



# Biochemical and molecular investigation of non-rhizobial endophytic bacteria as potential biofertilisers

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## Abstract

This study was performed to isolate non-rhizobial endophytic bacteria from the root nodules of *Glycine max* (soybean), *Vigna radiata* (mung bean) and *Vigna unguiculata* (cowpea). The bacteria were characterized for plant growth promoting properties such as indole acetic acid production, phosphate and zinc solubilisation, nitrogen fixation and hydrogen cyanide production. Phylogenetic identification was performed using the Neighbour-Joining method on 16S rRNA gene sequences. The impact of salt tolerant isolates on some properties of wheat cv. Chamran was evaluated by a completely randomised factorial design. Nine isolates having some characteristics related to plant growth promotion were identified as *Staphylococcus hominis* 7E, *Streptomyces* sp. 11E, *Bacillus* sp. 13E, *Acinetobacter* sp. 19E, from mung bean, *Bacillus endophyticus* 1E from cowpea, *Staphylococcus hominis* 9E, *Bacillus endophyticus* 14E, *Brevundimonas* sp. 16E and *Kocuria* sp. 26E from soybean nodules. Isolates 7E and 19E caused maximum growth inhibition of *Fusarium* on PDA plate. All isolates were able to grow at salinity levels of mixtures containing up to 400 mM of NaCl, CaCl<sub>2</sub> and MgCl<sub>2</sub>, but their growth was inhibited by increasing salinity level. Only the growth of isolate 14E increased at three levels of salinity compared with control. Some isolates, i.e. 7E, 14E, 19E and 26E had higher colony diameter at 45 °C after 48 h of incubation compared to the growth at 30 and 40 °C. Inoculation of soil with isolate 1E and isolate 26E caused to ameliorate salinity stress in wheat and increased the weight of 1000-grains as compared with non-inoculated treatments.

**Keywords** Nodule · Endophyte · Legumes · Plant growth · Salinity

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## Introduction

Herbaceous woody plants which produce seeds in pods are known as legumes such as peas, beans, alfalfa, vetches, soybeans and clovers. Rhizobia are bacteria that fix nitrogen inside the root and stem nodules of leguminous plants; therefore, legumes could grow well in nitrogen-free soil (Wang et al. 2017). The establishment of a symbiotic relationship between nitrogen-fixing prokaryotes and plants reduces the requirement for nitrogenous fertilisers. Non-rhizobial bacteria may be present inside the nodules of legume plants despite the specificity of the interactions between rhizobial bacteria and legumes (Zakhia et al. 2006; Velázquez et al. 2013). The non-rhizobial endophytic bacteria in healthy nodules have been documented from different genera, including *Agrobacterium*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Enterobacter*, *Micromonospora*, *Mycobacterium*, *Paenibacillus*, *Pseudomonas* and *Stenotrophomonas* (Velázquez et al. 2013). The role of endophytic bacteria within nodules is not well-understood.

However, the improvement of plant growth, health, and nodulation has been reported through the simultaneous association of endophytic bacteria with rhizobia (Rajendran et al. 2008; Bai et al. 2003; Tariq et al. 2012). Therefore, the isolation and identification of such non-rhizobial endophytic bacteria which possess plant growth promotion traits, salinity tolerance and the capability to grow at high temperatures are important to formulate new biofertilisers for legumes (and even non-legume plants) cultivated in arid and semi-arid areas. This study was performed to isolate and identify potential plant growth-promoting non-rhizobial bacteria from the root nodules of *Glycine max* (soybean), *Vigna radiata* (mung bean), and *Vigna unguiculata* (cowpea) and to evaluate their viability at high levels of salinity and temperatures. The impacts of two isolates on wheat growth and yield were investigated in greenhouse conditions.

## Materials and methods

### Isolation of nodule endophytic non-rhizobial bacteria

To investigate the nodule non-rhizobial endophytic bacteria, root nodules of *Glycine max* (soybean), *Vigna radiata* (mung bean) and *Vigna unguiculata* (cowpea) were collected for isolation. The crops were grown in fields in the southwest region of Iran (specifically, in the Safi Abad Agricultural and Natural Resources Research and Training Center, Khuzestan Province, Dezful County). A total of 210 nodules were randomly collected during the flowering of the three studied species—7 nodules from every 10 plants in each species (i.e., 70 nodules for each species)—and kept on silica gel until transport to the lab. The nodules were surface sterilised with 70% ethanol for 1 min and then with 2% hypochlorite for 5 min. They were then washed several times with sterile distilled water (Leite et al. 2017). To check the success of the sterilisation method, 100 µl from the final rinsed water were cultured on a nutrient agar plate and incubated at 28 °C for 72 h. To ensure disinfection of the nodules' surface, the disinfected samples were placed in 5 ml of sterile water and shaken for 5 min. Then, a loop of the suspension was spread on a nutrient agar medium and incubated for 48 h at 28 °C. No growth appeared on the nutrient agar medium, indicating that epiphyte and saprophytic bacteria were unable to grow after disinfection of the nodule surface (Schulz et al. 1993). The sterilised nodules were crushed under aseptic conditions, and 10 µl of the suspension were inoculated on nutrient agar (Vincent 1970) incubated at  $28 \pm 2$  °C for 72 h. The purification of bacterial colonies was performed according to the difference in morphology.

