Original Research Article

Soybean Bacterial Endophytes against Anthracnose Disease Collected from Karnataka State, India: An *In-vitro* Study

ABSTRACT

Endophytes trigger various defence mechanisms within their host plants, engaging primary and secondary protective pathways. This investigation primarily aimed to isolate bacterial endophytes from diverse agroecological regions in Karnataka. Subsequently, these endophytes were assessed for their inhibition against *Colletotrichum truncatum* using the *in-vitro* streak plate technique.A total of 43 bacteria isolated from soybean plants and key endophytes showing the inhibition against *Colletotrichumtruncatum* were in different zones namely, DHW-9(87%),BID-2(85%), BID-13(85%), BID-14(82.50%), DHW-15(80%), BID-15(75%),and BID-16(75%) exhibited notable efficacy against *C. truncatum* in decreasing order.Among these, the DHW-9 *(Stenotrophomonasmaltophilia*strainP4-32) bacterial endophytes isolated from the North Transition Zone (Dharwad) were highly effective against the pathogen, possibly due to employing many direct and indirect mechanisms. Furthermore, the inhibition potential of the bacterial endophytes varies with and within the place of agroecological zones.In conclusion, it has been observed that the bacterial endophyte DHW-9 inhibited the progression of anthracnose disease caused by *C. truncatum* in controlled *in vitro*. Hence, it is imperative to conduct additional experiments, including pot and field studies, to explore its potential to enhance the growth and yield of soybean plants.

Keywords: Soybean, Bacterial endophyte, Anthracnose,*Collectotrichum truncatum*, Biocontrol, Agroecological zones,Growth inhibition percentage.

1. INTRODUCTION

Soybean (*Glycine max* (L.) Merril) is a valuable crop used for both human and animal consumption due to its notable protein content of 40% and oil content of 20% by Abdelghanyet al. [1]. In agriculture, it is crucial to have disease-free seeds for planting. Soybean is susceptible to various stress factors, both from living organisms and environmental conditions, at different stages of its growth, from seed germination to full maturity. Diseases, in particular, lead to substantial losses in yield each year by Rajput et al.[2].Maintaining the health of soybean plants is vital for a profitable harvest.One of the main challenges in increasing soybean productivity is its vulnerability to different fungal diseases like anthracnose, charcoal rot, *Rhizoctonia* root rot, yellow mosaic virus, and rust.These diseases significantly impact soybean output, with anthracnose being a growing global concernby Singh et al.[3].

The *Colletotrichumtruncatum* is prevalent in almost all soybean-growing regions worldwide. It manifests symptoms on the stem, leaves, pods, and seeds. Warm temperatures (30-35°C) and rain mist, whichprovide moisture for 12 hours or more, create favourable conditions for the disease by Rajput et al.[2]& Nataraj et al.[4]. Anthracnose can lead to substantial losses,

especially during the rainy season. This disease has caused yield losses in several countries across Asia, Europe, and South and North America, reaching up to 50% in Thailand and 100% in India by Jones et al. [5]. In India, soybean anthracnose caused 16-25% yield loss by Nataraj et al.[6]. Recently,Rajput et al.[7]revealed that a 1% increase in soybean anthracnose disease severity reduced the yield of 115 kg/ha of soybean grain.Various disease management techniques mitigate anthracnose occurrences, including cultural practices, crop rotation, chemical fungicides, soil solarization by Adrees et al.[8], and biological control. However, the unregulated application of chemical fungicides has detrimental effects on the environment and presents hazards to human health and the native soil microorganismsRaniet al.[9]. Thus, sustainable agricultural practices for managing *C. truncatum* disease are imperative. Alternative methods for anthracnose control involve using biocontrol agents like *Bacillus* spp. by Sabate et al.[10]and*Pseudomonas*sp.by Moin et al.[11] and protective measures by Bernardo-Mazariegos et al.[12]. Bioagents with biocontrol properties are emerging as a potentially eco-friendly alternative to harmful conventional fungicides.

