Siderophore-Producing *Spinacia Oleracea* Bacterial Endophytes Enhance Nutrient Status and Vegetative Growth Under Iron-Deficit Conditions

Sankalp Misra[1,2] · Pradeep Semwal[2,3] · Deen Dayal Pandey[2] · Shashank Kumar Mishra[2] · Puneet Singh Chauhan[2,3]

Abstract

Microbial-assisted biofortification has emerged as a sustainable approach to improve food security by enhancing the nutrient content as well as yield of agricultural crops. In the present study, we have examined the potential of siderophore-producing plant growth-promoting endophytic bacterial strain in improving nutrient content and vegetative growth of *Spinacia oleracea* (spinach) under iron-deficit conditions. A total of 234 isolates, 13 from the root, 15 from the stem, and 17 from the leaves were isolated and tested positive for siderophore production. In vitro, results related to plant growth-promoting (PGP) traits have revealed the endophyte *Enterobacter quasihormaechei* (NBRI FY32) isolated from Fe-deficit spinach plant exhibited multifarious PGP traits with the highest siderophore-producing ability and the only bacterial strain possessing phytase activity as well as oxalate degradation ability. Furthermore, the results from the greenhouse experiment demonstrated that, under both Fe-sufficient and -deficit conditions, inoculation with NBRI FY32 significantly improved the overall plant vegetative parameters compared to the corresponding uninoculated control. Also, NBRI FY32 inoculation has significantly enhanced nitrogen (N), phosphorus (P), potassium (K), sodium (Na), calcium (Ca), zinc (Zn), manganese (Mn), and Iron (Fe) content in plants under Fe-sufficient and -deficit conditions. In addition, NBRI FY32 showed maximum colonizing and survivability in spinach root and shoot under Fe-sufficient and -deficit conditions. Thus, the findings of this study demonstrate for the first time that siderophore-producing endophyte *E. quasihormaechei* (NBRI FY32) having multiple PGP attributes along with high competence and colonization can fortify spinach plant by enhancing nutrient levels in the host plant.

Keywords Endophytes · Nutrient content · *Enterobacter quasihormaechei* · Plant growth · Colonization

✉ Puneet Singh Chauhan
puneet@nbri.res.in; puneetnbri@gmail.com

1 Faculty of Biosciences, Institute of Biosciences and Technology, Shri Ramswaroop Memorial University, Deva Road, Lucknow, Barabanki, Uttar Pradesh 225003, India

2 Microbial Technologies Division, Council of Scientific and Industrial Research-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

3 Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India

Introduction

Biofortification of agricultural crops is one of the sustainable ways to tackle food security problems by enhancing the nutrient level in crop plants (Huang et al. 2020). In addition, biofortified crops have demonstrated a significant positive effect on human health (Praharaj et al. 2021). Among various biofortification approaches, microbe-mediated solubilization and mobilization of nutrients in the plant is recognized as one of the efficient ways to ameliorate nutrient deficiency in the human diet (Singh et al. 2017). In the past few decades, rhizospheric microorganisms have been considered to enhance the accumulation of micronutrients in different crops (Abaid-Ullah et al. 2015; Prasanna et al. 2015). However, in recent years, the focus has switched to the endophytes, which are part of the interior microbiome. Endophytic microbes reside inside the host plant tissue without causing any injury and increase the growth of plants

through indirect and direct mechanisms (Rana et al. 2021). The direct mechanisms include phytohormone production such as auxin, cytokinin, and gibberellin, ACC deaminase activity and nutrient solubilization such as phosphate, nitrogen fixation, and sequestration of iron by bacterial siderophores (Adeleke et al. 2021; Lipková et al. 2021; Rana et al. 2021). The indirect mechanisms involve the prevention of the negative effects of phytopathogenic microbes by the production of siderophores, cell wall degrading enzymes, antibiotics, hydrogen cyanide, induced systemic resistance, and quorum quenching (Macedo-Raygoza et al. 2019; Lipková et al. 2021). The exploitation of siderophore-producing plant growth-promoting bacterial endophytes could prove to be an important strategy for enhancing plant growth and nutrient content in agricultural crops. Some of the bacterial genera namely *Enterobacter*, *Bacillus*, *Acidovorax*, *Burkholderia*, *Variovorax*, *Curtobacterium*, *Pantoea*, *Pseudomonas*, *Agrobacterium*, and *Klebsiella* have been reported as plant growth-promoting endophytes (Rana et al. 2021). However, the scientific community is continuously searching for new PGP and biocontrol endophytic bacterial strains in an effort to increase plant growth and lower the necessity for agrochemicals. The exploitation of PGP endophytes capable of improving plant health and enhancing nutrient content under nutrient-deficient conditions will definitely present a major path toward sustainable agriculture. Recently, several reports have well established the function of endophytic bacterial strains toward the biofortification of different crop plants (Makar et al. 2021; Verma et al. 2021; Vishwakarma et al. 2021). In addition, bacterial endophytes enhance other phyto-beneficial attributes including photosynthetic pigments, vegetative growth, osmotic regulation, modification of root architecture, and improved mineral absorption (Cochard et al. 2022; Sundram et al. 2022). Furthermore, bacterial endophytes also play a crucial role in increasing the bioactive constituents in host plants (Li et al. 2023). Furthermore, in comparison to plant growth promoting rhizobacteria (PGPR), endophytes exhibited improved adaptations against abiotic stresses, that results in enhanced plant growth (Pillay and Nowak 1997). However, certain endophytes proved to be unsuccessful under normal environmental conditions because of their inability to colonize the host system (Bagheri et al. 2021). Therefore, for the successful application of bacterial endophytes under normal as well as stressed conditions, it is a prerequisite that bacterial endophytes should demonstrate effective colonization inside the host plant (Tharek et al. 2021).

The present study aimed to isolate and characterize PGP attributes of endophytic bacteria from spinach plants under Fe-sufficient and Fe-deficit conditions. Furthermore, there are no data available on the endophyte-mediated biofortification of spinach plants under Fe-deficit conditions. Therefore, we further aimed to compare the potential application of two endophytic bacterial strains having different siderophore-producing abilities for enhancing plant growth and nutrient content under Fe-sufficient and Fe-deficit conditions. To the best of our knowledge, this is the first report illustrating multiple PGP attributes of *Enterobacter quasihormaechei* as an endophyte for conferring plant fitness and biofortification under Fe-deficit soil conditions.

Materials and Methods

Plant Material Preparation

A loose loam soil having a pH of 7.2 and 0.9 mg Fe kg^{-1} soil of DPTA-extractable Fe was used for the pot experiments. After air drying, the soil was powdered, and chemical fertilizers were applied at the recommended rate. Five *Spinacia oleracea* L. (Spinach) seeds were sowed per pot and soil moisture was maintained at 15% (wt/wt) with deionized water. After 16 d growth, three uniform-sized seedlings were thinned per pot. The pots were divided into two groups after 30 days of development in a growth chamber. In one pot set, the leaves were sprayed with 100 mM FeEDTA solution every other day (Fe-sufficient), whereas in the other, deionized water was used (called Fe-deficit).