### Characterisation of isolates for traits related to plant growth promotion

Qualitative and quantitative assessments of phosphate dissolution activity of the isolates were accomplished using Pikovskaya solid and broth mediums based on tricalcium phosphate, respectively (Pikovskaya 1948). About 10 µl of overnight culture of each isolate with the same bacterial population ( $6 \times 10^8$  Cfu ml<sup>-1</sup>) was dropped on a solid medium in a plate and incubated for 96 h at 30 °C. Transparent areas around the colonies were considered as an indicator of the phosphorous-dissolution ability of the isolates. The amount of soluble phosphorus released by phosphorus-solubilising bacteria was determined using broth medium. An overnight culture of bacteria with the same population ( $6 \times 10^8$  Cfu ml<sup>-1</sup>) was inoculated (2% v/v) in a culture medium. Flasks were incubated at 30 °C under shaking conditions (140 rpm) for 96 h. After that, the suspension inside each flask was centrifuged for 10 min at 10,000 rpm to remove cells and debris. The phosphorus content of the centrifuged samples was measured using an ammonium molybdate vanadate reagent and reading the absorbance at 470 nm (Jeon et al. 2003) via spectrophotometer (PD-303UV, Apel). The screening of isolates to dissolve zinc was done using a solid medium containing 0.1% zinc oxide by inoculating 10 µl from an overnight culture of isolates as a drop on the medium. The creation of a clear halo around the colony after 96 h of incubation at 30 °C was considered as zinc solubilisation (Saravanan et al. 2004). The ratio of total diameter (colony + halo zone) to colony diameter was considered as the zinc solubilising index (SI) (Edi-Premono et al. 1996). To quantify the solubilised Zn by isolates, broth culture media containing 0.1% zinc oxide were inoculated with 2% v/v from the overnight culture of bacteria with the same population ( $6 \times 10^8$  Cfu ml<sup>-1</sup>). The inoculated flasks were incubated in a shaker incubator (120 rpm) for 96 h at 28 °C. Thereafter, the pH of each flask was measured, and the contents were centrifuged at 10,000 rpm for 10 min to remove bacterial residues and cells (Saravanan et al. 2004). The samples were then diluted to a ratio of 1:10, and the zinc concentration in the solution was determined using an atomic absorption spectrophotometer (SavantAA, GBC).

A qualitative evaluation of the isolates' capability to produce an auxin hormone was carried out by culturing the isolates onto a Luria bertani tryptophan (LBT) medium containing (g l<sup>-1</sup>): (5) glucose, (5) sodium chloride, (5) yeast extract, (10) tryptone, (0.5) tryptophan, and (20) agar (Bric et al. 1991). The isolates were cultured linearly onto the plate containing the already mentioned medium and covered with filter paper; then, the plates were incubated

at 30 °C for 24 h to grow the isolates. The filter paper was removed and moistened with a few drops of Salcovsky's reagent. The formation of a pink colour and its growing intensity from pale to deep pink, indicated auxin production by the isolates (Bent et al. 2001).

The isolates were further screened for nitrogen-fixing capability on a Dobereiner nitrogen-free medium and incubated at  $30 \pm 2$  °C for 48 h (Döbereiner et al. 1972). The change of colour of the medium from green to yellow due to the activity of bacteria indicated the ability of the isolates to fix nitrogen.

Further analysis was performed on the isolates by considering their potential to produce hydrogen cyanide. A single colony of each tested bacterial isolate was streaked on a tryptone soy agar medium, supplemented with glycine ( $4.4 \text{ g l}^{-1}$ ). Then, the soaked filter paper in the sodium picrate (0.5% picric acid and 2% sodium carbonate) was fitted to the underside of the plate lids and sealed with para-film tape to prevent the escape of hydrogen cyanogen.

The plates were incubated for 120 h at 30 °C. The production of hydrogen cyanide was evaluated by changing the colour of the filter paper. The filter colours varied from cream (low HCN production), to light brown (medium HCN production), dark brown (high HCN production), or brick (high HCN production) and scored from 1 to 4, respectively (Bakker and Schippers 1987).