Endophytes are effective biological control agents in sustainable agriculture by Dalal et al.[13]. Some endophytes interact directly with the host plant, making them more efficient against plant pathogensandact as biocontrol agents in sustainable agricultural production,Brunda et al.[14]. According to Chauhan et al.[15],endophytes can trigger defence responses during pathogen attacks and influence host plants' physiological traits. Although the exact nature of the relationship between plants and endophytes is not fully understood, it has been observed that specific isolates benefit their hosts by producing new chemicals and antifungal substances, thereby reducing disease progression.Furthermore, endophytes have demonstrated their ability to support plants in absorbing and utilizing nutrients by providing phytohormones, molecules, enzymes, and siderophores, an antimicrobial agent. Moreover, endophytes confer additional advantages to plants, including nitrogen fixation, increased tolerance to high temperatures and drought, adaptation to osmotic stress conditions, and other benefits by Khan and Doty.[16]. In the present study, we studied whether the bacterial endophytes isolated from the different agroecological zones showed differential inhibition potential against soybean anthracnose pathogen *C. truncated*. The soil and climatic conditions largely influence the bacterial endophyte composition of the plant. Therefore, we hypothesized that the bacterial endophytes isolated from the different agroecological zones have other and will have differential inhibition potentials.

2. MATERIALS AND METHODS

2.1*Colletotrichumtruncatum* inoculum preparation

The culture of *Colletotrichum truncatum* was isolated on potato dextrose agar (PDA) plates composed of Potato extracts (200 g), Agar (20 g), Dextrose (20 g), and Distilled water (1000 ml). To maintain pure cultures of *C. truncatum*, a small portion from the original culture was

transferred to a new PDA plate through successive sub-culturing. These plates were then incubated at a temperature of 27±10°C for 15 days.

2.2Isolation of soybean bacterial endophytes

The bacterial endophytes were isolated from the stem and root of soybean plants cultivated in diverse agroecological regions within Karnataka (KA). In total, 43 bacterial endophytes were collected from various zones across Karnataka (refer to Table 1), encompassing the North East Transition Zone (Bidar) and North Transition Zone (Dharwad)**.** Since morphological characteristics play a crucial role in taxonomic classification, attributes such as texture, margin, form, elevation, and colour of the bacteria were examined (Table 2). To isolate endophytic bacteria, soybean plants were collected during the flowering stage. The fresh root and shoot samples underwent a thorough washing with running tap water to eliminate any adhering soil particles, then immersed in 70% ethanol for one minute. The plant samples were then treated with 1% sodium hypochlorite for five minutes and then rinsed in 70% ethanol for another one minute. Finally, the samples underwent three rinses with sterile distilled water, each lasting for two minutes, before being left to air dry. Post-pretreatment, the samples were homogenized in sterile distilled water using a sterilized mortar and pestle. The root and shoot samples were crushed separately using two distinct mortar and pestle sets. About 1 ml of the crushed sample was serially diluted, and 1 ml (equivalent to 1000 microlitres) of aliquots from dilutions ranging between 10^{-1} to 10^{-6} were evenly spread onto King's B medium plates (Composition: Protease peptone-20.0 g, K_2HPO_4 (anhydrous) -1.5 g,Glycerol- 10.0 ml, Agar-18.0 g, $MgSO_4.7H_2O$-1.5 g, pH- 7.2) using a sterilized glass L-rod. This plating process was carried out in triplicates, and all plates were then kept in an incubator at 28°C for 48 hours. Following incubation, distinct bacterial colonies displaying different colour, texture, consistency, and size variations were selected and streaked onto nutrient agar medium using the streak plate method. These plates were once again incubated at 28°C for 48 hours. A subset of representative isolates was chosen from each sample based on the differences in cultural morphology for further investigations.

2.3Assessing growth inhibition percentage of the *C. truncatum* by the potential bacterial endophytes

The antagonistic potential of 42 bacterial endophytes against *C. truncatum* was assessed using the dual culture method, following the procedure outlined by Dennis and Webster [17]. On Soybean Casein Digest Agar (Tryptone Soya Agar) (Composition- Tryptone- 15gm/lit, soya peptone- 5.0gm/lit, sodium chloride- 5.0gm/lit, agar- 15gm/lit)medium. Petri dishes were filled with approximately 20ml of Soybean Casein Digest Agar and allowed to solidify. A sterile cork borer was used to cut a 5 mm agar disc from the fungal pathogen, which was then placed near the periphery of the SCA plate. The bacterial isolate was aseptically streaked at the centre. Plates without an antagonist were used as controls for the pathogen. The incubation occurred at 25 ± 10°C for seven days, and two replications were maintained for each treatment. The magnitude of antagonistic activity demonstrated by the biocontrol agents upon contact with the pathogen was assessed by recording the colony diameters in two directions, and an average

was in both the dual culture plate and the control plate. The calculation of the growth inhibition percentage followed the formula outlined by Vincent[18]:

$$I = (C - T)/C \times 100$$

Here,

I represent the percentage inhibition of mycelium growth, C denotes the linear growth of the fungus in the control group (in centimetres), and T the linear growth of the fungus in the treatment group (in centimetres).

