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Bioprospecting beneficial endophytic bacterial communities associated with Rosmarinus officinalis for sustaining plant health and productivity

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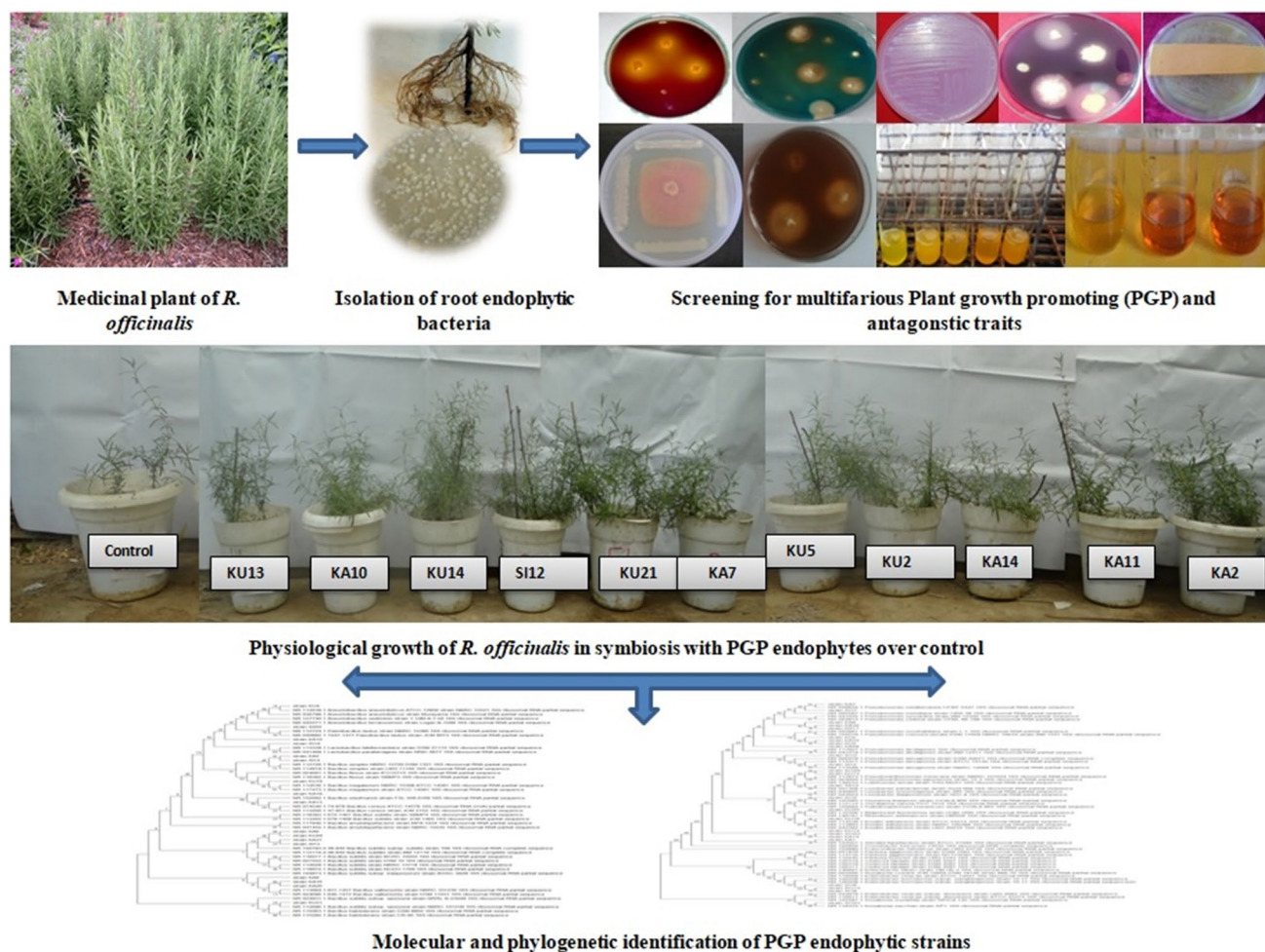
Abstract