### Characterisation of the antifungal activity, salinity, and temperature tolerance of isolates

The antifungal activity of isolates was investigated using the dual culture method (Berg et al. 2005) against *Fusarium oxysporum*, which was obtained from the microbial collection of Shahid Chamran University of Ahvaz, Iran. The test was done in triplicate on a potato dextrose agar plate. A plug of a new culture of fungus was placed onto the centre of the medium in the plate, and then bacterial strains that grew overnight were streaked 2 cm from the mycelia plug on both sides. Thereafter, the plates were incubated at 28 °C until the fungus covered the control plate. The radial growth of fungal mycelium was measured, and the inhibition of fungal growth was estimated by comparing it with a control plate.

To investigate the tolerance of isolates with different levels of salinity, a drop from the overnight culture of each isolate with the same population ( $6 \times 10^8 \text{ Cf} \mu\text{l}^{-1}$ ) was placed in triplicate on a nutrient agar plate containing 100, 200, and 400 mM of NaCl,  $\text{CaCl}_2$ , and  $\text{MgCl}_2$  mixtures at a ratio of 3:2:1. These were equivalent to electrical conductivities of ( $\text{dSm}^{-1}$ ) 12.3, 24, and 46.2, respectively.

The inoculated plates were incubated at 30 °C for 96 h, and the growth colonies' diameters were measured and compared with the diameters of colonies in control plates without adding any salt. The inhibition of the colonies' growth

due to salinity was calculated as  $(100 \times \text{colony diameter in control plate} - \text{colony diameter in salt supplemented plate} / \text{colony diameter in control plate})$ .

The growth of isolates at different temperatures (28, 35, and 40 °C) was considered by inoculating a drop of the overnight culture of each isolate with the same population ( $6 \times 10^8 \text{ Cf} \mu\text{l}^{-1}$ ) onto nutrient agar plates at three replications. The colony diameter of the isolates was measured, and a mean comparison was done using Tukey's ( $P < 0.05$ ) analysis.

### Identification of non-rhizobial endophytic bacteria

Some standard biochemical tests (oxidase, catalase, and gram staining) were performed to identify the isolates based on Bergey's Manual of Systematic Bacteriology. The phylogenetic identification of non-rhizobial endophytic bacteria was done by amplifying extracted DNA through the boiling method (Queipo-Ortuño et al. 2008).

Primer 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and reverse primer 1429R (5' GGT TAC CTT GTT ACG ACT T 3') were used for PCR (Zainudin et al. 2013). The amplification was initiated at 95 °C for 5 min, followed by 35 cycles each containing denaturation (95 °C, 30 s), annealing (55 °C, 40 s), elongation (72 °C, 60 s), and a final extension (72 °C, 10 min). The reaction products were sequenced. The gene sequences were compared with other sequences in the GenBank databases using NCBI BLAST at <https://www.ncbi.nlm.nih.gov/blast/Blast.cgi> and ClusralW2 software.

The phylogenetic tree was constructed using MEGA software (Kumar et al. 2018) through the 16S rRNA gene sequence alignment of the studied samples using the Neighbour-Joining method (Saitou and Nei 1987) based on the Kimura 2-parameter model (Kimura 1980).

### Evaluation of isolated bacteria on some wheat properties under salinity stress

For testing the efficiency of two selected bacteria according to their salinity tolerance at  $12 \text{ dSm}^{-1}$ , their effects were observed on various properties of wheat cv. Chamran under different salinity levels of soil as in a completely randomised factorial design. The experimental factors consisted of bacterial inoculation (B0 without inoculant, B1 inoculation with isolate 1E, B2 inoculation with isolate 26E, and B3 inoculation with mix of isolates 1E and 26E) and three levels of salinity (S0  $3.1 \text{ dSm}^{-1}$ , S1  $6.3 \text{ dSm}^{-1}$  and S2  $9.7 \text{ dSm}^{-1}$  from the source of NaCl,  $\text{CaCl}_2$ , and  $\text{MgCl}_2$  at ratio of 3:2:1, respectively). Soil collected from the research area of the Agriculture Faculty of Shahid Chamran University of Ahvaz was added to 36 pots and supplemented with nitrogen and phosphorous fertilisers (350 kg/ha urea, 100 kg/ha triple superphosphate). The results of the chemical analysis of the

soil were as follows: Electrical conductivity = 2.90 dSm<sup>-1</sup>, pH 7.7, organic matter = 0.71%, available P = 10.1 (mg kg<sup>-1</sup>), available K = 290 mg kg<sup>-1</sup> and total nitrogen = 0.07% with a clay loam soil texture. About 1 ml of the overnight culture of each isolated sample (diluted to 10<sup>6</sup> CFU ml<sup>-1</sup>) was inoculated under wheat seeds (*Triticum aestivum*) Chamran cultivar. The soil moisture was kept at 75% of the soil field capacity using distilled water. During the experiment, the chlorophyll index (Spad) was measured, and root dry weight, phosphorous concentration, the Na<sup>+</sup>/K<sup>+</sup> ratio of roots and 1000-grain weight were determined. The data were analysed via statistical software (version 8.1). Mean comparisons were made using Tukey's test at 5%.