2.4Molecular characterization of endophytic bacteria

We have identified distinct bacterial endophytes using 16s rRNA sequencing due to their ubiquitous presence, conserved segment, and appropriate size (1500 bp) suitable for computational analysis. Typically, bacterial DNA is extracted using the CTAB (cetyltrimethylammonium bromide) techniqueRajput et al.[19].The purity and amount of genomic DNA wereestimated in the nanodrop.The polymerase chain reaction was used to amplify the 16S rRNA gene, employing universal primers: 41f (5'-GCTCAAGATTGAACGCTGGCG-3') and 1488r (5'-GTTACCTTGTTACGACTTCADD-3'), following the method outlined by Maatallah et al.[20]. A 30 µl PCR reaction mixture consisting of Nuclease free water of PCR grade nuclease - 9.0 µl,2 x multiplex PCR master mix - 15.0 µl,1.5 µl of Forward primer,1.5 µl of Reverse primer, BSA-1.5µl, Genomic DNA 1.5 µl was prepared.The samples were amplified using a Polymerase Chain Reaction (PCR) technique that included 5 minutes of initial denaturation at 94 °C, 30 seconds of annealing at 58 °C, and 1 minute and 30 seconds of elongation at 72 °C. This cycle was repeated 34 times before stopping at 4°C. The PCR products were then separated on a 1% agarose gel at 90 volts. Using the BLAST tool, the resultant sequences were matched to the GenBank database, and a multiple sequence alignment was performed using CLUSTAL W. Finally, the bacterial endophytes' DNA sequences were submitted to the NCBI database to obtain accession numbers.

2.5 Data Analysis

The data was statistically analyzed using R Studio. Graphs were prepared using GraphPad Prism software (San Diego, CA, USA).

3. RESULTS

3.1 Diversity of soybean bacterial endophytes

We focused on the antagonistic bacterial endophytes isolated from diverse agroecological zones within Karnataka. A total of 43 bacterial endophytes were isolated from the stem and root tissue of soybean plants from different locations throughout Karnataka.The diversity of bacterial endophytes was calculated using the Shannon-Weiner Diversity Index and Simpson Index. According to our findings, the agroecological zones had a significant ($p<0.001$) effect on endophytic bacterial diversity. TheShannon diversity indexfor root and shoot was significantly higher in the north transition zone region (1.56, 1.62).Similarly, The Simpsons- weiner diversity

index for root and shoot was significantly higher in the north transition zone region (0.42, 0.47)(Table 1).

3.2 Identification of potential endophytic bacterial through streak plate technique

The endophytic bacterial isolates collected from different agroecological zones had shown differential inhibition potential. Moreover, within the same region, the inhibition potential differed for the different bacterial isolates against *C. truncatum*. Among 20 bacterial endophytes isolated from the north transition zone region, maximum antifungal activity against *C. truncatum.* 85.00 % was obtained from BID-2 and BID-13, which was not showing significant with isolate BID-14(82.50%), BID-15(75.00 %) and BID-16(75.00 %) and showing significant higher than remaining isolate (Fig-1). Among 23 bacterial endophytes isolated from the northeasttransition zone region, maximum antifungal activity against *C. truncatum*87.00 % was obtained from DHW-9, which was not showing significant with isolate DHW-15 (80.00%), DHW-18 (67.00 %) and DHW-23 (67.00 %) and showing significantly higher than remaining isolate (Fig 2).

