



RESEARCH ARTICLE

Characterisation of a brinjal-associated bacterial endophyte with antagonistic potential against *Ralstonia solanacearum*

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Abstract

Bacterial wilt, caused by *Ralstonia solanacearum*, is a major constraint to brinjal production and requires the implementation of sustainable management strategies. In the present investigation, a bacterial endophyte, KnEB54, was isolated from the tissues of healthy brinjal plants and assessed for antagonistic efficiency against *R. solanacearum*. Morphologic examination of KnEB54 revealed smooth circular, cream-coloured, small colonies. Biochemical profiling using the VITEK® 2 compact automated bacterial identification system revealed a unique metabolic profile with the ability to utilise a wide range of carbohydrates and various enzymatic activities, which is evidence of high ecological adaptability. Molecular identification by 16S rRNA sequencing revealed 98.64 % similarity with *Enterobacter soli*. *In vitro* antagonistic assays confirmed that KnEB54 effectively suppressed the growth of *R. solanacearum*, indicating its potential as a biocontrol agent. These results have demonstrated that the endophytic *E. soli* strain KnEB54 is a promising isolate for biological control of bacterial wilt of brinjal. Nevertheless, to establish its practical relevance in sustainable agriculture, comprehensive greenhouse and field trials, along with detailed investigations into its functional attributes, remain imperative.

Keywords: bacterial wilt; biocontrol; endophyte; *Ralstonia solanacearum*

Introduction

Bacterial wilt, caused by *Ralstonia solanacearum* species complex, is one of the most devastating plant diseases and represents a globally significant threat to agricultural productivity, characterised by its ability to colonise the vascular tissues of a diverse array of host plants (1). The disease inflicts severe yield losses, with annual losses estimated to exceed USD 1 billion, significantly impacting global crop productivity (2). The pathogen has a broad host range of more than 450 plant species such as tomato, chilli, potato and brinjal (3, 4). Conventional methods of managing this disease include mostly the use of antibiotics, such as streptomycin and tetracycline, but there are multiple limitations of the practice, including the emergence of antimicrobial resistance, as well as environmental and health risks. Therefore, biological management practices can be used as a substitute for chemical management in sustainable agriculture. These biocontrol agents provide host plants with broad-spectrum benefits of nitrogen fixation, antimicrobial compounds, plant growth promoters and induction of immune resistance in plants (4).

Endophytes are microorganisms that reside inside plant

tissues without causing significant damage to the plant. These microorganisms reside entirely within plant tissues and grow in the roots, stems and may also be present in the leaves of the host plant without causing any apparent damage (5). Bacterial endophytes may compete for an ecological niche that resembles that of *R. solanacearum*. Thus, they can be used as biocontrol agents against bacterial wilt disease. In addition to this, these microorganisms can confer advantages to the host by promoting growth, enhancing nutrient acquisition, producing phytohormones, secreting antimicrobial metabolites, competing for ecological niches and stimulating host defence responses (6).

Previous research has established the association of endophytic bacteria with brinjal and other crops, demonstrating their significant potential to enhance plant growth and manage soil-borne pathogens (7). Endophytes are recognised for their ability to suppress pathogens through multiple mechanisms. These include the production of antimicrobial metabolites, competition for nutrients and ecological niches and the induction of host defence responses. This is supported by evidence of endophytic bacterial

colonisation and the suppression of *R. solanacearum* in eggplant (8). Despite these advancements, there remains a lack of detailed strain-specific characterisation of endophytic isolates, particularly concerning their functional characteristics, antagonistic efficacy and the underlying mechanisms of action. Furthermore, there is limited information regarding their ecological fitness and field applicability in the literature.

Therefore, it is essential to comprehensively understand the morphological, biochemical and functional attributes of individual endophytic strains to assess their potential as effective and sustainable biocontrol agents in agriculture. In this context, the present study was conducted to describe a bacterial endophyte associated with brinjal and evaluate its antagonistic potential against *R. solanacearum*.