Sampling and Isolation of Siderophore-Producing Bacteria

After 60 d of growth, plant material (leaf, stem, and root) was sampled from each pot, washed with tap water, and surface sterilized with 5% sodium hypochlorite for 5 min followed by washing 4–5 times with sterile distilled water. The sterile samples were diluted serially in 0.85% saline solution and the final suspension was spread on agar plates with 20 mM FeCl$_3$ or 1% (v/v) CAS (chromeazurol S) indicator and incubated at 26–28 °C (Schwyn and Neilands 1987). The microbial colonies with an orange halo having a diameter > 5 mm were selected as siderophore secretors. Pure cultures of the endophytic bacterial isolates were maintained in 30% glycerol at − 80 °C.

Quantitative Estimation of PGP Attributes of Selected Isolates

Auxin Production

Auxin (IAA) production by selected isolates was determined according to Bric et al. (1991). 24 h old grown cultures were centrifuged at 10,000 rpm for 10 min followed by the addition of 100 µl of orthophosphoric acid (10 mM) and 4 ml of Salkowski's reagent in 2 ml of bacterial supernatant and

incubated for 30 min at room temperature. Absorbance was recorded at 530 nm.

Phosphate Solubilization

Phosphate solubilization was quantified by using NBRIP medium for the selected isolates as per the protocol of Nautiyal (1999). Briefly, 50 µl of bacterial culture (overnight grown in nutrient broth) was inoculated in test tubes containing 5 ml NBRI-BPB medium followed by incubation at 28 ± 2 °C on an incubator shaker (180 rpm) for 48 h. Finally, the cells were harvested at 10,000 rpm for 10 min by centrifugation and the supernatant was used to estimate the amount of solubilized phosphate spectrophotometrically at 600 nm (Nautiyal 1999).

Siderophore Production

Siderophore quantification was performed by cultivating overnight grown bacterial strains on sodium succinate medium (SSM) emended with 20 µM Fe at 26–28 °C in an incubator shaker and absorbance was measured at 400 nm (Meyer and Abdallah 1978; Sah and Singh 2016).

Biofilm Formation

Biofilm production was measured using a microtiter plate assay, as described by Srivastava et al. (2008). Briefly, bacterial cultures were grown in a microtiter plate, and the supernatant was removed. The plate was then washed with phosphate-buffered saline and dried. Next, the plate was stained with crystal violet, and the excess stain was removed with ethanol. The biofilm was then quantified by measuring the absorbance of the crystal violet at 590 nm.

Determination of Oxalate Degradation Activity

For estimation of oxalate degradation activity, selected strains were inoculated onto MRS (Hi Media, India) agar plates containing 50 mmol L^{-1} of calcium oxalate. After incubation at 28 °C for 48 h, the presence of clear areas around bacterial colonies was considered as an indication of oxalate degradation activity by the bacterial strains.

Determination of Phytase Activity

The selected bacterial endophytes were streaked on a phytase screening medium (PSM) and incubated at 28 °C for 24 h to evaluate phytase production ability. The translucent area of the plate will give a visual indication of extracellular phytase production by the bacterial strains (Kerovuo et al. 1998).

Selection and Identification of Bacterial Endophytes on the Basis of 16S rRNA Sequencing Analysis

Selected high siderophore-producing endophytic bacteria were identified based on the 16S rRNA gene sequencing analysis. For the isolation of genomic DNA, GenElute™ Bacterial Genomic DNA Kit was used. PCR was carried out in 50 µl vol containing 1X PCR buffer, 1.0 U *Taq* polymerase, 200 mM of each dNTP, 1.50 mM $MgCl_2$, 10 µM of each primer (27F: 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1492R: 5′-GGT TAC CTT GTT ACG ACT T-3′), and 20−30 ng of bacterial genomic DNA. The PCR product was purified using a QIAquick® PCR purification kit (Qiagen, Germany) followed by sequencing (3730XL DNA analyzer, Applied Biosystems, USA) (Misra et al. 2017).

Evaluation of Selected Bacterial Strains for Enhancing Vegetative Parameters and Bioavailable Content of Nutrients in Spinach Under Greenhouse Condition

Plant vegetative Parameters

Following the characterization of selected bacterial strains based on their phytase-producing and oxalate degradation ability, endophytic bacterial strains differing in siderophore producibility were evaluated for their capability of plant growth promotion using spinach as a host plant under greenhouse conditions. The experiments were carried out at the CSIR-National Botanical Research Institute, Lucknow, India (latitude/longitude 11°24'N/79°44'E), with 18 plant replicates evenly distributed in 06 pots (03 plant replicates per pot) containing 2.0 mm sieved field soil (2.0 kg soil per pot). Spinach seeds were surface sterilized using 0.1% $HgCl_2$, followed by thorough washing with sterile distilled water and bacterized as described by Nautiyal (1997). The following were the bacterial strain-specific treatments for each host plant: $T1$ = full Hoagland solution (positive control); $T2$ = iron-free Hoagland solution (negative control); $T3$ = bacteria (siderophore positive) + Fe; $T4$ = bacteria (siderophore positive) without Fe; $T5$ = bacteria (siderophore negative) + Fe; $T6$ = bacteria (siderophore negative) without Fe. Pots were treated with standard Hoagland's solution once per week; for the remaining growth period of plants, sterile water was used. Standard Hoagland's solution supplemented with 20 µM Fe will be used to irrigate the pots having Fe as a treatment. Soil moisture was maintained at 20% with Hoagland solution (supplemented with Fe and without Fe) in control treatments and 48 h grown bacterial culture ($\sim 10^{8-9}$ CFU ml^{-1}) in treatments receiving bacterial inoculation. Plants in all the treatments were simultaneously grown and harvested at the same time. After 30 days, each

treatment was used to measure shoot and root length, leaf number, and plant fresh and dry weight.

Plant Nutrient Content

Subsequently, only shoot samples of each condition were subjected to air drying for their further nutrient analysis, while due to less quantity of root samples, they could not be considered for estimation of their nutrient content. The air-dried shoot samples were ground to powder. K, P, Ca, and Na contents in the shoot were estimated using the tri-acid mixture (H_2SO_4: $HClO_4$:HNO_3; 1:10:4 ratio) by wet-digestion at 200 °C. Subsequently, the vanadomolybdophosphoric acid colorimetric (Evolution 201; Thermo Scientific, USA) method (Tandon 1993) was used to determine the shoot P content. Whereas, K, Na, and Ca contents in the shoot were estimated with the help of a flame photometer (FP114; Thermo Scientific, USA) using the ammonium acetate method (Hanway and Heidel 1952). N content in the shoot was quantified using Kjeldahl method (Jackson 1973) with the help of an automatic nitrogen estimation apparatus (Kel-Plus Classic DX VA; Pelican Equipments, Chennai, India). To measure, Fe, Mn, and Zn air-dried shoot samples (0.1 g) were finely ground and digested in 3 ml HNO_3 at 120 °C for 6 h (Dixit et al. 2016). Following this, the digested samples were analyzed using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500 cx, USA). Rhodium (Rh) and Stannous (Sn) were used as internal calibration standards in ICP-MS.