3.3Morphological characteristics of bacterial endophytes

The morphological characteristics of bacterial endophytes were detected, such as texture-circular, irregular, margin- entire,undulate, elevation-flat, convex, elevated, and colour- yellow, white, and so on (Table 2). The North Transition Zone showed55% and 45% circular and elevated forms, respectively. Additionally, all the endophytes had an entire shape margin and had elevation raised 35 %, convex 25%, flat 25 % and elevated 15 %. Similarly, the colourswereoff-white 35%, white 30%, dark-off white 15%, yellow 15%, and transparent 5%.In the North East Transition Zone, we found circular, thread and irregular forms were 52.17 %, 4.34 %, and 43.47 %, respectively. Similarly, the bacterial margin was of entire 42.17 %, undulate 39.13 %,bunch 4.34 %, and lobate 4.34 % type. Moreover, the elevation was raised by 43.47 %, flat 30.43 %, and convex 26.08 % type.The bacterialcolours were off-white type 30.43 %, white 21.73 %, transparent 21.73 %, yellow 13.04 %, pink 8.69 % and light pink 4.34 %(Table 2).

3.4Endophytic Bacteria Molecular Characterization

The strains DHW-9 and BID-2 were characterized through 16S rRNA gene sequencing. Subsequently, the obtained amplicons were sequenced, and the resulting nucleotide sequences were deposited in GenBank (NCBI accession codes OR616566 and OR616569). Analysis of the nucleotide sequences, including homology and BLAST analysis, confirmed that DHW-9 corresponds to *Stenotrophomonasmaltophilia* strain P4-32, while BID-2 corresponds to *Alcaligenesaquatili* strain BC5 (Fig 4).

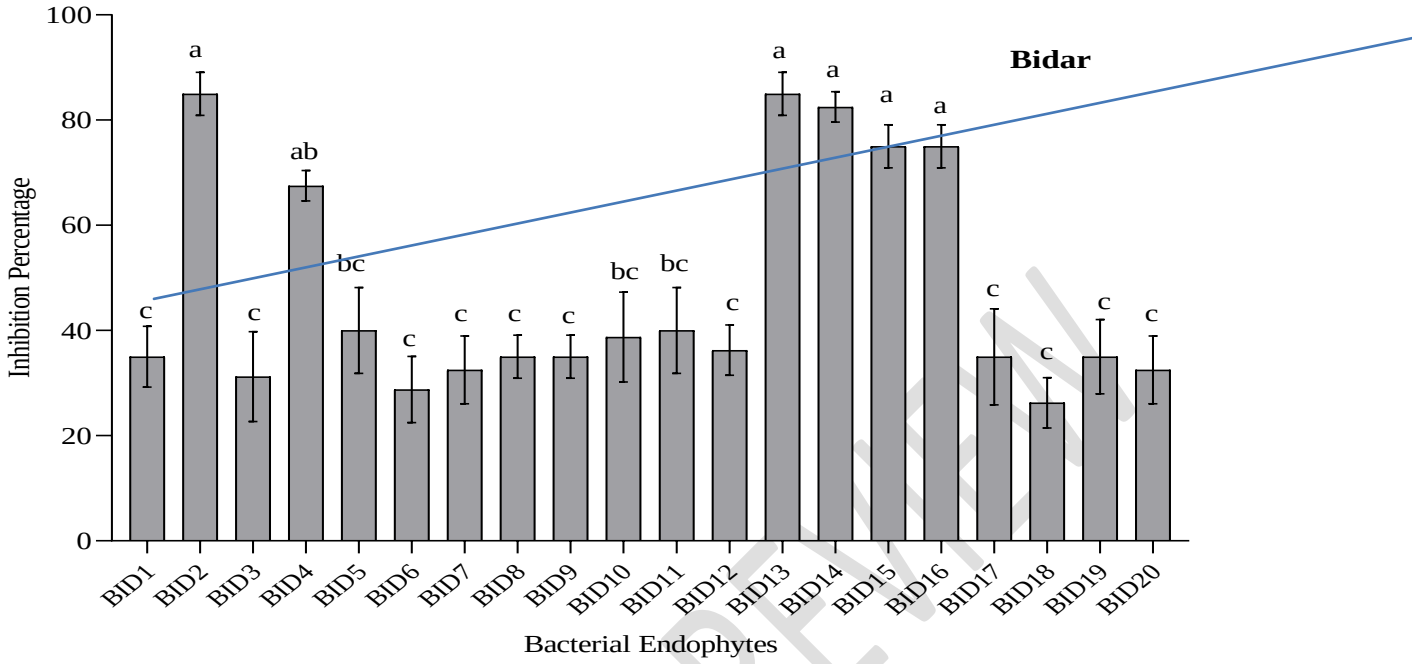

Fig 1. *In-vitro* evaluation for the antifungal activity of bacterial endophytes against *C. truncatum*of Bidar.