## Results and discussion

A total of 25 isolates from 3 different plants (4 belonging to cowpeas, 10 belonging to soybeans and 11 related to mung beans) were isolated. However, nine isolates with properties related to plant growth promotion were identified (Table 1). From these nine isolates, those with codes of 7E, 13E and 1E belonged to the root nodules of mung beans and cowpeas, indicated higher zinc SI and higher zinc dissolution in a broth medium. The pH of the growth medium after 5 days of incubation exhibited a reduction from 7.0 (control) to 5.5–6.9. The negative and significant correlation between pH and Zn values ( $r = -0.60^*$ ) of the broth medium demonstrated the isolates' ability to dissolve zinc from the source of zinc oxide. There was a perceptible decrease in the pH of the broth culture medium inoculated with isolates 13E, 7E and 1E (Table 1). The results indicated that the maximum soluble Zn (64.3 mg l<sup>-1</sup>) was achieved by isolate 13E when the pH was 5.9. In the present study, the results indicated a contradictory relationship between increased labile Zn and the reduced pH of the medium, suggesting that the solubility of Zn is increased at lower pH levels. Such phenomena

have been reported previously (Dinesh et al. 2018; Goteti et al. 2013).

All of the isolates could solubilise phosphorus. However, the isolates 16E, 9E and 7E, which were isolated from soybean and mung bean root nodules, showed the maximum potential of phosphate solubilisation in broth and onto a plate (higher phosphorus SI) in comparison to other isolates (Table 1). The correlation between pH and measured phosphorous was negative and significant ( $r = -0.83^{**}$ ). There was a drastic decrease in the pH of the inoculated medium with each isolate, as the pH ranged from 4.0 to 5.2. In this experiment, isolates 16E, 9E and 7E, which lowered the pH value of the medium to below 5.0, had a better capability of P solubilisation. However, in potent isolate 13E, pH did not fall below 5.0, suggesting that in addition to lowering pH, other mechanisms are involved in the phosphorous release process. It is well-known that several mechanisms are involved in inorganic phosphate solubilisation. The production of mineral-dissolving compounds such as organic acids, siderophores, protons, hydroxyl ions, and CO<sub>2</sub> have been considered as notable mechanisms (Alori et al. 2017). It has been reported that organic acid excretion by bacteria signifies a decrease in pH in the medium, which causes acidification in the surroundings of microbial cells. As a consequence, P ions are released via the substitution of H<sup>+</sup> for Ca<sup>2+</sup> (Goldstein 1994). Most of the isolated bacteria grown on an N-free medium changed the colour of the medium to yellow, which suggests that they were able to fix atmospheric nitrogen (Table 2). According to the results, six isolates showed auxin production ability by changing the paper colour to pink. Isolate 11E had a higher ability to produce auxin than the other isolates. All the isolates could produce HCN; isolates 13E, 19E, and 24E from the mung bean nodule received the highest score.

A phylogenetic tree with the sum of branch length of 0.81692635 has been illustrated in Fig. 1. The percentage of replicate trees in which the related taxa clustered together in the bootstrap test (1000 replicates) is displayed next to

**Table 1** Solubilising index (SI), dissolution amount of zinc and phosphorous and pH of the medium under the impact of non-rhizobial endophytic bacteria

Code	Isolation source	SI <sup>a</sup>	Zn (mg l <sup>-1</sup> )	pH <sup>a</sup>	SI <sup>b</sup>	P (mg l <sup>-1</sup> )	pH <sup>b</sup>
1E	Cowpea	1.6	35.8	6.2	1.5	74.2	5.2
7E	Mung bean	1.7	43.7	6.1	2.5	246.5	4.0
9E	Soybean	1.3	12.5	6.6	2.4	308.7	4.1
11E	Mung bean	1.3	10.5	6.8	1.1	20.3	5.5
13E	Mung bean	1.6	64.3	5.9	2.0	169.5	5.2
14E	Soybean	1.2	10	6.9	1.1	34.5	4.9
16E	Soybean	1.2	15.3	6.5	2.8	430.5	4.1
19E	Mung bean	1.3	6.5	6.9	1.2	85.6	5.1
26E	Soybean	1.2	11.5	6.8	1.4	84.2	5.0

