shoot/root weights, were recorded after harvest [28].

2.10. LC–MS analysis of active ingredients

The raw LC-MS data files were processed with MZmine 2.5 software. Processing of data with MZmine comprises as follows: data importing, detection of MS peak, building of chromatogram, deconvolution of chromatogram, grouping of isotopes, alignment of peaks, filtering MS row, and gaps filling. Each dataset was kept in .csv layout and submitted to univariate analysis on the Metabo Analyst 4.0 web server by choosing the “statistical analysis” component [32].

Statistical analysis

Duncan's multiple range tests (DMRTs) were used to compare the difference between the mean values of different parameters at $P < 0.05$. Plant growth parameter was compared using ANOVA and Duncan’s means comparison.

3. Results

3.1. Isolation of Endophytes

Eighty bacterial endophytes were successfully isolated from various parts of *Bacopa monnieri* plants, including the roots, shoots, and leaves. These isolates were purified and designated with the corresponding names. Selection was based on unique morphological characteristics, and these selected isolates were checked for plant growth-enhancing attributes. On the basis of plant growth attributes, two bacterial endophytes were selected for subsequent characterization and identification. The selection of endophytes was carried out on the basis of the following qualitative screening procedures.

3.2. IAA Production

All the isolates showed visible color alterations, ranging from very light pink to dark red, after addition of Salkowski solution, which is indicative of the synthesis of the IAA. Bacterial isolates, *Shinella oryzae and Achromobector dendritificanse* exhibited proficiency in IAA production, synthesized 300 µg mL^{-1} and 310 µg mL^{-1}respectively.

3.3. Phosphate/Zinc Solubilization

The endophytic potential to solubilize phosphate/zinc was assessed via the agar plate method using NBRI-BPB agar media. Evaluation involved observing distinct clear zones surrounding the colonies. The endophytic bacteria *Shinella oryzae and Achromobector dendritificanse* demonstrated phosphate solubilization capabilities, with solubilization indices recorded at 2.4 mm and 2.5 mm, respectively. Similarly, zinc (Zn) solubilization by *Shinella oryzae, Achromobector dendritificanse* was 3.6, 3.5 and, respectively (Table 1).

3.4. HCN synthesis

In the qualitative evaluation of hydrogen cyanide (HCN) generation by bacterial endophytes demonstrated HCN production, yielding positive qualitative outcomes with varying intensities. Specifically, *Shinella oryzae* exhibited good HCN production (+++), while *Achromobector*

dendritificanse demonstrated a moderate level of production (++). Spectrophotometric quantification further revealed that *Shinella oryzae* generated a substantial quantity of HCN, with the highest recorded optical density value of 0.044, followed by *Achromobector dendritificanse*, with a value of 0.030 (Table 1).

3.4 Ammonia production

The bacterial isolates *Shinella oryzae* and *Achromobector dendritificanse* exhibited ammonia production after the addition of Nessler's reagent (Table 1).

3.5 Siderophore production

Under limited iron availability, the synthesis of siderophores was shown by developing a yellowish/orange area around the colony on CAS agar by fungal and bacterial isolates. Quantitative estimation revealed that the bacterial isolates *Shinella oryzae* and *Achromobector dendritificanse* also displayed quantitative siderophore production rates of 41.91 psu and 42.70 psu, respectively. (Table 1)

3.6 Enzyme production assay

Endophytes exhibited varied enzymatic activity. Bacterial endophytes *Shinella oryzae* and *Achromobector dendritificanse* were positive for amylase and phytase. On the other hand, *Shinella oryzae* produced tannase, catalase, while *Achromobector dendritificanse* synthesized lipase (Table 3).

3.7. Characterization of promising isolates

The bacterial endophytes were found within the genera *Shinella* and *Achromobacter* by conducting pairwise sequence alignment of the 16S regions in bacteria. The sequence of *Shinella* and *Achromobacter* was 99.19% similar to that of *Shinella oryzae, Shinella zoogloeoides* and *Achromobacter denitrificance* and *Achromobacter ruhlandii,* respectively. Phylogenetic tree of isolate *Shinella* sp. and *Achromobacter* sp. found by maximum likelihood method based on the kimura two parameter method are depicted in Figure 1 and 2.