Materials and Methods

Isolation of endophytic bacteria

Healthy brinjal plants were collected from the farmers' field (20°12'8.96" N, 83°54'45.51" E) and brought to the laboratory of the Department of Plant Pathology, Odisha University of Agriculture and Technology (OUAT). The plants were washed under running tap water to clean soil and dirt and then cut into 1 cm pieces from leaf, stem and root. These cut pieces were sterilised in by sequential immersion in ethanol 70 % for 1 min, 1 % sodium hypochlorite NaOCl for 3 min and 70 % of ethanol for 30 sec, followed by three washes in distilled water and blotted dry on sterile filter paper under aseptic conditions. The surface-disinfected tissues were subjected to endophytic microbiome extraction by macerating in a pre-sterile pestle-mortar in a laminar flow cabinet. Macerated tissues were collected in 100 mL tubes and were serially diluted up to 10^{-5} following the 10:1 dilution method. Aliquots of 1.0 mL from each serial dilution (10^{-1} to 10^{-5}) of each biological replicate were plated in triplicate using the pour plate method. For each plate, 1.0 mL of the diluted sample was aseptically transferred into a sterile petri dish, followed by the addition of approximately 15–20 mL of molten nutrient agar, cooled to approximately 45 °C. The plates were gently swirled to ensure uniform mixing and allowed to solidify. All plates were incubated at 28 ± 2 °C for 48 hr. Colonies were differentiated based on size, colour and morphology, with each morphotype counted separately. A single representative colony from each morphotype was isolated and purified by streaking on fresh nutrient agar plates.

Isolation of the pathogen

Brinjal plants exhibiting bacterial wilt symptoms were collected from the All India Coordinated Research Project (AICRP) farm at OUAT, located at 20°16'34.63" N, 85° 47'7.12" E and transported to the laboratory of the Department of Plant Pathology, OUAT. The diseased plants were first washed with tap water, cut a few inches above the root on the stem, placed in clear sterile water and examined for any oozing. The bacterial ooze from the cut ends was streaked onto nutrient agar (NA) plates, which were incubated at 28 °C for a period of 24 hr. Bacterial ooze from the cut ends was streaked onto NA plates and incubated at 28 °C for 24 hr. Following isolation, the *R. solanacearum* isolates were purified by streaking a single colony from each isolate onto triphenyl tetrazolium chloride (TZC) medium (9). These isolates were then sub-cultured in TZC slants and incubated at 28 °C for 48 hr.

In vitro evaluation of isolated endophyte bacteria against *Ralstonia solanacearum*

The antibiotic activity of the isolated endophytic bacteria against the *R. solanacearum* isolate was evaluated in the laboratory using a disc diffusion assay with slight modifications (10). A 1 mL suspension of *R. solanacearum* (10^7 – 10^8 colony-forming units (CFU)/mL) was prepared by suspending a loopful of bacteria, which had been cultured on TZC agar for 48 hr at 28 °C, in 10 mL of sterilised distilled water. This suspension was then mixed with 10 mL of melted nutrient agar at 55 °C. The agar mixture was poured into a petri dish, allowed to solidify and air-dried. A sterile filter paper disc (0.5 cm in diameter) was dripped with 50 µL of the endophytic bacteria suspension (10^7 – 10^8 CFU/mL) and placed at the centre of the agar and filter papers treated with sterile distilled water served as controls. After incubating at room temperature (28 ± 2 °C) for 24 hr, the diameter of the inhibition zone created by the endophytic bacteria was measured.