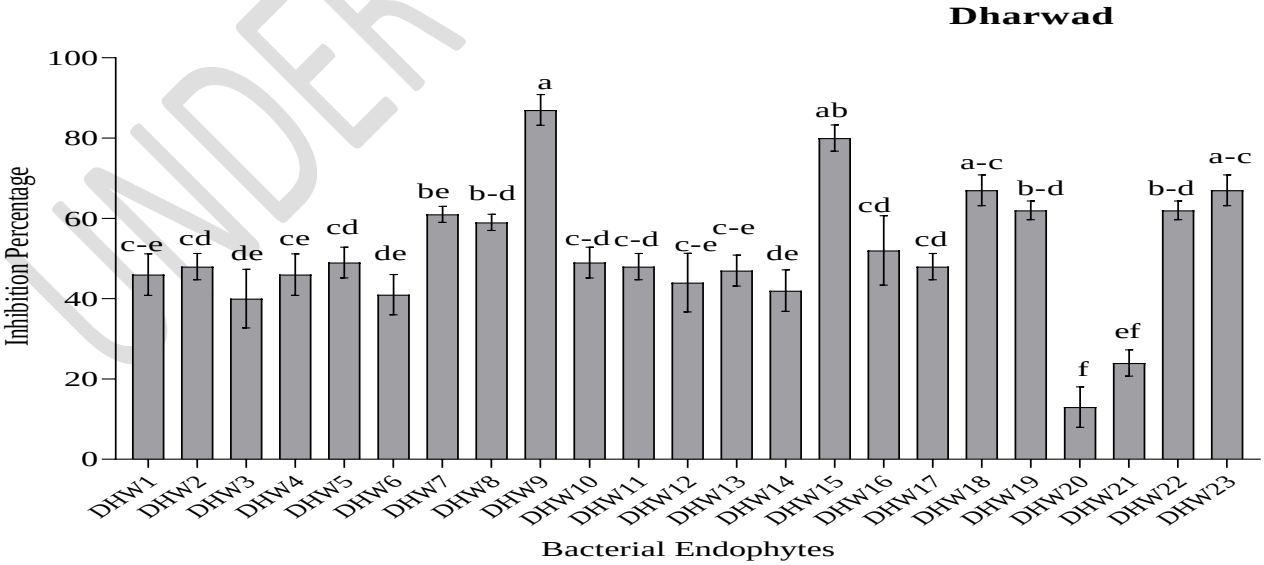

Fig 2. *In-vitro* evaluation for the antifungal activity of bacterial endophytes against *C. truncatum*ofDharwad