Table 1. Plant growth endorsing qualities of bacterial endophytes

Endophytic Bacterial/ Fungal Isolates	HCN Production		Phosphate / Zn Solubilization	Siderophore Production		IAA Production (µg/ml)	Ammonia Production
	Qualitative	Quantitative (OD)		Qualitative	Quantitative %Siderophore Unit (PSU)		
Bacterial Isolates							
Shinella oryzae	+	0.044	4.1±0.72 / 3.6±0.68	+	41.91±0.46	300±0.98	+
Achromobacter denitrificance	+	0.030	3.4±0.65 / 3.5±0.65	+	42.70±0.42	290±0.91	+

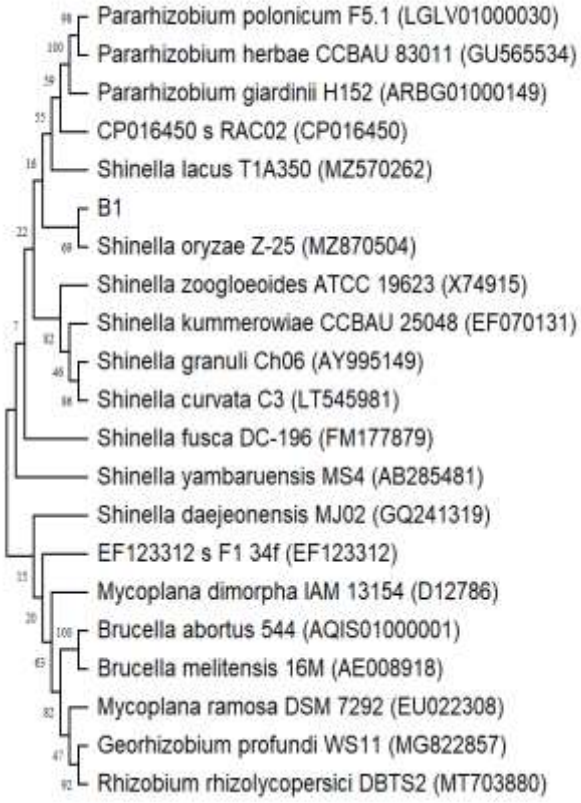

Figure 1. Phylogenetic tree of isolate *Shinella sp.* found by maximum likelihood method based on the kimura two parameter method.

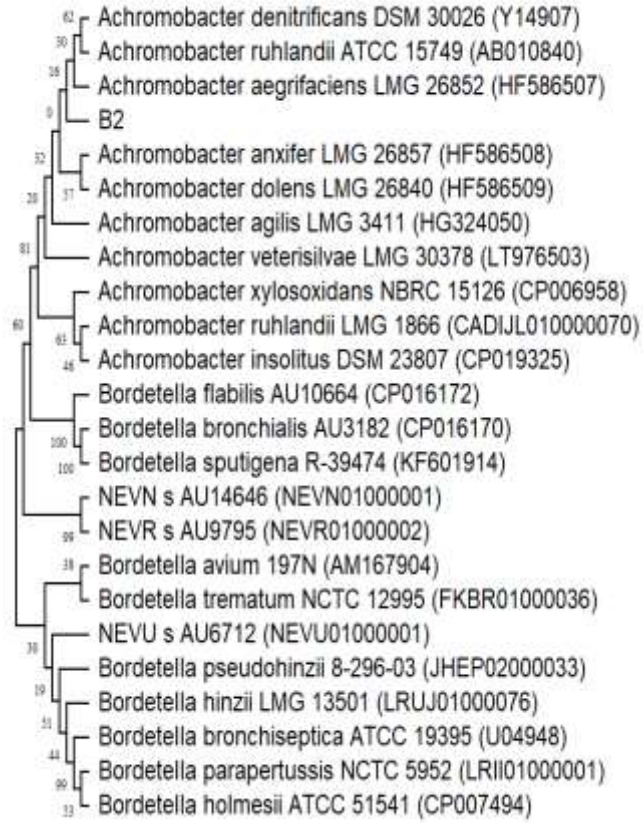

Figure 2. Phylogenetic tree of isolate *Achromobacter.* found by maximum likelihood method based on the kimura two parameter method.