Morphological and biochemical characterisation of the endophytic bacteria

The isolated endophyte bacteria were cultured in nutrient agar medium and incubated at 28 °C for 24 hr. Then, the single colony characters were carefully observed. Biochemical analysis was done using the VITEK® 2 compact automated bacterial identification system (BioMérieux, France). Isolated colonies were streaked on nutrient agar plates and incubated at 28 °C for 24–48 hr for obtaining pure cultures. Gram staining procedure was performed (Smith and Hussey, 2005) and based on the Gram reaction, an appropriate VITEK identification card (gram-negative, gram-positive, or Bacillus) was chosen. Pure bacterial culture was inoculated into 3 mL of sterile VITEK saline (0.45 % NaCl) and swirled to achieve a uniform suspension of colonies. A DensiCHEK turbidity meter was used to adjust the inoculum density to 0.50–0.63 McFarland. The VITEK® 2 bacterial suspension was loaded into VITEK® 2 cassettes, which were then placed in the VITEK® 2 compact system for automatic filling, incubation and identification. The system analysed the biochemical reactions performed by the isolate KneB54.

Molecular characterisation of the endophytic bacteria

Genomic DNA was extracted from pure bacterial broth cultures using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Catalogue No. D6005, from Zymo Research) following the manufacturer's protocol. To amplify the 16S rRNA gene, the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. Polymerase chain reaction was performed using a thermal cycler (Applied Biosystems MiniAmp plus, USA) and a 25 µL reaction mixture was prepared following the protocols described in previous studies, with slight modifications, including the addition of 10 pmol each of forward and reverse primers, 2.5 mM MgCl₂, 200 µM each of the four deoxyribonucleotide triphosphates (dNTPs), 0.5 U of Taq DNA polymerase, 1 × concentration of PCR buffer (Invitrogen, Life Technologies, Brazil) and 50–100 ng of isolated bacterial genomic DNA. The template was denatured by heating at a pre-temperature of 95 °C for 5 min. This was followed by 39 cycles of denaturation, 30 sec at 94 °C, annealing 45 sec at 59 °C and 1 min elongation at 72 °C, with a final extension of 7 min at 72 °C. The amplified DNA was separated by electrophoresis in an 0.8 % agarose gel run in 1 × Tris–Acetate–Ethylenediaminetetraacetic acid (TAE) buffer at 50 V for 30–45 min until the DNA fragments were well migrated. The gel was photographed using a gel documentation system. Amplified

16S rRNA gene products were purified using the PureLink Quick PCR Purification Kit (Invitrogen, USA, Catalogue number: K310001) following the manufacturer's protocol (https://documents.thermofisher.com/TFS-Assets/LSG/manuals/purelink_pcr_man) and quantified using a nanodrop spectrophotometer (Eppendorf, Germany). The PCR products were subjected to Sanger sequencing and the obtained sequence was subjected to basic local alignment search tool (BLAST) analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The 16S rRNA gene sequence was deposited in the GenBank database (<https://submit.ncbi.nlm.nih.gov/subs/genbank/>) and an accession number was obtained.

Results

Isolation of endophytic bacteria and pathogens

Endophyte bacteria were isolated from the leaves of the collected brinjal plants and designated as KnEB54. A bacterial pathogen was isolated from the collected bacterial wilt samples on triphenyl tetrazolium chloride (TZC) medium and designated as ODRS8.

In vitro evaluation of isolated endophyte bacteria against *Ralstonia solanacearum*

The isolated endophyte bacterium was evaluated for its antagonistic activity against the bacterial wilt pathogen (Fig. 1) and the inhibition zone was recorded to be 40.5 mm (Mean of three replications).

Morphological characterisation of endophytic bacteria

The colonies of isolate KnEB54 were small, smooth, circular and cream coloured (Fig. 2) and were gram-negative. The biochemical profile of isolate KnEB54 (Table 1) showed a metabolically active bacterium with a high ability to consume wide range of carbohydrates and amino acid substrates. The isolate showed positive reaction for several sugars, including D-glucose, D-maltose, D-mannitol, D-mannose, sucrose, D-trehalose and D-sorbitol, as well as enzyme activities, such as the hydrolysis of a (2,3,5,6,1,3)-galactosyl-(1 > 6)- α -glucanase, the beta-N-acetyl-glucosaminidase and the γ -glutamyl transferase, indicating the efficient utilisation of carbohydrates and the hydrolysis of glycosides. Positive reactions for urease, citrate utilisation and decarboxylase activities (ornithine and general decarboxylase base) were an