The present study aimed to isolate and identify root endophytic bacteria with multifunctional plant growth promoting (PGP) traits from medicinal plant *Rosmarinus officinalis* grown in the North-Western Himalayas. A total of 42 strains were isolated, exhibiting variable degrees of PGP traits, including phosphate solubilization (10–375 μg/mL), indole-3-acetic acid (6–66 μg/mL), siderophore (32.37%–301.48% SU) production and antifungal activity in terms of percent growth inhibition (% GI) against *Fusarium oxysporum* (44.44%–77.77% GI), *Fusarium graminearum* (48.88%–71.42% GI) and *Rhizoctonia solani* (44.44%–77.7% GI). The 16S rDNA sequencing results showed lineage of these strains to 15 genera viz*., Aneurinibacillus*, *Bacillus*, *Beijerinckia*, *Cedecea, Ensifer*, *Enterobacter, Kosakonia*, *Lactobacillus*, *Lysobacter*, *Oxynema*, *Pseudomonas*, *Pantoea*, *Paenibacillus*, *Pseudoxanthomonas* and *Serratia*. Out of 42 strains, 11 potential strains were selected for in vivo growth studies of *R. officinalis*. The results showed that the inoculation of *Bacillus subtilis* KU21, *Pseudomonas aeruginosa* SI12, and *Cedecea lapagei* KU14 significantly increased the physical growth parameters of plant over uninoculated control viz., number of lateral of branches (43.95%–46.39%), stem height (29.04%–38.57%), root length (32.31%–37.14%), shoot (34.76%–40.91%) and root biomass (62.89%–70.70%). Physiological characteristics such as total chlorophyll (30.41%–30.96%), phenol (14.43%–24.55%) and carotenoids (34.26%–39.87%) content, also showed a relative increase as compared to uninoculated control; furthermore, the macronutrients (NPK) contents of the plant as well as soil also showed an increase. The developed module may be recommended for sustainable production of *R. officinalis* in the North-Western Himalayan region without hampering the soil health and fertility.

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Graphic abstract

Medicinal plant of *R. officinalis*

Isolation of root endophytic bacteria

Screening for multifarious Plant growth promoting (PGP) and antagonstic traits

Physiological growth of *R. officinalis* in symbiosis with PGP endophytes over control

Molecular and phylogenetic identification of PGP endophytic strains

Introduction

Plants develop an intricate association with a variety of microorganisms including rhizospheric, and endophytic, which have attracted the attention of scientific community due to their verified benefits (Cordero et al. 2017; Liotti et al. 2018). These microorganisms are natural inhabitants of host plant and can also develop in an endogenous fashion. This is true for endophytic microorganisms, which colonize plant tissues for at least a part of their life-cycle without any visible disease symptoms (Silva et al. 2019). Bacteria of endophyte community are diverse and able to disperse throughout plant tissues systemically (Bacon and White 2016). Such colonization provides many advantages as this community, independent of its environmental circumstances, can interact effectively with the plant (Santoyo et al. 2016). This improves plant growth and a defense against plant pathogens, which further enhances stress tolerance, and can promote the synthesis or development of bioactive compounds of interest (Khamwan et al. 2018; McMullin et al. 2018).

Various genera of endophytic bacteria have been isolated from diverse type of plants, facilitating the growth of host plants under different agro-climatic conditions (Afzal et al. 2019; Liu et al. 2017). These include *Bacillus* and *Brevibacillus* (ALKahtani et al. 2020), *Burkholderia* and *Enterobacter* (Adhikari and Pandey, 2020), *Enterobacter* (Panigrahi et al. 2019) *Acinetobacter* and *Pantoea* (Faria et al. 2021).