3.8. Spot inoculation test for compatibility of endophytes

To check the compatibility of the selected bacterial isolates, the bacteria (*Shinella oryzae and Achromobacter denitrificance*) isolates were spot inoculated near each other onto Petri plate media and subjected to incubation at 30°C. After five days of incubation, no zone of inhibition was observed among the cultivated cultures. This showed that the selected bacterial endophytes can be used as a consortium and can be exploited as bioinoculants after making a consortium

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3.9. Plant Experiments

All three selected endophytes (*Shinella oryzae, Achromobacter denitrificance*) having plant growth promoting attributes were inoculated to *Bacopa monnieri* plants, which were harvested after 90 days under pot experimental conditions.

3.10. Plant Growth Parameters

Biopriming bacterial endophytes notably influenced both shoot and root lengths in a plant growth experiment. Inoculation with endophytes also influenced the fresh and dry biomass of the plants in comparison to those of the uninoculated plants. In particular, the shoot/root length (33.3/12.3 cm), fresh weight (20.1/9.1 g) and dry weight (5.3/2.9 g) of plants inoculated with the bacterial isolate (*Shinella* sp.) were measured. For (*Achromobacter* sp.), shoot/root length (32.1/11.5 cm), fresh weight (19.2/8.3 g) and dry weight (4.5/2.3 g) were measured. In the T4 treatment (*Shinella oryzae and Achromobacter denitrificance*), shoot/root length decreased (30.6/10.8 cm).

In summary, substantial enhancement in the growth of plants and the extension of root structures were evident upon inoculation with endophytic bacteria across all *Bacopa monnieri* plants, demonstrating the potential of these microbial agents for enhancing plant growth. The *Bacopa monnieri* plant content analysis showed that the biopriming of plants with potential endophytes with plant growth-promoting qualities also increased the amount of active ingredients. The presence of effective endophytes within host plants contributes significantly to the synthesis of beneficial pharmaceutical compounds in plants.

3.11. LCMS analysis

The whole plant ethanol extract was analyzed using LC–MS, and the resulting phytocompounds are detailed in Table 2. The characterization of these compounds was based on their molecular structures, masses, and retention times. The ethanol extract of *Bacopa monnieri* was found to contain important phytochemicals. With reference to component Bacopaside I, treatments T2, T3 and T4 produced 1.3%, 3.2% and 0.5% more than control treatment i.e. T1. Ingredient Bacopasise II, treatments T2, T3 and T4 produced 1.5%, 1.6% and 0.11% more than control treatment (T1). With reference to compound Brahmic acid, treatments T2, T3 and T4 produced 1.4%, 1.7% and 0.3% respectively, higher than treatment (T1). Considering compound stigmasterol, treatments T2, T3 and T4 produced 1.6%, 1.3% and 0.3% more than the control treatment (T1). In case of compound Apigenin, treatment T2, T3 and T4 produced 0.2%, 0.4% and 0.05% respectively, higher than the uninoculated treatment. Component Eblin lactone, treatments T2, T3 and T4 produced 0.6%, 3.2%, and 0.5% respectively, higher than the control (T1). Out of the 3 treatments inoculated with the endophytes, treatment T3 (*Achromobacter denitrificance*) exhibited better results as compared to other treatment i.e. T2, *Shinella oryzae* and T4, *Shinella oryzae and Achromobacter denitrificance* in modulating plant physiology for production of higher content of secondary metabolites (Table 2). The aforementioned compounds were prominently identified in the treatment plants grown in soil.

Table 2. *Bacopa monnieri* ethanolic extracts analysis by LC-MS.