<sup>a</sup>The results of zinc solubilisation test

<sup>b</sup>The results of phosphate solubilisation test



**Table 2** Some plant growth promotion traits and antifungal activity of non-rhizobial endophytic bacteria

Code	Isolation source	Auxin test	HCN test	N-fixation	Fungus growth inhibition (%)	Genus	Accession No
1E	Cowpea	– <sup>a</sup>	1	++	23	<i>Bacillus endophyticus</i>	MN227286
7E	Mung bean	++	1	– <sup>c</sup>	30	<i>Staphylococcus hominis</i>	MN227287
9E	Soybean	++	2	–	2	<i>Staphylococcus hominis</i>	MN227288
11E	Mung bean	++++ <sup>b</sup>	1	+ <sup>d</sup>	7	<i>Streptomyces</i> sp.	MN227289
13E	Mung bean	++ <sup>b</sup>	4	+	7	<i>Bacillus</i> sp.	MN227290
14E	Soybean	–	1	++ <sup>d</sup>	2	<i>Bacillus endophyticus</i>	MN227291
16E	Soybean	–	3	+++ <sup>d</sup>	5	<i>Brevundimonas</i> sp.	MN227292
19E	Mung bean	–	4	+	35	<i>Acinetobacter</i> sp.	MN227293
26E	Soybean	+	2	+++	16	<i>Kocuria</i> sp.	MN227295

<sup>a</sup>No colour change of paper

<sup>b</sup>Auxin production by changing the paper colour varied from pale pink (+) to deep pink (++++)

<sup>c</sup>Lack of bacterium growth on N-free medium

<sup>d</sup>Growth of bacterium on N-free medium and the intensity of the medium colour change to yellow

the branches (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter model and are in the units of the number of base substitutions per site. This analysis involved 34 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1607 positions in the final dataset.

Neighbour joining tree of the partial sequences of 16S rRNA genes from isolates indicated that isolates belonged to four genera (Fig. 1) from the groups of Firmicutes, Actinobacteria and Proteobacteria. The isolates 7E, 11E, 13E and 19E showed similarity to *Staphylococcus hominis*, *Streptomyces* sp., *Bacillus* sp. and *Acinetobacter* sp., respectively. Isolate 1E, according to 16S rRNA genes sequences, was closely related to *Bacillus endophyticus* and bacteria with codes of 9E, 14E, 16E and 26E isolated from soybean nodules indicated similarity to *Staphylococcus hominis*, *Bacillus endophyticus*, *Brevundimonas* sp. and *Kocuria* sp., respectively.

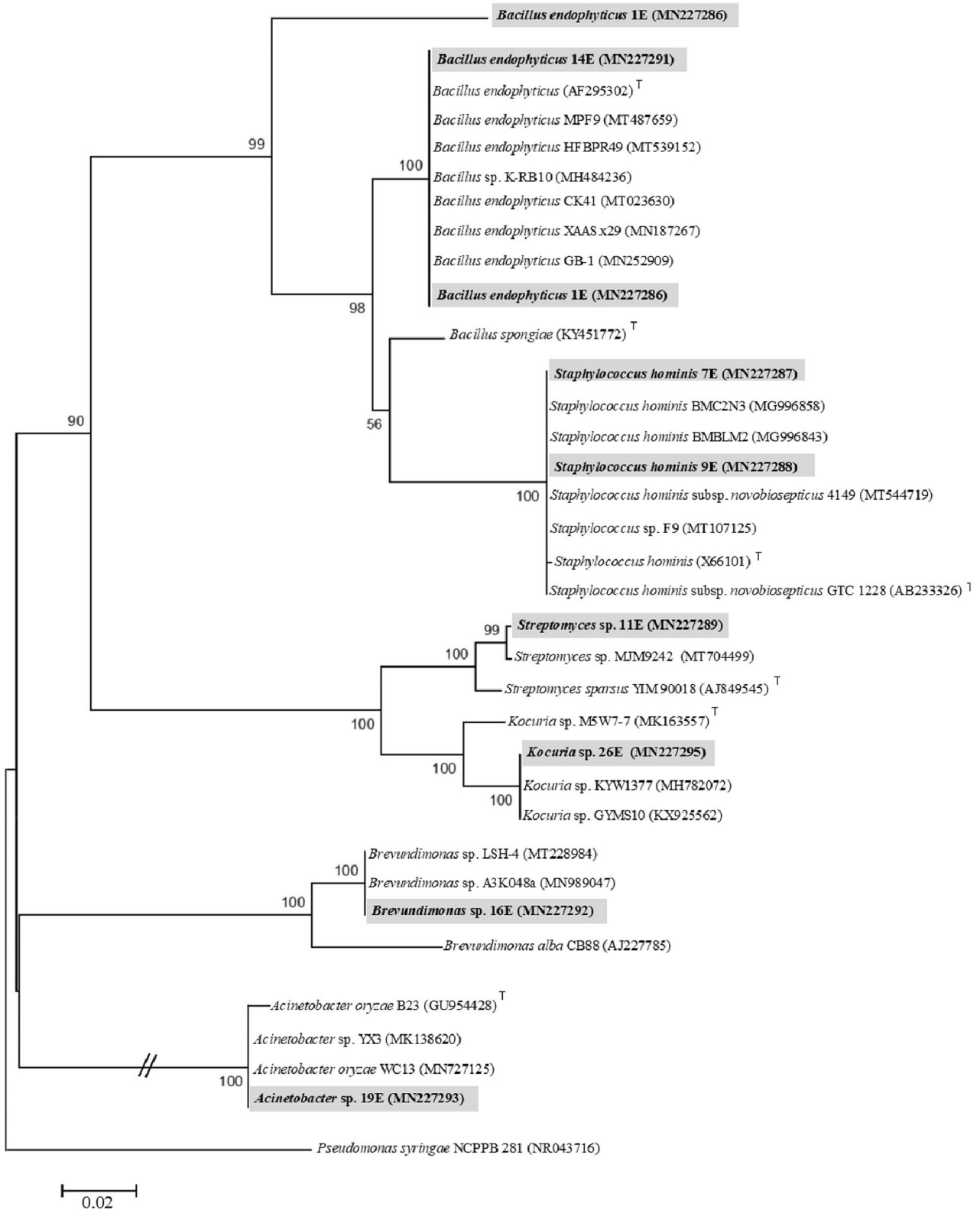
Most reported non-rhizobial bacteria in nodules of cowpea and soybean were related to the genera of *Enterobacter*, *Chryseobacterium*, *Sphingobacterium*, *Bacillus*, *Paenibacillus*, *Acinetobacter*, *Ochrobactrum* and *Pseudomonas* (Leite et al. 2017; De Meyer et al. 2015; Oliveira-Longatti et al. 2014; Zhao et al. 2018; Tariq et al. 2014; Zakhia et al. 2006).