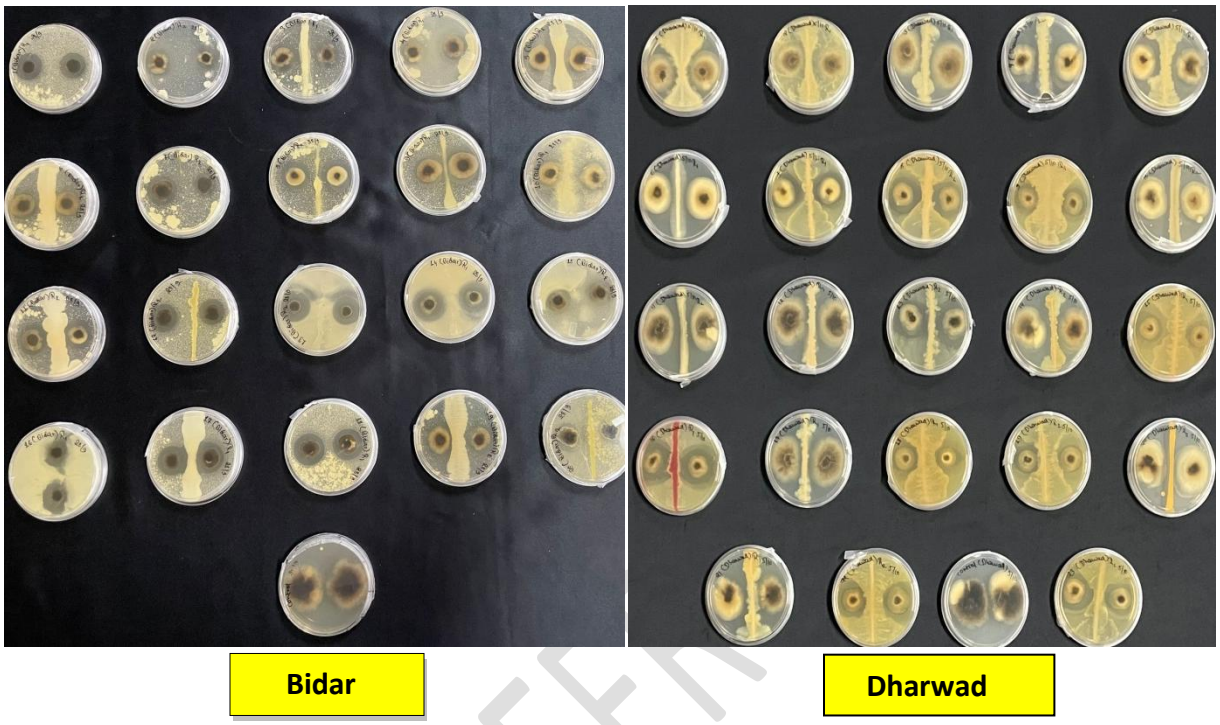

Fig 3. *In-vitro* evaluation for the antifungal activity of bacterial endophytes against *C. truncatum.*

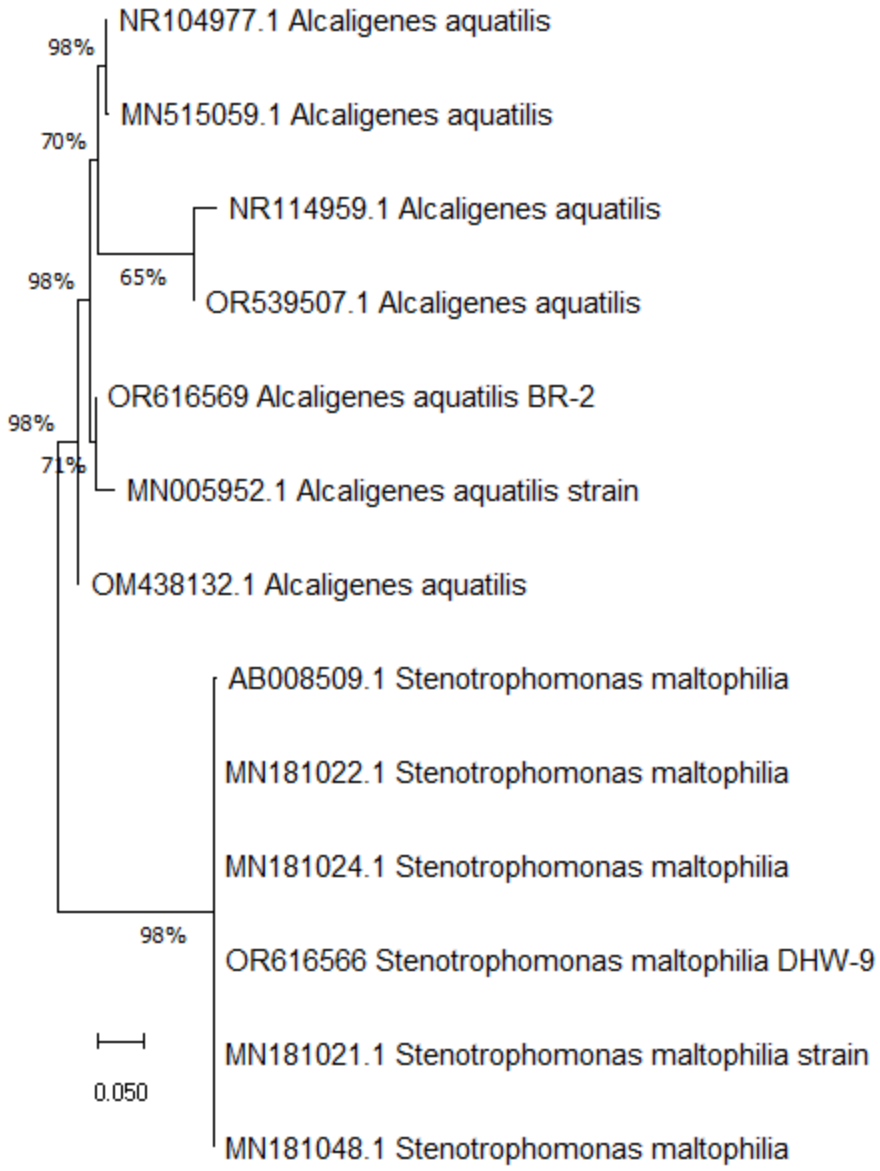

Fig 4 The phylogenetic relationships by neighbor joining method of the *Stenotrophomonasmaltophilia* strain P4-32 (DHW-9) and *Alcaligenesaquatilis* strain BC5 (BID-2) based on 16SrRNA gene analysis. Evolutionarydistances were calculated using the Mega-11 software.