S.NO.	Compounds	Control	*Shinella oryzae*	*Achromobacter denitrificance*	*Shinella oryzae* + *Achromobacter denitrificance*
1.	**Bacopaside I - M/Z**	979.1	979.2696	979.2759	979.2709
	RT	23.98	24.07	24.07	24.07
	Peak area	Peak not detected	2256918.711 **(1.3%)**	5254762.31 **(3.2%)**	4909584.571 **(0.5%)**
2.	**Bacopaside II- M/Z**	929.1	927.6531	927.6476	927.6465
	RT	24.1946	24.3225	24.3225	24.3225
	Peak area	Peak not detected	2612176.239 **(1.5%)**	2488107.997 **(1.6%)**	1115452.213 **(0.11%)**
3.	**Brahmic acid - M/Z**	504.35	504.1368	504.1348	504.1339
	RT	22.985	24.0701	24.0701	24.0701
	Peak area	Peak not detected	2625369.778 **(1.4%)**	2756424.937 **(1.7%)**	2746084.372 **(0.3%)**
4.	**Stigmasterol-M/Z**	412.69	412.69	412.69	412.3046
	RT	28.0503	28.1186	28.1175	28.0907
	Peak area	Peak not detected	2851508.676 **(1.6%)**	2104518.862 **(1.3%)**	2796499.471 **(0.3%)**
5.	**Apigenin-M/Z**	270.05	270.0863	270.0836	270.0834
	RT	26.0316	26.4168	26.4157	26.4056
	Peak area	Peak not detected	384304.54 **(0.2%)**	599046.55 **(0.4%)**	560326.73 **(0.05%)**
6.	**Eblin Lactone-M/Z**	454.3412628	454.3412628	454.3412628	454.3412628
	RT	24.06813945	23.99117817	24.07011736	24.07011
	Peak area	Peak not detected	1061834.946 **(0.6%)**	5254762.31 **(3.2%)**	4909584.571 **(0.5%)**

Table 3. Extracellular enzymes production by endophytes

		Enzymes						
Bacterial endophytes	**Lipase**	**Amylase**	**Urease**	**Gelatinase**	**Tannase**	**Phytase**	**Catalase**	**Cellulase**
Shinella oryzae	-	+	-	-	+	+	+	-
Achromobacter denitrificance	-	+	-	-	+	+	-	-

4. Discussion

Historically, humans have utilized natural resources, especially plants, to formulate different medicines to cure diseases [33]. Most of the active metabolites produced by plants are commercially available as herbal medicines, which are modern factories for the production of beneficial metabolites [34]. *Bacopa monnieri* is a small creeping medicinal herb commonly known as 'Brahmi', mostly

known for the production of Bacoside A and other crucial active ingredients used as nootropic drugs since the ancient period for its beneficial effects on overall consumer health [35]. Due to the large demand and short supply, raw material or powder-based medicines of this species are adulterated with dummy plants [36]. Therefore, enhancing plant growth along with the addition of active ingredients could be a solution for fulfilling the pharmaceutical demand of *Bacopa monnieri.* To achieve this goal, the exploitation of beneficial microbes might play a significant role in modulating host plant development and active ingredients[37]. There are several reports suggesting that endophytes with plant growth-promoting attributes play a substantial part in plant growth improvement and active ingredients production [38], [39], [40], [41], [42], [43], [44]. To meet the market demand for the active ingredient of *Bacopa monnieri*, various researchers have worked on the enhancement of active ingredients [45], [46], [47]. These compounds are synthesized by plants, but associated microorganisms modulate the host plant, which results in increased amounts of active phytochemicals [48], [49].

This study emphasizes the role of the *Bacopa monneri* endophyte in enhancing the host plant's growth parameters and active ingredients [47]. The isolated endophytes exhibited properties of phytohormone indole acetic acid synthesis, ammonia excretion and phosphate and zinc solubilization along with siderophore and HCN production [28]. The production of indole acetic acid by endophytes helped to enhance overall shoot and root growth. As is evident from our results, compared with the uninoculated plants, the inoculated plants showed greater shoot and root length and biomass [20]. A greater root biomass facilitated the uptake of more nutrients, resulting in better plant growth than in uninoculated plants. Furthermore, the synthesis of ammonia by endophytes enhances the nitrogen content in plants [50]. Moreover, the endophytic production of organic acids results in the solubilization of minerals such as phosphate and zinc; therefore, the plant absorbs more minerals from the soil, resulting in better plant growth compared to that of the uninoculated control [27]. Iron-chelating low-molecular-weight secondary metabolites are siderophores, produced by diverse groups of microbes that facilitate iron-limited scavenging. The endophytic production of siderophores also mobilizes insoluble phosphate and also enables plant development by delivering iron and zinc to *B. monnieri* [51].