We observed diverse bacterial genera associated with soybean nodules. We detected two new isolates, *Brevundimonas* and *Kocuria*, in soybean nodules, which have not been reported so far. Potential metal-resistant *Kocuria* sp. CRB15 has been isolated from the rhizospheric region of *Saccharum spontaneum* with hydrogen cyanide production capacity and P solubilisation (39.37 µg ml<sup>-1</sup>) (Hansda and Kumar 2017). A multi-trait plant growth-promoting bacterium, *Brevundimonas* sp., has been isolated from the

rhizosphere of *Saccharum* L. and grown in an arid region. Its beneficial effect on the growth of Bt-cotton was ascertained (Kumar and Gera 2014). *Staphylococcus hominis*, which is known to have plant growth promotion characteristics, has been isolated from the tissue of maize (Marag and Suman 2018). In the present study, four potential non-rhizobial bacteria with plant growth promotion properties were isolated from mung bean root nodules and were identified as being from different genera and as having variations in terms of the studied characteristics. Their P and Zn dissolution ability differed especially. Diverse bacterial genera—*Staphylococcus*, *Bacillus*, *Streptomyces* and *Acinetobacter*—were associated with mung bean nodules. The genus *Streptomyces* from the phylum Actinobacteria has been identified as plant growth-promoting bacteria in the rhizosphere and in association with roots that exhibit antifungal activity (Schrey and Tarkka 2008; Rey and Dumas 2017).

The plant growth promotion traits of bacteria operate through a complex phenomenon that influences plant growth and health by enhancing a plant's nutrient acquisition (such as the uptake of P, Zn and Fe), hormone production, or regulating plant hormone levels, and diminishing the repressive impacts of pathogenic microorganisms (Dhole et al. 2016; Bashan et al. 2008). Zinc-solubilising bacteria belonging to various species of *Acinetobacter*, *Bacillus*, *Enterobacter* and *Staphylococcus* have been reported (Yaish et al. 2015). In our study, *Acinetobacter* sp. possesses a strong ability to produce HCN, and it could inhibit the growth of pathogenic fungi. However, only a few reports showed that *Acinetobacter* strains fix nitrogen, dissolve phosphorous and inhibit the growth of pathogenic fungi (Zhao et al. 2018; Sarode Prashant et al. 2009).