Table 1. The diversity index of soybean growing agroecological region by Shannon-Weiner diversity and Simpson's index method.

Region	Shannon Weiner Diversity		Simpsons index	
	Shoot	Root	Shoot	Root
NorthTransition Zone	1.56[a]	1.62[a]	0.42[a]	0.47[a]
North East Transition Zone	1.28[b]	1.45[b]	0.31[b]	0.30[b]

Table 2. Morphological characteristics of bacterial endophytes isolated from different agroecological zones of Karnataka state.

Region	Morphology	Shape	Percentage	Region	Morphology	Shape	Percentage
North East-Zone	Form	Circular	55	North East Transition Zone	Form	Circular	52.17
		Elevated	45			Thread	4.34
		Entire	100			Irregular	43.47
	Margin	Raised	35		Margin	Entire	52.17
	Elevation	Convex	25			Bunch	4.34
		Flat	25			Undulate	39.13
		Elevated	15			Lobate	4.34
		White	30		Elevation	Convex	26.08
	Colour	Off-white	35			Flat	30.43
		Transparent	5			Raised	43.47
		Dark off-white	15		Colour	Off-white	30.43
		Yellow	15			Pink	8.69
						White	21.73
						Transparent	21.73
						Yellow	13.04
						Light pink	4.34

4.DISCUSSION

Anthracnose, caused by various lineages of the hemibiotrophic fungus *Colletotrichumtruncatum,* poses a significant threat to soybean productivity. While anthracnose losses have been overlooked, they could affect up to 50% of grain production. The robust survival of C. truncatum has impeded the effective management of this disease, the absence of suitable resistant soybean varieties, and the persistence of conventional fungicides in seed treatments. Therefore, there is a pressing need to explore alternative, environmentally sound approaches for managing anthracnose, including the potential use of biocontrol agents. Considering these challenges and the ecological aspect, our study aimed to identify a potent

biocontrol agent against *C. truncatum* and assess its effectiveness in disease management and its capacity to promote plant growth. In our research, 43 bacterial endophytes were isolated from the stem and root tissue of soybean plants from different locations throughout Karnataka, from the north transition zone and northeast transition zone region. The diversity index for root and shoot was significantly higher in the north transition zone region. Our results indicated that soybean roots have more diverse endophytes rather than the aerial part (shoot) of the soybean plant.Almida et al. [21] observed variations in bacterial populations across different plant tissues and cultivars in all of their examined field sites. Besides plant tissues and genotypes, environmental factors like temperature and soil type were identified as potential influencers of the endophytic bacteria. In a separate investigation by Egamberdiyeva and Hoflich[22], focused on plant growth-promoting bacteria isolated from wheat grown in diverse soil and temperature conditions, it was found that *Mycobacterium* sp*., P. fluorescens,* and *P. agglomerans* obtained from a semi-continental climate notably enhanced the root and shoot growth of winter wheat at 16°C as opposed to 26°C in loamy sand.In their 2022 study, Sharma et al. [23] selected *Cordiadichotoma*, a medicinal plant indigenous to India's Jammu region, to isolate and characterize culturable endophytic bacteria and assess their antibacterial attributes. Established surface sterilization protocols were employed to isolate 33 visibly distinct endophytic bacteria from various parts of the plant, including the roots, stem, and leaves. Notably, the diversity index was considerably higher for the roots compared to the stem and leaf tissues within these plant parts.

In their 2021 estimation, Pacheco et al.[24] isolated a total of 315 bacterial endophytes from leaves, roots, and stems. These endophytes were subsequently characterized through 16S ribosomal gene sequencing. Among the identified genera, Stenotrophomonas, Bacillus, and Pseudomonas were the most prevalent. Each examined tissue revealed distinct bacterial species found throughout the plant, including S. maltophilia, Paenibacilluscastaneae, and P. fluorescens. While there were no significant disparities in biodiversity indices, it was observed that root tissues had the highest abundance of bacterial endophytes.