Root Colonization Activity of Endophytic Bacterial Strains

In order to evaluate the colonization ability of selected endophytic bacterial strains in plant root and shoot cultivated in sterilized soil, a spontaneous strain of NBRI FY32 and NBRI DS5 having rifampicin resistance (Rif^R) was isolated on nutrient agar (NA) plates containing 250 µg rifampicin ml^{-1} (Nautiyal 1997). One gram of plant tissue (root and shoot) was cleaned under tap for 2 min to eliminate loosely adhered soil particles followed by surface sterilization using 5% sodium hypochlorite for 5 min and washing 4–5 times with autoclaved distilled water. The surface sterilized samples were crushed in 5 ml of 0.85% saline solution and serially diluted, then spread onto NA plates. NA plates were used to recover a mixed population of endospheric bacteria, and NA plates were supplemented with 50 µg of rifampicin ml^{-1} for NBRI FY32 and NBRI DS5. The average colonization of NBRI FY32 and NBRI DS5 ($Log_{10}CFU$/g root/shoot dry weight) was estimated from three plant samples. When root and shoot homogenates of uninoculated controls were plated from sterilized soils, no naturally occurring Rif^R bacteria were observed.

Statistical Analysis

Means were tested for variance homogeneity to assess the variation among obtained values. These means were then compared using ANOVA, followed by the Duncan test to determine significance ($p \leq 0.05$).

Results

Isolation and Screening of Endophytic Bacteria Based on Siderophore Production

In the present study, isolation of siderophore-producing endophytic bacteria was performed from iron sufficient as well as deficit conditions (Fig. 1). Out of total, 234 isolates, 13 from the root, 15 strains from the stem, and 17 strains from leaves were tested positive for siderophore production (Fig. 2). Further, these selected endophytic bacteria were characterized for different plant growth promoting attributes such as indole acetic acid (IAA) production, biofilm formation, phosphate (P) solubilization, and siderophore production.

Quantification of PGP Attributes

IAA production by selected bacterial isolates was exhibited in the range of 10.73 µg ml^{-1} to 23.22 µg ml^{-1} (Table 1). However, NBRI BR5 (23.22 µg ml^{-1}) and

Fig. 1 Representation of spinach leaf under Fe-sufficient and Fe-deficit condition in greenhouse condition
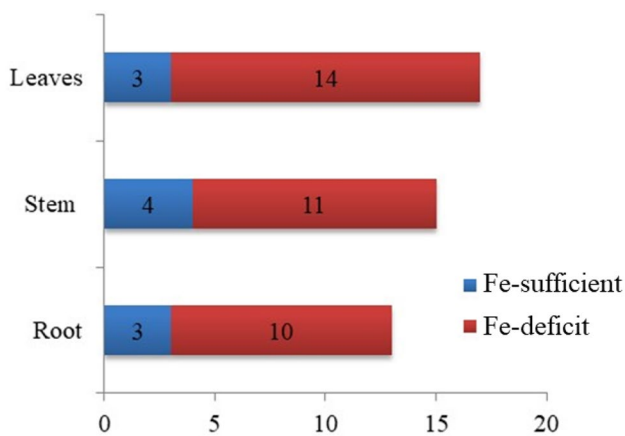
Fig. 2 Distribution of siderophore-producing endophytic bacterial isolates from different parts of spinach plant under Fe-sufficient and Fe-deficit condition

NBRI EY30 (18.67 µg ml^{-1}) were noted as the highest IAA producers under Fe-sufficient and Fe-deficit conditions, respectively. NBRI AN3 (12.30 µg ml^{-1}) and NBRI DK24 (10.73 µg ml^{-1}) were considered to be the lowest IAA producers under Fe-sufficient and Fe-deficit conditions, respectively (Table 1).

Regarding Phosphate solubilization, 45 selected bacterial isolates exhibited in the range of 21.71 to 48.79 µg ml^{-1} (Table 1). The most effective phosphate solubilizer bacterial isolate under Fe-sufficient and Fe-deficit conditions was found to be NBRI CK1 (42.71 µg ml^{-1}) and NBRI EY26 (48.79 µg ml^{-1}), respectively (Table 1). NBRI CS2 (24.22 µg ml^{-1}) and NBRI DL3 (21.71 µg ml^{-1}) demonstrated the least ability for the same under Fe-sufficient and Fe-deficit conditions, respectively (Table 1).

Regarding siderophore production, the selected 45 bacterial isolates demonstrated siderophore production in the range of 28.11 µM to 118.05 µM. NBRI AN3 (112.03 µM) and NBRI FY32 (118.05 µM) exhibited the highest quantitative value for siderophore production under Fe-sufficient and Fe-deficit conditions, respectively. The lowest siderophore-producing ability was exhibited by NBRI CR8 (29.78 µM) and NBRI DL3 (28.11 µM) under Fe-sufficient and Fe-deficit conditions, respectively, among selected 45 isolates (Table 1).

In the matter of biofilm formation, NBRI BR8 and NBRI DS8 demonstrated maximum ability from Fe-sufficient and Fe-deficit conditions, respectively, whereas, NBRI AK1 and NBRI BR5 demonstrated the lowest biofilm formation capability under Fe-sufficient condition, and NBRI EY30 under Fe-deficit condition (Table 1).

Estimation of Phytase-Producing and Oxalate Degradation Activity of Selected Endophytic Bacterial Strains

Further, we have selected 09 high siderophore-producing endophytic bacterial strains having multifarious PGP attributes and evaluated them for having phytase-producing and oxalate degradation activity. In the current study, the selected 09 bacterial strains were initially evaluated for qualitative estimation of phytase-producing and oxalate degradation activity. It was found that out of nine bacterial strains, only NBRI FY32 has the capability to produce phytase and degrade oxalate by forming a halo zone in the medium (Fig. 3a and b). Moreover, eight bacterial strains i.e., NBRI AK1, NBRI AN3, NBRI DK24, NBRI EL3, NBRI EN27, and NBRI EK29 were unable to demonstrate phytase-producing as well as oxalate degradation activity (Table 2). However, NBRI EY30 and NBRI DL4 exhibited only phytase-producing ability (Table 2).