Table 1. Biochemical characterisation of potential bacterial endophyte bacteria KnEB54 using VITEK® 2

Sl. No.	Biochemical details	Abbreviation	KnEB54
1	Ala-Phe-Pro-Arylamidase	APPA	–
2	Adonitol	ADO	–
3	L-Pyrrolydonyl-Arylamidase	PyrA	–
4	L-Arabitol	IARL	–
5	D-Cellobiose	dCEL	+
6	Beta-Galactosidase	BGAL	+
7	H ₂ S Production	H ₂ S	–
8	Beta-N-Acetyl-Glucosaminidase	BNAG	+
9	Glutamyl Arylamidase pNA	AGLTp	–
10	D-Glucose	dGLU	+
11	Gamma-Glutamyl-Transferase	GGT	+
12	Fermentation/Glucose	OFF	+
13	Beta-Glucosidase	BGLU	–
14	D-Maltose	dMAL	+
15	D-Mannitol	dMAN	+
16	D-Mannose	dMNE	+
17	BETA-Xylosidase	BXYL	+
18	BETA-Alanine Arylamidase pNA	BAlap	–
19	L-Proline Arylamidase	ProA	+
20	Lipase	LIP	–
21	Palatinose	PLE	–
22	Tyrosine Arylamidase	TyrA	+
23	Urease	URE	+
24	D-sorbitol	dSOR	+
25	Saccharose/Sucrose	SAC	+
26	D-Tagatose	dTAG	–
27	D-Trehalose	dTRE	+
28	Citrate (Sodium)	CIT	+
29	Malonate	MNT	–
30	5-Keto-D-Gluconate	5KG	–
31	L-Lactate Alkalinisation	ILATk	+
32	Alpha-Glucosidase	AGLU	+
33	Succinate Alkalinisation	SUCT	+
35	Phosphatase	PHOS	–
36	Glycine Arylamidase	GlyA	+
37	Ornithine Decarboxylase	ODC	+
38	Lysine Decarboxylase	LDC	–
39	Decarboxylase Base	ODEC	+
40	L-Histidine Assimilation	IHISa	–
41	Coumarate	CMT	–
42	Beta-Glucuronidase	BGUR	–
43	O/129 Resistance (comp. vibrio.)	O129R	+
44	Glu-Gly-Arg-Arylamidase	GGAA	–
45	L-Malate Assimilation	IMLTa	–
46	Ellman	ELLM	–
47	L-Lactate Assimilation	ILATa	+



Fig. 1. Antagonistic activity of KnEB54 against *Ralstonia solanacearum*.



Fig. 2. Pure culture of endophytic bacteria KnEB54.

additional indication of its adaptability to different nutrient conditions. However, the isolate was negative for several arylamidases, lipase, H_2S production, as well as utilisation of some substrates, such as adonitol, arabitrol and malonate, suggesting selective metabolic capabilities. Overall, this biochemical pattern corresponds to a versatile endophytic bacterium showing potential functional traits for plant interactions and antagonistic activity.

Molecular analysis of the isolate KnEB54 showed distinct bands at around 1500 bp, indicating the successful amplification of the target 16S rRNA gene, which is an important marker in bacterial identification (Fig. 3). The KnEB54 isolate showed 98.64 % sequence similarity with that of *Enterobacter soli* (NCBI Accession no. PX116799). Members of this genus are increasingly known to be endophytes exhibiting plant growth-promoting properties and resistance to abiotic stress as well as biocontrol activities.