Several studies have shown that roots associated bacterial strains can promote plant growth by mechanisms such as phytostimulation, biofertilization, and biocontrol (Gaiero et al. 2013; Abbamondi et al. 2016). Such association has direct physiological effects on plant growth and production, such as: nitrogen fixation, phosphate

solubilization (P-solubilization), production of ammonia, siderophores, phytohormones and hydrolytic enzymes (Silva et al. 2019; Glick 2012). These effects provide for the need of plant nutrition in a sustainable manner, thereby reducing the use of chemical fertilizers and pesticides. This in turn preserves the soil biological diversity, thus providing an alternative to the conventional methods of cultivation and compelling the researchers to look for eco-friendly and sustainable agriculture production methods. To this end, the use of plant growth promoting rhizobacteria (PGPR) has emerged as an attractive approach in rosemary production (Sadegh Kasmaei et al. 2019). They are often used as plant growth enhancers under both normal and stressful conditions (Ahemad and Kibret, 2014).

Medicinal plants choose endophytes in a particular way because the selection may be dependent on the secondary metabolites provided by the plant and the composition of the root exudates; therefore, these microbial communities diversify based on their nutritional requirements, the form of soil, and the atmosphere in which they are located (Maggini et al. 2018).

Rosmarinus officinalis (rosemary) is a member of Labiatae family and is one of the most popular medicinal herbs rich in polyphenols (carnosic acid and rosamarinic acid) and flavanoids. It is native to Mediterranean region and cultivated worldwide in cool regions at elevations of 1000–3000 m above sea level (masl) i.e. semiarid and sub humid bioclimatic regions. In Himachal Pradesh, rosemary is grown at an elevation range of 1050–2100 masl falling in the mid-hills sub humid and high hills temperate wet zone. Owing to its numerous biological activities (antibacterial, antiproliferative, anti-inflammatory, and antioxidant), this plant has gained more interest from commercial point of view (Bourhia et al. 2019). However, to date only few studies have explored the endosphere of rosemary regarding plant health (Ahmed et al. 2019). Some studies have been carried out previously to evaluate the impact of commercially available PGPR (*Pseudomonas fluorescens*) on growth parameters and quality of bioactive compounds (flavonoids, phenolic acids, especially rosmarinic acid) of *R. officnalis* (Dehghani et al. 2019; Sadegh Kasmaei et al. 2019). So far, no published study has correlated the effect of native PGP bacterial endophytes to the growth of *R. officnalis* in North-Western Himalayas. The hypothesis underlying the present study is that the bacterial endophytes of this plant are promising bio-inoculants that can improve plant health. The outline of this study concentrates on assessment of functional diversity of culturable root bacterial endophytes of *R. officinalis* and in vivo studies of potential strains on the productivity of *R.officinalis* and soil functions.

Materials and methods

Collection of root samples

Root samples of *R. officinalis* seedling were collected during the summer (June 2017) from rosemary cultivating sites (Kangra, Kullu, Solan, and Sirmour) of Himachal Pradesh. Two year old plants (in vegetative state) were selected for sampling from all locations. Geographical coordinates and environmental conditions of sampling sites are presented in Table S1. A total of twenty-four (3 samples × 8 sites) composite root samples were obtained from all the locations and stored in plastic bags at 4 °C for further assaying of bacterial community structure.

Isolation of bacterial endophytes

Surface sterilization of roots was performed for isolation of bacterial endophytes following the standard method (de Favaro et al. 2012) with slight modification as follows: root samples were washed under running water, sterilized with 70% ethanol for 45 s, and 2% sodium hypochlorite for 5 min followed by washing 4–5 times with sterile distilled water. The surface sterility of roots was cross-checked by plating 100 μL of the final wash and incubated overnight at 30 ºC. No growth on plate indicated absence of microorganisms on the root's surface. One gram of surface sterilized root sample was placed in 9 mL of sterilized distilled water and was ground to produce slurry using pestle and mortar under aseptic conditions. The root suspension was diluted in ten-fold series and bacterial count was determined by standard spread plate technique. An aliquot of 100 μL of suspension (10^{-2}-10^{-4} dilutions) was spread on nutrient and tryptic soy agar medium (Collins and Lyne 1984; Zhao et al. 2015) and incubated at 30 ºC till the appearance of bacterial colonies (up to 5 days). Isolated bacteria were enumerated as colony forming units per gram of roots (cfu/g root).