16S rRNA-Based Identification of Selected Endophytic Bacterial Strains

All nine selected potent siderophore-producing endophytes were identified through partial 16S rRNA gene sequencing (~ 1400 bp amplicon) which revealed that most of the endophytic isolates belonged to the Bacillaceae family. Among the Bacillaceae family, one of the isolates belonged to each *B. zanthoxyli*, *B. massilionigeriensis*, and *P. flexa*, while two belonged to each *P. megaterium* and *P. aryabhattai*, respectively. Apart from *Bacillus* species, the rest of the endophytic bacterial isolates were affirmed to *Lysinibacillus fusiformis* (01) and *Enterobacter quasihormaechei* (01) (Fig. 4). The sequences of these nine endophytic bacterial isolates were submitted to the database of NCBI (GenBank) along with their accession numbers (Table 2). For phylogenetic reconstruction analysis, the Neighbor-joining statistical method with the Jukes-Cantor model was used.

Inoculation Effect of Endophytes in Plant Growth and Nutrient Enhancement Under Greenhouse Conditions

Effect of Endophyte Inoculation on Spinach Vegetative Growth

The spinach (*Spinacia oleracea* L.) plant was used as a model plant for determining growth promotion by using two endophytic bacterial strains under both Fe-sufficient and Fe-deficit conditions. In this study, NBRI FY32 was selected as a high-siderophore-producing endophyte with phytase-producing and oxalate degradation ability, whereas NBRI DS5 was selected as a non-siderophore-forming endophyte.

Table 1 Endophytic bacterial isolates with plant growth promoting attributes

Isolates	Plant growth promoting traits			
	Biofilm[a]	IAA[b]	P-Solubilization[c]	Siderophore[d]
Fe-sufficient				
NBRI AK1	0.25 ± 0.02	13.93 ± 0.08	34.08 ± 0.11	110.05 ± 0.22
NBRI AK2	0.51 ± 0.02	12.65 ± 0.08	28.75 ± 0.09	108.00 ± 0.16
NBRI AN3	0.82 ± 0.17	12.30 ± 0.09	35.71 ± 0.15	112.03 ± 0.14
NBRI AN4	0.28 ± 0.01	19.93 ± 2.43	36.14 ± 0.17	105.12 ± 0.20
NBRI AY5	0.40 ± 0.06	12.88 ± 0.12	35.21 ± 0.08	35.23 ± 0.10
NBRI AY6	0.68 ± 0.13	14.98 ± 0.04	33.48 ± 0.17	64.55 ± 0.14
NBRI BR5	0.25 ± 0.03	23.22 ± 0.08	32.41 ± 0.04	32.11 ± 0.08
NBRI BR8	2.66 ± 0.33	14.91 ± 0.08	29.73 ± 0.12	67.54 ± 0.15
NBRI BR9	0.47 ± 0.04	14.07 ± 0.12	26.30 ± 0.18	59.26 ± 0.13
NBRI BY11	0.54 ± 0.09	15.05 ± 0.08	33.61 ± 0.10	71.33 ± 0.15
NBRI CR8	1.98 ± 0.66	23.03 ± 0.08	32.74 ± 0.23	29.78 ± 0.08
NBRI CS1	1.33 ± 0.65	13.42 ± 0.10	26.93 ± 0.17	54.00 ± 0.17
NBRI CS2	0.27 ± 0.06	17.59 ± 0.06	24.22 ± 0.15	61.22 ± 0.15
NBRI CK51	0.71 ± 0.07	15.87 ± 0.10	42.71 ± 0.10	33.67 ± 0.09
Fe-deficit				
NBRI DS6	0.39 ± 0.03	11.92 ± 0.12	28.43 ± 0.11	58.43 ± 0.12
NBRI DS7	0.58 ± 0.08	18.43 ± 0.06	36.64 ± 0.11	32.56 ± 0.07
NBRI DS8	2.27 ± 0.55	13.84 ± 0.06	30.05 ± 0.19	75.65 ± 0.17
NBRI DL1	0.77 ± 0.10	17.48 ± 0.12	35.25 ± 0.19	107.66 ± 0.18
NBRI DL2	1.08 ± 0.15	14.33 ± 0.1	24.83 ± 0.15	36.54 ± 0.06
NBRI DL3	0.23 ± 0.02	13.25 ± 0.14	21.71 ± 0.16	28.11 ± 0.08
NBRI DL4	0.93 ± 0.19	16.17 ± 0.06	38.52 ± 0.27	115.43 ± 0.21
NBRI DL6	0.50 ± 0.04	13.39 ± 0.08	32.33 ± 0.11	67.28 ± 0.14
NBRI DL7	0.48 ± 0.02	12.16 ± 0.06	25.52 ± 0.11	78.34 ± 0.15
NBRI DK24	0.93 ± 0.02	10.73 ± 0.10	27.60 ± 0.15	114.12 ± 0.23
NBRI EL1	0.49 ± 0.19	11.13 ± 0.12	23.94 ± 0.11	109.55 ± 0.19
NBRI EL2	0.36 ± 0.03	14.30 ± 0.10	35.49 ± 0.08	63.60 ± 0.13
NBRI EL3	0.43 ± 0.06	12.25 ± 0.16	30.18 ± 0.09	116.11 ± 0.23
NBRI EN27	0.99 ± 0.28	17.52 ± 0.12	40.78 ± 0.06	115.20 ± 0.18
NBRI EK28	0.31 ± 0.01	18.32 ± 0.12	27.71 ± 0.15	71.34 ± 0.13
NBRI EK29	0.23 ± 0.06	16.89 ± 0.10	32.96 ± 0.17	117.10 ± 0.22
NBRI EY26	0.53 ± 0.08	12.97 ± 0.10	48.79 ± 0.11	66.10 ± 0.17
NBRI EY30	0.18 ± 0.01	18.67 ± 0.08	29.14 ± 0.09	114.26 ± 0.19
NBRI FN34	0.30 ± 0.02	14.47 ± 0.18	31.66 ± 0.14	31.77 ± 0.10
NBRI FY32	0.68 ± 0.31	12.67 ± 0.07	32.67 ± 0.19	118.05 ± 0.26
NBRI FY33	0.26 ± 0.01	14.23 ± 0.10	27.69 ± 0.11	28.76 ± 0.07
NBRI GK37	1.22 ± 0.21	12.48 ± 0.10	37.35 ± 0.13	33.11 ± 0.09
NBRI GN40	0.28 ± 0.04	16.36 ± 0.05	28.49 ± 0.09	37.55 ± 0.06
NBRI HN46	0.45 ± 0.04	11.22 ± 0.08	32.18 ± 0.11	38.13 ± 0.06
NBRI IK47	1.08 ± 0.18	16.57 ± 0.10	32.00 ± 0.15	62.22 ± 0.11
NBRI IN50	0.50 ± 0.08	16.57 ± 0.08	34.30 ± 0.13	33.10 ± 0.08
NBRI IY48	0.71 ± 0.05	14.32 ± 0.06	36.23 ± 0.12	36.66 ± 0.09
NBRI IY49	0.64 ± 0.04	16.22 ± 0.03	31.33 ± 0.10	28.45 ± 0.07
NBRI OK77	0.53 ± 0.04	12.25 ± 0.08	45.09 ± 0.18	69.44 ± 0.13
NBRI ON80	0.27 ± 0.02	16.33 ± 0.06	37.35 ± 0.14	73.21 ± 0.17
NBRI ON81	0.62 ± 0.03	16.66 ± 0.04	37.28 ± 0.11	68.35 ± 0.15
LSD (P = 0.05)	0.015	0.016	0.016	0.016