Discussion

Endophytic bacteria are important biological agents that colonise inside the plant tissues and compete for the space and nutrition of the vascular pathogen, alternatively triggering systemic resistance

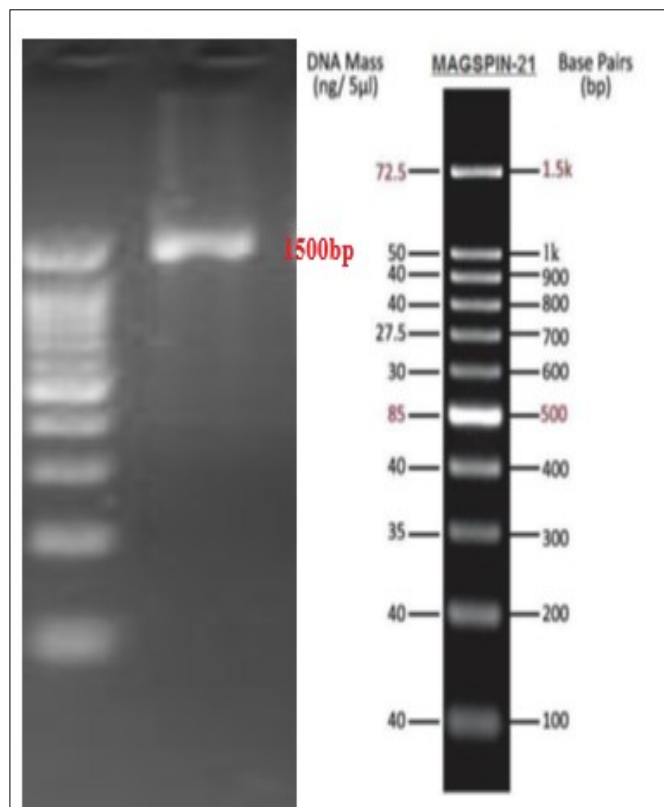


Fig. 3. Gel electrophoresis photograph of successful amplification of bacterial 16S rRNA gene of *Enterobacter soli* KnEB54 with a 100bp DNA ladder as size reference. Bands at ~1500 bp confirmed successful amplification of target sequences.

against soil-borne pathogens such as *R. solanacearum* (11). These microbial endophytes, such as *Bacillus*, *Pseudomonas*, *Enterobacter*, etc., successfully colonise internal ecological niches without inducing any deleterious physiological response, thereby enhancing the growth and drought tolerance of the plants as well as the biocontrol of vascular wilt (12). Endophytes have been found to show potential in the control of bacterial wilt in solanaceous crops through a wide range of mechanisms. These include production of antimicrobial compounds that inhibit pathogen *R. solanacearum*, triggering of systemic resistance in host plants and competition for nutrients and colonisation sites in plant tissues (13). Certain endophytic bacteria and fungi are effective in limiting the incidence of bacterial wilt in solanaceous crops such as tomatoes, potatoes and brinjal by using these various strategies (7). Isolated endophytic bacteria from the internal tissue and rhizosphere parts of brinjal plants and some isolates of *Pseudomonas* spp. displayed strong antagonistic activity against *R. solanacearum*. The present study shows that the endophytic isolate KnEB54, identified as *E. soli*, possesses significant antagonistic ability against resistant bacteria and can be used as a biocontrol agent. The inhibition observed in this study indicates that KnEB54 has strong competitive or inhibitory characteristics that could directly impair pathogen establishment, as opposed to only the endophytic suppression that has been reported in general. The results agree with the previous studies that reported *Enterobacter* spp. as antagonists of *R. solanacearum*, including *Enterobacter tabaci* UPMBC and *Enterobacter* sp. XB99R, but emphasises the value of *E. soli* as a relatively under-exploited species in this regard (12, 14).