In this study, novel active ingredients of *B. monnieri*, viz. Bacopaside I, Bacopaside II, Brahmic acid, Stigmasterol, Apigenin and Eblin Lactone were found to be enhanced in endophyte-treated plants. With reference to the novel compounds detected in *B. monnieri*, out of the 3 treatments inoculated with the endophytes, treatment T3 (*Achromobacter denitrificance*) exhibited better results as compared to other treatment i.e. T2 (*Shinella oryzae)* and T4 *(Shinella oryzae and Achromobacter denitrificance)* in modulating plant physiology for production of higher content of secondary metabolites. The reason could be the better performance of these two endophytes with the host plant. However, in the case of treatment T4 a consortium of two bacteria the production of active ingredients was lower than that in the individual treatment, such as *Achromobacter denitrificance*. This could be due to that none of the three endophytes could complement each other to enhance plant growth and synthesize active ingredients. On the other hand, the endophytes grew very well on media in Petri plates; in close association, there was no sign of inhibition, but under natural conditions, they likely did not synergize with each other. Furthermore, when we inoculated the plants with as consortia (treatment T4- *Shinella oryzae and Achromobacter denitrificance*), the plant growth parameters did not appreciably improve, similar to those of the active ingredient content, compared to those of the other treatment (T3- *Achromobacter denitrificance*) but were greater than those of the uninoculated treatment (T1). The potential endophytes induced multifarious metabolic modifications at cell level which fine-tuned the diverse pathways happening in the plant cell for creating a "synergistic" situation for the host.

Sterile soil environments offer inoculated microbial activity, which potentially alter plant-microbe interactions critical for nutrient uptake and modulating genome / nucleic acid to synthesize proteins in the form of active ingredients [52]. On the basis of molecular analysis, two endophytic bacteria, *Shinella oryzae and Achromobector dendritificanse*, interacted with *B. monnieri* plants. Previous investigations have revealed that plant growth-promoting endophytes increase the number of active phytochemicals in *Teucrium polium* [53] and *P. incisa* [54]. This study emphasizes the potential of

endophytes, specifically *Shinella oryzae* and *Achromobacter denitrificans*. The isolation and application of these endophytes in enhancing plant growth along with active phytochemicals in *Bacopa monnieri* has been reported for the first time. Nonetheless, comprehensive field trials are imperative to assess the practical applicability of these endophytes in an agricultural setting, thereby confirming their efficacy as real plant growth promoters and active ingredient enhancers in *Bacopa monnieri* cultivation on a commercial scale [55]. Furthermore, this research highlighted the significance of endophytes in the biosynthesis of pharmaceutically valuable Phyto molecules. Additionally, this study also aims to motivate the scientific investigation of potential endophytes with various medicinal plants for the increased production of significant Phyto molecules for the pharmaceutical and phytochemical sectors. This approach may also contribute to reducing economic costs and discouraging the use of dummy or fake medicinal plants to fulfill pharma industry requirements. Further exploration of endophytes sourced from *Bacopa* plants for the production of important phytochemicals for the pharmaceutical and phytochemical industries is needed. Conversely, there is inadequate knowledge about the diversity of the endophytic microbiome related to *B. monnieri* plants, which could function as plant growth-endorsing agents [35]. However, no detailed information about the endophytic microbial diversity associated with the pharmaceutically imperative *Bacopa monnieri* is available.

5. Conclusions

The bacterial endophytes *Shinella oryzae* and *Achromobacter denitrificance,* were isolated from *Bacopa monnieri.* These endophytes demonstrated plant growth-promoting abilities, including the production of phytohormones, siderophores, hydrogen cyanide (HCN), and ammonia, phosphate and zinc solubilization. Inoculating *Bacopa monnieri* plants with these endophytes resulted in elevated plant biomass and active ingredient content compared to those of uninoculated controls. This study provides the first documentation of the synthesis of Bacopaside I, Bacopaside II, Brahmic acid, Stigmasterol, Apigenin and Eblin Lactone using endophytes derived from *B. monnieri.* whereas they were absent in the control treatment. These efficient bacterial and fungal endophytes could be harnessed for increased production of active ingredients. Thus, our findings provide a basis for potentially improving *B. monnieri* cultivation and enhancing novel phytochemicals by exploiting potential native endophytes.

Author Contributions

Conceptualization, writing original draft preparation, Himani, GR, Viv K.; software and formal analysis, BN, CS, Vijay K; data curation.; review, and editing, Viv K, Vij K, and VR. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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