Values are means ± SE of three replications. Least squared differences (LSD = 0.05)

[a]Biofilm was measured at optical density at 590 nm

[b]Indole acetic acid (IAA) production and

[c]phosphate (P) solubilization is expressed as $\mu g\ ml^{-1}$

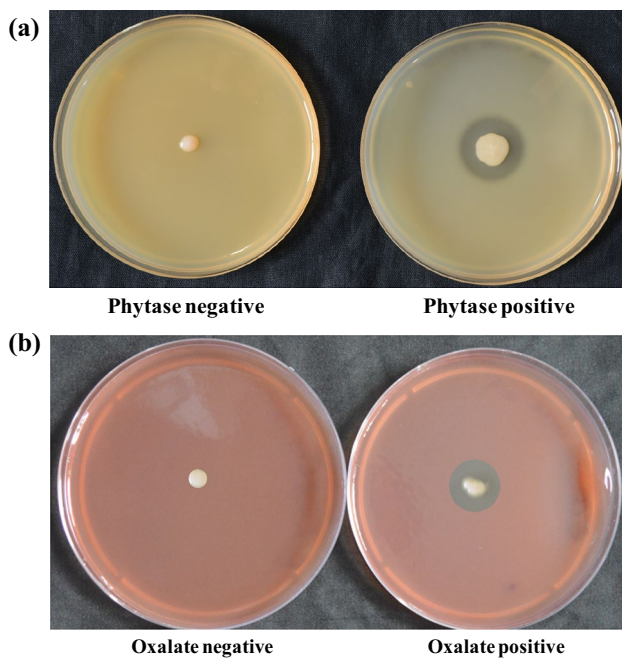[d]Siderophore production is expressed as μM

Fig. 3 Qualitative estimation of **a** phytase-producing ability, and **b** oxalate degradation ability of endophytic bacterial strain

Greenhouse results demonstrated that inoculation with NBRI FY32 has significantly enhanced the overall plant vegetative growth in comparison to respective uninoculated controls under both Fe-sufficient and Fe-deficit conditions (Table 3 and Fig. 5). However, plants under Fe-deficit control condition recorded significantly least values for all the concerned vegetative parameters among all the treatments (Table 3). NBRI DS5 only resulted in significantly high values for root length and fresh and dry plant weight as compared to control plants under Fe-sufficient conditions (Table 3). NBRI FY32 has demonstrated significant enhancement in root and shoot length, number of leaves, and plant fresh and dry weight by 3.38%, 21.07%, 16.34%, 19.36%, and 11.53%, respectively, under Fe-sufficient conditions, while 33.46%, 46.12%,

26.10%, 38.82%, and 27.14% under Fe-deficit condition, respectively (Table 3).

Effect of Endophyte Inoculation on Spinach Nutrient Content

In the present study, the phyto-beneficial impact of endophytic bacterial strains on spinach was verified under Fe-sufficient and Fe-deficit conditions by evaluating the nutrient content level. Almost all the nutrients in the spinach shoot were significantly enhanced upon inoculation of NBRI FY32 under both Fe-sufficient and Fe-deficit conditions (Table 4). Nitrogen (N), Potassium (K), Phosphorus (P), Sodium (Na), Calcium (Ca), Manganese (Mn), and Zinc (Zn) contents were found to be significantly higher in plants inoculated with NBRI FY32 under Fe-sufficient condition, while plants without any bacterial inoculation recorded significantly minimum value for the same under Fe-deficit condition (Table 4). Iron (Fe) content was observed to be significantly higher in plants inoculated with NBRI FY32 under Fe-deficit conditions, whereas plants receiving no bacteria demonstrated significantly minimum Fe content under Fe-deficit conditions (Table 4). In the present study, K, N, P, Na, Ca, Mn, and Zn contents were increased by 24.36%, 22.28%, 32.23%, 6.60%, 13.15%, 2.66%, and 7.71%, respectively, in plants inoculated with NBRI FY32 as compared to control plants under Fe-sufficient condition. However, under Fe-deficit conditions, K, N, P, Na, Ca, and Zn contents were enhanced by 24.95%, 29.35%, 21.04%, 8.80%, 6.01%, and 20.17%, respectively, in plants treated with NBRI FY32.

Colonization of Endophytic Bacterial Strains in Spinach

Simultaneously, we observed the colonization ability of endophytic bacterial strains along with the heterogeneous bacterial population in spinach shoots under Fe-sufficient and Fe-deficit conditions. NBRI FY32 showed maximum

Table 2 Differential biochemical attributes of selected bacterial strains with their 16S rRNA-based binomial nomenclature

Strains	Accession number*	Identification by 16S rRNA	Phytase	Oxalate
NBRI AK1	OQ459845	*Priestia flexa*	−	−
NBRI AN3	OQ459842	*Lysinibacillus fusiformis*	−	−
NBRI EY30	OQ459846	*Priestia megaterium*	+	−
NBRI FY32	OQ459841	*Enterobacter quasihormaechei*	+	+
NBRI DL4	OQ459848	*Bacillus massilionigeriensis*	+	−
NBRI DK24	OQ459847	*Bacillus zanthoxyli*	−	−
NBRI EL3	OQ459849	*Priestia megaterium*	−	−
NBRI EN27	OQ459844	*Priestia aryabhattai*	−	−
NBRI EK29	OQ459843	*Priestia aryabhattai*	−	−

*Accession number represents NCBI (GenBank) nucleotide sequence deposits

"+" and "−" represent the presence and absence of properties by the respective isolate

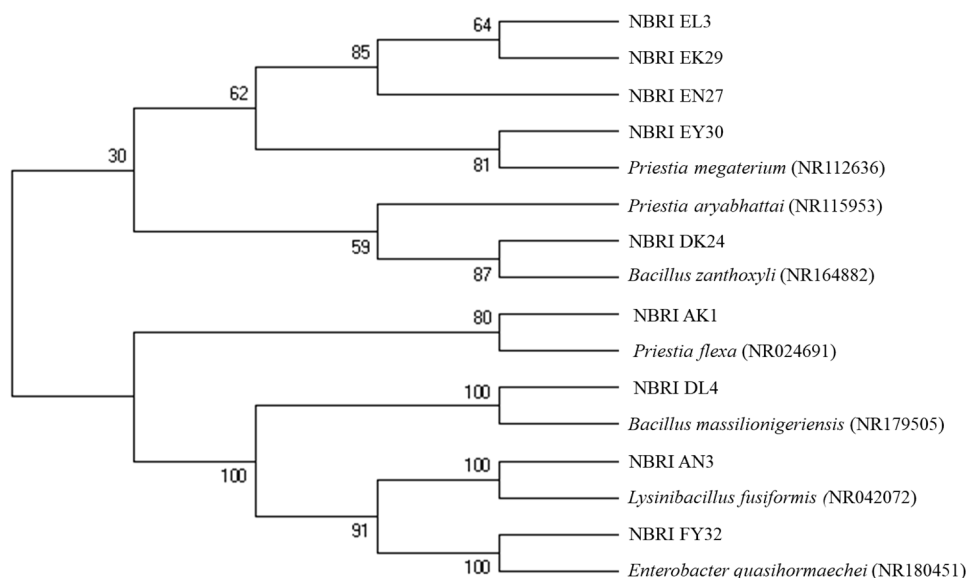**Fig. 4** Molecular phylogenetic analysis of selected endophytic bacterial strains