A total of 42 distinct morphotypes were selected and purified on nutrient and tryptic soy agar medium. The pure culture of these strains was preserved on petri plates at 4 °C for further analysis.

Morpho-biochemical characterization of endophytic bacteria.

Microscopic examination of endophytic bacteria was done together with biochemical characterization according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). Antibiotic resistance pattern of isolates was determined by testing their tolerance to intrinsic levels of various antibiotic kits (Hexa universal 1, 2 and Hexa Pseudo 1, 2) (Himedia, India) using disc diffusion method (Bakthavatchalu et al. 2012).

Identification of endophytic bacteria

16S rDNA sequence analysis was employed for molecular identification of isolated bacteria. Genomic DNA was extracted using the conventional method (Sambrook et al. 1989) followed by PCR-mediated amplification with a set of universal primers (16SF: 5'- AGAGTTTGATCCTGGCTC AG-3'and16SF: 5'-AAGGAGGTGATCCAGCCGCA-3') (Mehta et al. 2013). PCR reaction mix of 25 μL was prepared with 50 ng of template DNA, 20 pmol of each primer, 0.2 mM dNTPs, and 1U Taq polymerase in 1X PCR buffer. The reaction was cycled 35 times as denaturation at 94 ℃ for the 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 1 min 30 s followed by a final extension at 72 ℃ for 10 min. The PCR product was analyzed by gel electrophoresis on 1.2% (w/v) agarose gel. A band of ~ 1400 bp was excised from the gel and purified using a gel extraction kit (RBCs Real Genomics, New Taipei City, Taiwan) and was sequenced by GeNei™ Laboratories (Bengaluru, India). Based on 16S rDNA sequences, phylogenetically related bacteria were aligned using a BLASTn search (Altschul et al. 1997). Multiple alignments with sequences of related taxa were implemented using ClustalW (Higgins et al. 1994). A neighbor-joining phylogenetic tree was constructed with other 16S rDNA sequences of the related taxa retrieved from the GenBank database using MEGA X software.

In vitro screening for traits involved in plant growth promotion

Isolated bacteria were further authenticated by subsequent in vitro experiments to see whether they exhibited qualities which identified them as possible plant growth promoting bacterial endophytes (PGPBEs). Each in vitro screening test was conducted in triplicates. P-solubilization activity was determined by the method of Pikovskaya (1948). Quantitative production of indole-3-acetic acid (IAA) was estimated using the colorimetric method described by Gorden and Palleg (1957). To test the efficacy of endophytic bacteria as nitrogen fixers, loop full of 24 h old culture of each isolate were streaked on nitrogen free agar medium (Table S2) and incubated for 72 h at 30 ℃. Colonies showing growth on inoculated medium after being transferred ten times in the same medium were potent nitrogen fixers (Jensen 1987). The ability of isolates to produce siderophore and hydrocyanic acid (HCN) was also assessed by Schwyn and Neilands (1987) and Bakker and Schipper (1987) methods, respectively. For lytic enzyme activity, spot inoculation was done on minimal agar medium (Table S2) amended with 0.3% colloidal chitin for chitinase (Robert and Selitrennikoff 1988), starch agar medium for amylase (Shaw et al. 1995) and skim milk agar plates for protease activity (Fleming et al. 1975).

Ammonia production was observed according to the method of Cappuccino and Sherman (1992).

The antagonistic activity of the bacterial isolates against test fungal pathogen viz., *F. oxysporum* (ITCC 7337), *F. graminearum* (ITCC 5334) and *R. solani* (ITCC 5308), was done by agar dual plate method (Vincent 1947). A loopfull of 48 h old culture of each isolate was streaked a little below the centre of the prepoured petriplates of malt extract agar (MEA) and incubated at 37 ℃. After 24 h, a 5 mm diameter actively growing test fungal pathogen was placed simultaneously on one side of the streak. The petri plate inoculated with the test pathogen only was kept as control for comparison. The plates were incubated at 24 ± 1 ℃ and % GI was calculated using following formula:

$$\%GI = \frac{C - T}{C} \times 100$$

where,

C = Growth of fungus in control.