However, the antagonistic impact of endophytic bacteria within nodules of soybeans, mung beans and cowpeas



**Fig. 1** Phylogenetic tree of studied samples and their closest type strains through 16S rRNA gene sequence alignment. The tree was rooted with *Pseudomonas syringae* (NR043716). The studied strains have been highlighted in grey and type strains have been indicated with a superscript T

for *Fusarium oxysporum* has not been studied. Remarkably, all isolates seemed to inhibit the mycelial growth of *Fusarium* on a PDA plate. The antifungal activity of root nodules' endophytic bacteria was revealed by Zhao et al. (2018) and Lin et al. (2013) against *Phytophthora sojae* and *Verticillium*, respectively. The inhibition of phytopathogens by endophytic bacteria might occur through different mechanisms such as competition, antibiosis, and fungal cell lysis (Zhao et al. 2018).

The characterised endophytic bacterial isolates in the present study showed varied bearing to salinity with a significant difference by Tukey pairwise comparison ( $P < 0.05$ ). Remarkably, all the isolates could grow at the tested level of salinity. However, increasing the salinity of the medium from 12.3 to 46.2 dSm<sup>-1</sup> inhibited the growth of the tested isolates. Isolates of *Bacillus endophyticus* 14E demonstrated different behaviours in salt-containing plates, and its growth increased at three levels of salinity compared with control. The growth inhibition percentage of isolate 14E indicated more growth of this isolate at salinity level of 100 mM (Table 3). Likewise, the salt tolerance and salt-induced

growth of some endophytic isolates from sugarcane were studied by Pirhadi et al. (2016). The isolates 7E (*Staphylococcus hominis*), 14E (*Bacillus endophyticus*), 19E (*Acinetobacter* sp.), and 26E (*Kocuria* sp.) had a higher growth rate at 45 °C than at 30 and 40 °C after 48 h of incubation (Table 4). Bacterial survival at high temperatures is important for selecting the best plant growth-promoting bacteria for use in arid and semi-arid regions.

The bacterial isolates of *Bacillus endophyticus* 1E and *Kocuria* sp. 26E significantly alleviated the harmful effects of soil salinity on wheat growth and significantly increased root dry weight, chlorophyll index, root phosphorous concentration, and 1000-grain weight when compared with non-inoculated plants in salt-stressed conditions (Table S1). Root dry weight (54%) and chlorophyll index (28%) significantly decreased in plants exposed to 9.7 dSm<sup>-1</sup> salinity when compared with control plants. Meanwhile, after the inoculation of soil by the mixture of both bacteria, significant increases in root dry weight and chlorophyll index were observed. The impacts of *Kocuria* sp. 26E on both measured parameters were greater than for *Bacillus endophyticus* 1E and led to greater increases in the measured parameters at each salinity level. Salinity stress (9.7 dSm<sup>-1</sup>) reduced the phosphorous concentration of roots (33%) when compared with unstressed plants. However, plants treated with a mix of bacteria yielded significantly greater values of root phosphorous concentration (42%).

Similarly, the mean value of 1000-grain weight demonstrated a significantly higher value for plants treated with bacterial isolates than for uninoculated plants. The salinity stress (9.6 dSm<sup>-1</sup>) decreased 1000-grain weight (39%) in comparison with control plants. However, the application of bacteria ameliorated salt stress in wheat, as the 1000-grain weight showed a 29% enhancement at 9.7 dSm<sup>-1</sup> due to the mixed inoculation of bacteria when compared with salt-stressed plants without bacterial inoculation.

The results showed that salinity stress increased the Na<sup>+</sup>/K<sup>+</sup> ratio in wheat plants. However, bacterial inoculation reduced this ratio when compared with uninoculated plants. The highest reduction in the Na<sup>+</sup>/K<sup>+</sup> ratio was 48%, which was observed in plants inoculated with mix

**Table 3** The impact of different salinity levels on mean inhibition growth of isolates compared to the control plates (none added salts into the medium)

Isolates code	Salinity level (mM)		
	100 (EC = 12.3 dS m <sup>-1</sup> )	200 (EC = 24 dS m <sup>-1</sup> )	400 (EC = 46.2 dS m <sup>-1</sup> )
1E	4.54 <sup>mn</sup>	50.08 <sup>ij</sup>	68.95 <sup>b-h</sup>
7E	33.44 <sup>jk</sup>	61.28 <sup>f-i</sup>	75.50 <sup>a-f</sup>
9E	26.35 <sup>kl</sup>	57.22 <sup>ghi</sup>	63.75 <sup>d-i</sup>
11E	80.88 <sup>a-d</sup>	86.60 <sup>ab</sup>	91.08 <sup>a</sup>
13E	62.33 <sup>e-i</sup>	75.27 <sup>a-g</sup>	88.33 <sup>a</sup>
14E	-66.66 <sup>o</sup>	-12.50 <sup>n</sup>	-8.333 <sup>n</sup>
16E	56.92 <sup>hi</sup>	77.06 <sup>a-f</sup>	90.62 <sup>a</sup>
19E	65.84 <sup>c-i</sup>	80.20 <sup>a-e</sup>	83.17 <sup>abc</sup>
26E	13.50 <sup>lm</sup>	50.01 <sup>ij</sup>	60.70 <sup>f-i</sup>