Table 3 Measurement of spinach vegetative parameters grown under Fe-sufficient and Fe-deficit conditions

Treatment	Plant growth parameters				
	Shoot length (cm)	Root length (cm)	No. of leaves	Fresh plant wt. (g)	Dry plant wt. (g)
$T1$	35.07 ± 0.67^c	5.13 ± 0.55^b	6.33 ± 0.33^{bc}	5.58 ± 0.04^b	0.69 ± 0.02^b
$T2$	24.73 ± 0.52^a	3.27 ± 0.49^a	5.66 ± 0.33^a	3.86 ± 0.03^a	0.51 ± 0.03^a
$T3$	36.30 ± 0.76^{cd}	6.50 ± 0.83^e	7.33 ± 0.33^c	6.92 ± 0.03^f	0.78 ± 0.02^d
$T4$	37.17 ± 0.75^d	6.07 ± 0.43^d	7.66 ± 0.33^d	6.31 ± 0.02^d	0.70 ± 0.01^b
$T5$	33.70 ± 0.84^b	5.50 ± 0.42^c	6.00 ± 0.00^b	6.50 ± 0.03^e	0.74 ± 0.02^c
$T6$	35.67 ± 0.51^c	5.37 ± 0.50^{bc}	6.33 ± 0.33^{bc}	6.11 ± 0.03^c	0.71 ± 0.02^{bc}

Parameters are expressed as mean values of three replicates $\pm$ SE which were compared by analysis of variance (ANOVA), followed by the Duncan test. Statistically significant differences (signified by different letters) were then determined at $p \le 0.05$, using the software SPSS ver 20.0. Values in the columns with the same letter are not significantly different ($p \le 0.05$) by the Duncan test. Four different treatments refer to: $T1$ = full Hoagland solution (positive control), $T2$ = iron-free Hoagland solution (negative control), $T3$ = NBRI FY32 + Fe, $T4$ = NBRI FY32 without Fe, $T5$ = NBRI DS5 + Fe, and $T6$ = NBRI DS5 without Fe

Fig. 5 Representation of bacterial strains inoculation on spinach under Fe-sufficient and Fe-deficit condition in greenhouse. In this figure, $T1$ = full Hoagland solution (positive control); $T2$ = iron-free Hoagland solution (negative control); $T3$ = bacteria (siderophore posi- tive) + Fe; $T4$ = bacteria (siderophore positive) without Fe; $T5$ = bac- teria (siderophore negative) + Fe; $T6$ = bacteria (siderophore negative) without Fe

Table 4 Nutrient content in spinach shoot grown under Fe-sufficient and Fe-deficit conditions

Shoot nutrient	T1	T2	T3	T4	T5	T6
Potassium (K)	3140.00 ± 30.55^b	3051.63 ± 4.41^a	4151.70 ± 15.89^e	4066.50 ± 7.26^d	3168.40 ± 9.74^b	3366.50 ± 14.27^c
Nitrogen (N)	5.79 ± 0.10^{bc}	4.67 ± 0.01^a	7.45 ± 0.09^e	6.61 ± 0.16^d	5.44 ± 0.15^b	6.14 ± 0.18^c
Phosphorus (P)	4370.83 ± 50.94^a	4390.20 ± 30.44^a	6450.30 ± 70.55^e	5560.00 ± 40.24^d	4862.45 ± 48.61^c	4662.23 ± 50.34^b
Sodium (Na)	5095.00 ± 80.81^b	4960.00 ± 70.63^a	5455.00 ± 69.53^c	5438.35 ± 74.01^c	5125.35 ± 44.58^{bc}	5077.65 ± 81.51^b
Calcium (Ca)	$11,960.00 \pm 73.63^a$	$12,015.00 \pm 50.40^a$	$13,771.70 \pm 71.66^d$	$12,783.30 \pm 91.14^b$	$12,813.30 \pm 91.14^{bc}$	$12,893.30 \pm 91.14^c$
Manganese (Mn)	143.73 ± 11.30^c	142.30 ± 13.12^b	147.66 ± 10.23^d	136.15 ± 14.38^a	146.23 ± 13.82^{cd}	142.33 ± 16.18^b
Iron (Fe)	174.00 ± 11.30^b	79.24 ± 9.82^a	182.31 ± 21.66^c	191.20 ± 1.15^e	175.32 ± 17.45^b	185.14 ± 18.21^d
Zinc (Zn)	170.27 ± 13.54^b	141.10 ± 17.35^a	184.50 ± 21.59^d	176.75 ± 20.25^c	172.53 ± 16.52^{bc}	172.71 ± 10.28^{bc}

Parameters are expressed as mean values of six replicates $\pm$ SE which were compared by analysis of variance (ANOVA), followed by Tuckey's (HSD) test. Statistically significant differences (signified by different letters) were then determined at $P \leq 0.05$, using the software SPSS 16.0. Four different treatments refer to $T1$ = full Hoagland solution (positive control), $T2$ = iron-free Hoagland solution (negative control), $T3$ = NBRI FY32 + Fe, $T4$ = NBRI FY32 without Fe, $T5$ = NBRI DS5 + Fe, and $T6$ = NBRI DS5 without Fe

K, P, Na, and Ca are expressed as µg gm^{-1}

N is represented in percentage (%)

Mn, Fe, and Zn are measured in ppm

Table 5 Endophytic bacterial population (Log$_{10}$ CFU g^{-1}) in spinach root and shoot grown under Fe-sufficient and Fe-deficit conditions

Treatment	Heterogenous		Rifampicin resistant	
	Root	Shoot	Root	Shoot
T1	4.33 ± 0.08	2.61 ± 0.07	0	0
T2	4.28 ± 0.05	2.60 ± 0.06	0	0
T3	4.44 ± 0.07	2.62 ± 0.05	2.06 ± 0.04	1.71 ± 0.04
T4	4.35 ± 0.04	2.48 ± 0.04	1.85 ± 0.06	1.33 ± 0.06
T5	4.34 ± 0.08	2.53 ± 0.05	1.66 ± 0.06	1.36 ± 0.05
T6	4.32 ± 0.05	2.61 ± 0.06	1.88 ± 0.05	1.32 ± 0.04