T = Growth of fungus in treatment.

Stimulation of plant growth

To test the efficacy of endophytic strains for stimulating plant growth, a pot experiment was conducted for a period of four months (March–June, 2018) at Department of Basic Sciences, College of Horticulture, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni-Solan, Himachal Pradesh (India).

Eleven isolates with best ex situ PGP traits were selected. The potting mixture was prepared by mixing sand, soil and farm yard manure (FYM) in a ratio of 1:2:1. The following mixture was then subjected to intermittent sterilization i.e. three successive autoclave cycles of 1 h each at 100 ℃ with 24 h of incubation between each cycle (tyndallization). The pH of potting mixture was determined in 1:2.5 (soil:water) suspension and the electrical conductivity (E.C.) of the supernatant liquid was recorded and expressed in dSm^{-1} (Jackson 1973). Furthermore, organic carbon (O.C.) was determined by chromic acid titration method of Walkley and Black (1934). Available N, P, and K contents of soil were determined following standard procedures (Tandon 2009).

The properties of the potting mixture were: pH 7.01; E.C. 0.69 dSm^{-1}; O.C. 1.12%; available N, P and K contents 290.32, 23.40 and 315.45 kg/ha, respectively. The soil used for pot (20.00 cm diameter and 16.00 cm deep) experiment belongs to Entisols order as per United States department of Agriculture Soil Taxonomy. 4 kg of potting mixture was filled in the pots before commencement of experiment. Two months old seedlings of *R.officinalis* were procured from the Department of Forest Products, College of Forestry, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni-Solan, Himachal Pradesh (India) and were

washed under running tap water to remove the soil adhering to the roots. Pure cultures of bacterial isolates were inoculated into 100 mL flask containing nutrient broth and grown aerobically overnight on a rotary shaker (Thermo Scientific™ Sorvall™ ST 8) at 30 ℃ (120 rpm). Bacteria were subsequently pelleted by centrifugation at 6000 rpm for 10 min. The pellets were washed with sterile distilled water three times, and the concentration of cells adjusted to 1×10^8 cfu/mL by dilution. This liquid formulation was used as inoculum (Mortensen 1997). Roots of seedlings were immersed in prepared inoculum for about 1 h before plantation. Two seedlings per pot were planted and allowed to grow for four months. Booster doses of liquid bacterial cultures of the same cell density were applied at the rate of 20 mL/plant near the root zone with 15 days interval after planting (3 times). Seedlings were watered daily during the first two weeks of planting followed by irrigation once every two days. The following 12 treatments were arranged in a completely randomized block design (CRD) with three replications for each treatment: T1, control (uninoculated); T2, *Pseudomonas mediterranea* KA7; T3, *Pseudomonas oryzihabitans* KU5; T4, *Bacillus flexux* KA10; T5, *Pseudomonas aeruginosa* SI12; T6, *Pseudoxanthomonas japonensis* KU13; T7, *Pseudomonas putida* KU2; T8, *Cedecea lapagei* KU14; T9, *Pantoea agglomerans* KA14; T10, *Pseudomonas koreensis* KA11; T11, *Bacillus simplex* KA2; T12, *Bacillus subtilis* KU21.

Observations on plant growth parameters such as shoot and root length (cm), biomass (g), and the number of lateral branches per plant were recorded by following standard methods. Oven-dried plant samples were ground and sieved for the estimation of macronutrients (NPK). The total concentration of N in plant samples was determined using micro-Kjeldhal's method (Helrich 1990). Plant samples were digested in a diacid mixture of HNO_3:$HCLO_4$ (4:1) for P and K analysis (Jackson 1973). P concentration was tested in the digested sample (Jackson 1973). K in the digest was analyzed using the flame photometer (Biogen, Microcontroller Flame Photometer) (Jackson 1967). Total chlorophyll, carotenoids, and total phenol content (TPC) of leaf samples were determined using methods of Withem et al. (1971), Yang et al. (1998), and Faust and Mikulewics (1967), respectively.