Numbers followed by the same letter are not significantly different at  $P < 0.05$ , using tukey Pairwise Comparisons

**Table 4** Mean comparison for colony diameter (mm) of isolates at different incubation temperatures

Time	Temperature	1E	7E	9E	11E	13E	14E	16E	19E	26E
24 h	30 °C	3.65 <sup>a-m</sup>	2.15 <sup>h-p</sup>	1.95 <sup>k-p</sup>	2.4 <sup>g-o</sup>	1.85 <sup>l-q</sup>	1.7 <sup>n-q</sup>	2.0 <sup>j-p</sup>	1.8 <sup>m-q</sup>	2.5 <sup>g-o</sup>
	40 °C	4.5 <sup>a-f</sup>	4.0 <sup>a-h</sup>	2.2 <sup>h-p</sup>	3.3 <sup>c-n</sup>	3.8 <sup>a-k</sup>	2.95 <sup>f-n</sup>	3.1 <sup>d-n</sup>	3.4 <sup>b-n</sup>	3.25 <sup>c-n</sup>
	45 °C	3.0 <sup>e-n</sup>	1.65 <sup>n-q</sup>	0.5 <sup>pq</sup>	2.0 <sup>j-p</sup>	3.85 <sup>a-j</sup>	1.75 <sup>n-q</sup>	0.0 <sup>q</sup>	2.8 <sup>f-n</sup>	3.35 <sup>c-n</sup>
48 h	30 °C	4.1 <sup>a-g</sup>	2.65 <sup>f-n</sup>	2.1 <sup>i-p</sup>	3.25 <sup>c-n</sup>	2.3 <sup>g-p</sup>	3.1 <sup>d-n</sup>	3.1 <sup>d-n</sup>	3.1 <sup>d-n</sup>	3.7 <sup>a-l</sup>
	40 °C	5.3 <sup>a</sup>	4.85 <sup>a-e</sup>	3.85 <sup>a-j</sup>	3.95 <sup>a-i</sup>	5.4 <sup>a</sup>	3.95 <sup>a-i</sup>	4.95 <sup>a-d</sup>	4.95 <sup>a-d</sup>	5.3 <sup>a</sup>
	45 °C	4.45 <sup>a-f</sup>	5.25 <sup>ab</sup>	2.2 <sup>h-p</sup>	3.35 <sup>c-n</sup>	5.1 <sup>abc</sup>	5.4 <sup>a</sup>	0.75 <sup>o-q</sup>	5.4 <sup>a</sup>	5.4 <sup>a</sup>

Numbers followed by the same letter are not significantly different at  $P < 0.05$ , using tukey Pairwise Comparisons

of bacteria. Meanwhile, the lowest reduction was 28% in plants inoculated with *Bacillus endophyticus* 1E (when compared to plants exposed to 9.7 dSm<sup>-1</sup> salinity levels without inoculation) (Table S1). The salinity stress of plants can be reduced through Na efflux and K efflux as mentioned by Shabala and Cuin (2008). Increasing sodium concentration in soil induces sodium influx and restricts potassium uptake by the roots, which leads to water deficiency and osmotic pressure, thereby resulting in a reduction of photosynthesis and chlorophyll concentration (Keisham et al. 2018; Negrao et al. 2017). Potassium has a noticeable role in adjusting the osmotic status within cells and mitigating sodium's toxic effects on plants (Almeida et al. 2017). The present research indicated the ameliorative roles of halo-tolerant PGPB on plant growth under salt stress conditions (Khan et al. 2019; Kim et al. 2017) through different mechanisms, such as lowering the ratio of Na<sup>+</sup>/K<sup>+</sup> in various plants like maize and soybean (Khan et al. 2019; Ashraf et al. 2004; Nadeem et al. 2010).

The results of our study demonstrated different nodule endophytic isolates with various impacts in terms of the inhibition of phytopathogens and their tolerance of salinity and high temperatures.

Recently, endophytic bacteria have been used as a bio-source to improve plant growth and decrease the need for chemical fertilisers. They have also decreased toxins in sustainable agriculture. To our knowledge, the genera examined in this study (*Brevundimonas* and *Kocuria*) have not been reported as endophytes of legume nodules. The inoculation of isolated bacteria in the soil demonstrated that bacterial inoculation ameliorated salinity stress in plants and increased their 1000-grain weights. According to their plant promotion characteristics—especially phosphorus dissolution by *Brevundimonas*—they could have biotechnological applications as microbial inoculants.

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## Compliance with ethical standards

**Conflict of interests** The authors have declared no conflict of interests.

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