Parameters are expressed as mean values of six replicates $\pm$ SE. Four different treatments refer to $T1$ = full Hoagland solution (positive control), $T2$ = iron-free Hoagland solution (negative control), $T3$ = NBRI FY32 + Fe, $T4$ = NBRI FY32 without Fe, $T5$ = NBRI DS5 + Fe, and $T6$ = NBRI DS5 without Fe

colonizing and survival ability in spinach root and shoot with 2.06 Log$_{10}$ CFU g^{-1} and 1.71 Log$_{10}$ CFU g^{-1} under Fe-sufficient conditions (Table 5). Although, under Fe-deficit condition NBRI DS5 demonstrated maximum colonization in root with 1.88 Log$_{10}$ CFU g^{-1} while NBRI FY32 was observed to have maximum colonization in shoot with 1.33 Log$_{10}$ CFU g^{-1} (Table 5). Also, a heterogeneous endophytic bacterial population was observed higher in both the root and shoot of plants inoculated with NBRI FY32 under Fe-sufficient conditions (Table 5). Although under Fe-deficit conditions, the maximum heterogeneous population was found in plant shoot inoculated with NBRI DS5 with 2.61 Log$_{10}$ CFU g^{-1}, while plant inoculated with NBRI FY32 demonstrated maximum count in root with 4.35 Log$_{10}$ CFU g^{-1} (Table 5). However, no natural population of Rif^R bacterial endophytes was detected in roots well as in the shoot of plants receiving no bacterial treatment under Fe-sufficient and Fe-deficit conditions.

Discussion

Exploration of microbes possessing multifarious plant growth-promoting attributes and biofortification ability is indispensable to tackling the food security problem. Earlier studies have illustrated the crucial role of rhizobacterial strains in plant growth and nutrient enhancement under normal as well as stressed conditions (Bisht et al. 2019; Misra et al. 2019). However, recent reports have established a close association of endophytes with their host plants in comparison to microbes isolated from soil or other plants (Maggini et al. 2019). Researchers have well reported the important role of endophytes in the fortification of nutrients under normal conditions but limited reports are available under stress conditions (Zhang et al. 2019; Kaur et al. 2020; Rana et al. 2021; Sun et al. 2021). Although, earlier studies have reported the isolation of endophytic bacterial strains from different host crops under non-stress conditions (Lipková et al. 2021; Dubey et al. 2021). However, in the present study, bacterial endophytes were isolated from spinach plants under Fe-sufficient and Fe-deficit conditions.

Isolation and characterization of microbes for nutrient enhancement in host crops have successfully ameliorated nutrient deficiency in the human diet (Singh et al. 2017). Enhancing micronutrients in crop plants through conventional breeding, mineral fertilizers, and transgenic methods is reported but with less consistency (Murgia et al. 2012). Several endophytic bacteria have been reported to fortify the iron contents in food crops besides improving crop growth

through siderophore production (Zhang et al. 2019; Kaur et al. 2020; Rana et al. 2021). Recently, Sultana et al. (2021) showed that plant growth promoting *Bacillus aryabhattai* MS3 exhibited the highest siderophore production under salt stress and iron-limited condition.

Among different PGP attributes, the biofilm-forming abilities of microbes allow them to endure harsh environmental conditions by conferring tolerance against various abiotic stresses (Kamjumphol et al. 2013). In the context of biofilm formation, our result demonstrates that NBRI BR8 and NBRI DS8 from Fe-sufficient and Fe-deficit conditions represented maximum ability. The ability of Phyto-beneficial bacterial strains to form biofilms aids plants in defending themselves against various pathogenic diseases (Deng et al. 2011; Chen et al. 2012, 2013). This ability has provided bacteria the advantage of being isolated from harsh habitats including deserts and animal digestive systems (Matulová et al. 2011; Earl et al. 2012; Schyns et al. 2013). For adaptation, biofilm formation by endophytic bacterial strains is among the processes that need to be primarily modulated for getting access to plant internal tissues (Taulé et al. 2021; Pinski et al. 2019; Levy et al. 2018; Bogino et al. 2013). Recently, Jeong et al. (2021) reported that *Kosakonia cowanii*, a seed endophyte from a xerophytic invasive plant, *Lactuca serriola*, demonstrated drought tolerance to the host by producing a high concentration of biofilm constituents. Also, biofilm formation is required for successful colonization by an efficient endophyte to deliver its plant growth-promoting effect inside the host plant (Tharek et al. 2021).

Considering auxin production in the present study, we have reported IAA production by endophytic bacterial strains under control and iron-deficit conditions. Recently, several researchers have reported auxin production by bacterial endophytes under normal conditions (Lipková et al. 2021; Sun et al. 2021). However, several studies have demonstrated a reduction in auxin-producing ability by bacterial strains other than endophytes under stressed conditions (Tank and Saraf 2010; Soleimani et al. 2017; Sarkar et al. 2018).

Phosphorus is another essential micronutrient that is commonly immobilized and inaccessible to plants. In the present study, endophytic bacterial strains have demonstrated a wide range of P-solubilizing abilities. Earlier studies have demonstrated that *P*-solubilizing endophytic bacterial strains are able to promote plant growth under normal conditions (Dubey et al. 2021; Lipková et al. 2021). Moreover, endophytic bacteria with *P*-solubilization activity directly improved plant responses to abiotic stressors (Rajini et al. 2020).

Simultaneously, PGP endophytic bacteria demonstrate another important trait of siderophore formation, which allows bacteria to chelate the accessible form of iron (Fe^{3+}) in the rhizosphere, keeping it unavailable to undesirable microbes and limiting their proliferation (Zhang et al. 2019). In the present study, several endophytic bacterial strains demonstrated siderophore-producing ability which was found to be in accordance with previous reports (Jin et al. 2010; Tripathi et al. 2020). Recent reports have suggested the phyto-beneficial role of siderophore-producing endophytic bacterial strains by significantly promoting plant growth in terms of vegetative parameters and photosynthetic pigments (Yadav 2019; Lobo and dos Santos 2019; Rana et al. 2021). Besides enhancing plant growth, endophytic bacterial strains play a crucial role in fortifying the iron contents in plants through siderophore production (Kaur et al. 2020). The iron-chelated complex was translocated inside the plant by integral transporter proteins present on the plasma membrane of the root (Boukhalfa and Crumbliss 2002). Inoculations of maize and wheat plants with *Pantoea agglomerans* and *Arthrobacter sulfonivorans*, respectively, the siderophores-producing endophytes, have assisted in higher translocation of iron in shoots and roots (Singh et al. 2018; Rana et al. 2021). In addition, Sultana et al. (2021) have reported that Siderophore-producing bacterial strains other than endophytes have enhanced plant growth by ameliorating salt stress and Fe limitation concurrently.