These findings showed that bacterial endophytes isolated from healthy soybean tissues from shoots and roots suppressed the mean growth of the soybean plant disease *C. truncatum*. Seven of the 43 bacterial endophytes effectively prevented the growth of *C. truncatum*. The streak plate examine showed that DHW-9 (*Stenotrophomonasmaltophilia* strain P4-32) was the most effective against *C. truncatum* of the seven endophytes tested. Furthermore, bacterial endophytes produce cell wall-degrading enzymes such as cellulase, 1, 3-glucanase, and chitinase, which are responsible for their mycoparasitic abilities Singh and Gaur.[25]. Likewise, Feng et al. [26] found that the endophytic bacteria *Bacillus siamensis* WB1 demonstrated greater efficacy against fourteen distinct plant diseases. It also notably the germination of spores related to a disease that affects walnut trees, known as walnut anthracnose (*Colletotrichumacutatum)*. *Bacillus siamensis* WB1 induces siderophores and indole-3-acetic acid, as well as extremely efficient chemicals such as antifungal lipopeptides and extracellular hydrolytic enzymes.In a 2013 study, Abraham et al.[27] identified six bacterial endophytes from different parts of *Heveabrasiliensis* plants, including petioles, leaves, and root tissues. Notably, the bacterial endophyte EIL-2 exhibits a 62-64% inhibitory effect on *PhytophthoraMeadii* disease. EIL-2 was identified as *Alcaligenes sp*., while the other isolates

were classified as *Pseudomonas sp.* In Soumyamol et al.'s [28] research from 2023, these findings validate that the five identified bacterial isolates from the roots and leaves of rubber plants. Among these, *Stenotrophomonas* sp., when subjected to dual culture testing, demonstrated an impressive 62 percent inhibition of radial growth, which exhibits growth-promoting traits and holds promise as a biocontrol agent against *Colletotrichum spp*., a highly destructive pathogen affecting rubber crops. These bacterial endophytes were identified using a combination of 16S rRNA gene sequencing and biochemical study. Siderophore generation, cellulase production, citrate utilization, and oxidase production were all positive features in all five isolates. Similarly, the genus Stenotrophomonas has been employed as a potential microbial agent for biocontrol against various plant diseases that cause damage to commercial crops, as identified by Berg et al.[29] in 2002. As per the findings of Ryan et al.[30] in 2009, *Stenotrophomonas* sp. has shown antagonistic effects on plant diseases, along with exhibiting properties that promote plant growth. Certain species within the Stenotrophomonas genus, notably *Stenotrophomonasrhizophilia* and *Stenotrophomonasmaltophilia*, have shown favourable interactions with plants. This genus is gaining prominence as a source of biofertilizers. It is recognized for its production of osmoprotective compounds that play pivotal roles in cellular processes like DNA replication and cellular metabolism and serve as potent enzyme stabilizers Kumar et al. [31]. In their recent study, Ray and researchers [32] aimed to employ heat-treated *S. rolfsii* to induce the production of antifungal chemicals in the endophytic bacterium *Alcaligenes faecalis* BHU12. *In vitro* experiments revealed that the cell-free supernatant obtained from cultivating BHU 12 in the presence of freeze-crushed and autoclaved *S. rolfsii* substantially led to hyphal degeneration of *S. rolfsii* and suppression of sclerotial germination.Likewise, Fairman et al. [32] discovered that the *Stenotrophomonasmaltophilia* strain UPMKH2 effectively suppressed the development of rice blast disease and subsequently enhanced crop yield and its potential as a viable biocontrol agent.

5. CONCLUSION

This study shows strong evidence supporting the effectiveness of bacterial endophytes in controlling *C. truncatum* infection, which leads to anthracnose disease in soybean plants.The involvement of endophytes in stimulating plant defences and enhancing the growth of soybean plants. Our findings highlight the potential of DHW-9, followed by BID-2, BID-13, DHW-15,BID-14, BID-15, and BID-16,in inhibiting pathogen growth,possibly through the production of antifungal compounds and CWD enzymes. Further research should explore their effectiveness in real-world farming conditions, their impact on plant growth, and the specific mechanisms by which they counteract pathogens. This will improve our comprehension and utilization of endophytes as highly efficient agents in managing plant diseases.

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