Enterobacter sp. has been previously reported as an endophyte from many crop plants, like poplar (15), *Indigofera argentea* Burm. f. (16), banana (16), *Ocimum sanctum* L. (17) and legumes (6). However, very few reports have been there as

Enterobacter spp. as an endophyte from brinjal plants. The occurrence of *Enterobacter* spp. in the vascular tissues implies its potential to colonise in the internal tissues of the plant, similar to that of the pathogen *R. solanacearum*, thereby imparting a competitive advantage for the suppression of disease. This localisation enhances its potential as a biological control agent for combating bacterial wilt pathogens like *R. solanacearum*.

In this study, the isolated endophytic bacteria KnEB54 were identified as *E. soli* and exhibited good antagonistic activity against *R. solanacearum*. These findings are in line with previous findings where *E. tabaci* UPMBC1 was isolated from brinjal tissue and showed potential antagonistic activity against *R. solanacearum* (14). Similarly, previous researchers isolated *Enterobacter* sp. XB99R, which exhibited the biocontrol potential against *R. solanacearum* (8). The antagonistic activity observed in this study may be explained by multiple mechanisms generally linked to endophytic bacteria. These include production of antimicrobial compounds, competition for nutrients and colonisation sites and induction of systemic resistance in the host plant (13). Another important mechanism is the potential induction of systemic resistance in the host plant, where the endophytes stimulate the plant defence pathway, which increases the resistance against invading pathogens. Endophytic bacteria have been reported to activate induced systemic resistance (ISR) by means of signalling pathways involving jasmonic acid and ethylene, which results in increased plant defence responses (18). Moreover, the capacity of the endophytic bacterium to inhabit in the internal tissues, particularly in the vascular bundles, provides a strategic advantage, as it directly interacts and competes with *R. solanacearum*. This space overlap may help in the exclusion and suppression of niches during early development stages of pathogen invasion. Generally, these approaches indicate that KnEB54 is a holistic approach to suppressing *R. solanacearum* and has the potential to be used as a biological control agent.

Biochemical characterisation of KnEB54 revealed a metabolically versatile organism that is able to use a large variety of carbohydrates and express several enzymatic activities. Ability to use different substrates, positive reaction for enzymes such as α -galactosidase, α -glucosidase and urease suggest its versatility in adapting to different environmental conditions. Similar biochemical versatility has been reported in *Enterobacter* spp, which have been known to comprise plant growth-promoting traits and biocontrol features (19).

Although the findings of this research are promising, it is still limited to *in vitro* studies of antagonistic activity; therefore, its effectiveness under greenhouse and field conditions has yet to be validated. Similar limitations in translating endophytic biocontrol potential from laboratory to field conditions have been widely reported in the literature (15). The endophytic isolate KnEB54 (*E. soli*) showed significant antagonistic activity against *R. solanacearum*, consistent with reports of plant growth-promoting and biocontrol potential in *Enterobacter* species (18). Future research needs to focus on validating its effectiveness under field conditions and elucidating the molecular pathways involved in pathogen suppression.

Conclusion

In this study, a bacterial endophyte (KnEB54) has been successfully isolated from healthy tissues of brinjal and characterised by morphological, biochemical and molecular methods. The isolate was identified as *Enterobacter soli* by 16S rRNA gene sequencing and its metabolic profile appeared to be diversified to provide it with a high adaptability nature and ecological fitness in the plant tissues. Importantly, the isolate showed significant *in vitro* antagonistic activity against *Ralstonia solanacearum* and suggests its potential role in suppression of bacterial wilt. The multifunctional characteristics of the isolate, such as various substrate utilisation and enzymatic activities, further highlight its potential use in sustainable disease management strategies. Overall, *E. soli* KnEB54 is a promising candidate for biological management of bacterial wilt in brinjal and adds to the knowledge about endophytic bacteria in sustainable agriculture.

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Authors' contributions

DM and MKM conceived and designed the experiments. DM, LS, SS and AM performed the experiments. DM, MKM and AD analysed the data. AM, SS, BJ and SSP contributed reagents, materials and analysis tools. DM and LS wrote the manuscript. DM, LS, SSP and BJ revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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