Essential minerals including calcium, magnesium, iron, and zinc are chelated by phytic and oxalic acid under normal physiological conditions. Moreover, they also bind to proteins and amino acids and block digestion enzymes (Martins et al. 2017). Thus, phytic and oxalic acid should be hydrolyzed by enzymes for improved nutrient bioavailability since they are an anti-nutritive factor for food derived from plants. Endophytic bacteria possessing phytic and oxalic acid degrading abilities may convert, mobilize, and solubilize nutrients. In the present study, among the 09 selected PGP bacterial endophytes, only NBRI FY32 *Enterobacter quasihormaechei* demonstrated the presence of both phytic and oxalic acid degrading activities. Our study found to be in agreement with the findings of a recent study describing that an endophyte SSP2 *Enterobacter hormaechei* isolated from Chinese fir with high phosphate solubilizing activity has significantly promoted the vegetative growth and nutrient content of Chinese fir (Chen et al. 2021). Recently, Mei et al. (2021) reported that bacterial endophyte *Pantoea agglomerans* IALR1325 containing phytase activity has promoted phosphate solubilization and therefore, significantly enhanced pepper and tomato growth under controlled conditions.

Kost et al. (2014) explored the role of oxalate-degrading plant-beneficial species of the *Burkholderia* genus for successful plant colonization as an endophyte. In addition, this study suggested oxalate degradation by endophytic bacteria aided in heavy metal tolerance and biocontrol of plants. In the present study, we have also reported that NBRI FY32 *Enterobacter quasihormaechei* is an oxalate-degrading

endophyte. However, Kumar and Belur (2018) studied that endophytes usually colonize plant tissues by producing the necessary enzymes for the colonization of plant tissues.

Further, the present study demonstrated growth promotion in terms of vegetative parameters and nutrient content by the inoculation of siderophore-producing PGP endophytes under controlled conditions. Recent studies have demonstrated that endophytic bacterial strains can enhance plant growth directly or indirectly through several mechanisms (Mahmood and Kataoka 2020; Rana et al. 2021; Salehin et al. 2021). However, there are several reports demonstrating the role of bacterial endophytes in the growth promotion of plants under different abiotic stress conditions (Jiménez-Gómez et al. 2020; Kushwaha et al. 2020) but their role under Fe-deficit conditions is still scanty. In the present study, the results of the greenhouse revealed that plants inoculated with NBRI FY32 demonstrated significant enhancement in vegetative parameters and high nutrient content under both Fe-sufficient and Fe-deficit conditions.

Similar to our findings, several reports have also demonstrated that siderophore-producing endophytes positively influence plant growth by providing available iron to plants but these studies were restricted to non-stressed conditions (Zhang et al. 2019; Rana et al. 2021). Moreover, the effect of seed inoculation with endophytic bacterial strains on enhanced vegetative parameters of the plant was reported earlier by Sah et al. (2017) by performing a greenhouse experiment using RSP5 (high siderophore producer) and RSP8 (low siderophore producer). In the present study, NBRI FY32 was selected as a high siderophore-producing bacteria, while NBRI DS5 was non-siderophore-producing bacteria. Similar to our findings, Sah et al. (2017) reported that the lack of RSP5 and RSP8 in the control plant resulted in reduced iron transportation and plant growth. Microbes play a vital role in facilitating plant iron (Fe) absorption under Fe-limiting conditions (Jin et al. 2010). Correspondingly, the siderophore produced by NBRI FY32 in the current study was able to solubilize the insoluble forms of iron in the soil, making it accessible to plants. Nevertheless, the prior study revealed that the microbial community in the rhizosphere, which influences the composition of siderophore-secreting microorganisms in the rhizosphere, has a significant impact on Fe acquisition (Jin et al. 2010; Camejo et al. 2013).

The lack of nutrient availability may be a severe barrier to plant growth in many places across the globe, particularly in the tropics where soils are very deficient in nutrients. Plants get the majority of their mineral nutrients through the rhizosphere. The present study revealed that plants inoculated with NBRI FY32 resulted in enhanced nutrient content under Fe-sufficient and Fe-deficit conditions. Our results corroborate with the findings of earlier reports that have established the role of endophytic

bacterial strains in increasing nutrient content in strawberries, radishes, tomatoes, and lettuce (Makar et al. 2021; Verma et al. 2021; Vishwakarma et al. 2021). In a metagenomic analysis of the structure and functions of the bacterial endophyte community in *Panax ginseng* at various ages, Hong et al. (2019) found significant putative genes that may be involved in iron acquisition and siderophore formation and may contribute to bacterial activities in plants.

Furthermore, Rana et al. (2021) explained the ecological significance of endophytic bacterial strains by demonstrating their interaction more strongly with plant hosts than rhizospheric microbes through active colonization of microniches within the plant tissues. In the present work, spinach plants inoculated with NBRI FY32 supported the higher heterogeneous endophytic bacterial count. Since NBRI FY32 has exhibited oxalate-degrading activity in the present study, therefore it might be the rationale behind the active colonization of plant internal tissues. Verma et al. (2019) evaluated the endophytic bacterial count from wheat tissues (root and stem), and the results of the endophytic bacterial count from those tissues were found to be compatible with the present findings. Recently, Tharek et al. (2021) has analyzed the genome of *E. coli* USML2 and revealed genes essential for endophytic bacterial colonization such as flagella biosynthesis, chemotaxis, EPS, and biofilm formation which further proved the plant growth-promoting potentials of *E. coli* USML2 as an endophyte. Furthermore, as compared to other plant tissues, the count of bacterial endophytes in plant roots grown in non-sterile soil was reported to be higher (Sun et al. 2008). The enhanced colonization of bacterial endophytes in the apical zone of the root is due to various mechanisms of colonization and interaction with the host plant (Botta et al. 2013).

Conclusion

The role of siderophore-producing endophytic bacterial strains having multifaceted PGP attributes is essential for the confrontation of problems associated with the low nutrient content of spinach plants under nutrient-deficient conditions. The present study will assist in outlining the significant functions of siderophore-producing bacterial endophyte *E. quasihormaechei* (NBRI FY32) in Fe-sufficient as well as Fe-deficit conditions. The improvement of nutrient content in spinach plants under Fe-deficit conditions has evidenced the biofortification of host plants with certitude and in a safe way. Therefore, the present study will provide an advantage in screening out potential endophytic isolates with enhanced colonization for sustainable food crop development under nutrient-deprived conditions.

Author contribution Sankalp Misra: Methodology, Writing – original draft, Investigation. Pradeep Semwal: Formal analysis. Deen Dayal Pandey: Data curation. Shashank Kumar Mishra: Validation, Visualization. Puneet Singh Chauhan: Conceptualization, Supervision, Funding acquisition.

Declarations

Conflicts of interest All authors have no conflict of interest.

Ethics Approval Not applicable.

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