Isolation of endophytic bacteria from experimentally inoculated plants

To test whether the bacterial strains were capable of colonizing plant tissues, isolation of the total endophytic bacterial population was done after termination of the experiment. For this, root samples were collected from each treatment including control and standard serial dilution spread plate technique was employed as described in the previous section (isolation of bacterial endophytes). All bacterial endophytes recovered were compared with control data to distinguish growth of introduced bacteria from the presence of indigenous microorganisms. Colonies which showed morphological characteristics similar to treated strain were selected and identified biochemically as per Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The dominance of inoculated strain was calculated according to Simpson's index of dominance (D) as:

$$D = Pi^2$$

where, Pi is the relative abundance of isolates calculated according to the following equation Pi = ni/N.

n_i, is the number of inoculated strain colonies and N, is the total number of endophyte colonies.

Statistical analysis

All the experiments were conducted under the statistical framework with three replications per treatment along with appropriate controls replicated three times. The data obtained from the laboratory experiments and the net house was subjected to one-way analysis of variance (ANOVA) using SPSS version 16 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 7.0 (Microsoft, Redmond, WA, USA). The means and standard deviation of data were also calculated. Comparisons of treatment means were performed by the Fisher's Projected LSD (least significant differences) test at $P \leq 0.05$ level of significance. PCA (principal component analysis) was performed on pot experiment, to evaluate the relationship between effects of endophyte inoculation on several plant growth parameters. PCA was performed using PAST 3.0 software.

Results

Isolation and identification of endophytic bacteria

A total of 42 strains were isolated based on unique colony morphologies on medium used for isolation. Amongst these, 17 were Gram's positive while 25 were Gram's negative, varied from rods, cocci to coccobacilli. Endospore formation was observed in 16 isolates. Biochemically, low number of isolates were positive for indole (28.57%) and hydrogen sulfide (H_2S) (9.52%) production. Greater numbers of bacterial isolates were positive nitrate reduction (80.95%) and oxidase production (61.90%). All the isolates were positive for catalase except KU20 and KU25. 59.52% were positive for methyl red, 40.47% showed positive test for voges proskauer (VP); only 21.43% bacterial isolates were able to hydrolyse gelatin. Almost all the isolates were able to

ferment dextrose and sucrose, whereas a few isolates showed positive lactose fermentation test (Table S3).

Phylogenetic analysis based on 16S rDNA sequencing and alignment showed that these endophytes were affiliated to 2 phyla, i.e., Proteobacteria and Firmicutes, 15 genera viz., *Aneurinibacillus*, *Bacillus*, *Beijerinckia*, *Cedecea, Ensifer, Enterobacter, Kosakonia, Lactobacillus, Lysobacter, Oxynema, Pseudomonas, Pantoea, Paenibacillus, Pseudoxanthomonas* and *Serratia* and 32 species (Fig. 1a, b). The most dominant endophytic bacteria were reported in genera *Bacillus* and *Pseudomonas* representing 33.33% and 25.64% of the total isolates. A detailed account of the identities of these isolates has been presented in Table S4.

Beneficial Plant traits of bacterial strains

In vitro screening revealed that most of the strains exhibited multiple PGP activities (Fig. 2). All the strains substantially solubilized mineral P in PVK broth (70–375 µg/mL). All the strains of genera *Cedecea, Ensifer, Enterobacter, Lactobacillus, Oxynema, Paenibacillus* and *Serratia* showed IAA and siderophore production and ability to fix nitrogen. Majority of strains belonging to genera *Bacillus, Pseudomonas, Pantoea* and *Pseudoxanthomonas* were IAA (10, 10, 2 and 2 no. of strains) and siderophore (12, 11, 2 and 2 no. of strains) producers and fixed nitrogen (8, 10, 1 and 1 no. of strains), respectively. The strain of genus *Beijerinckia* produced siderophore and fixed nitrogen but was deficient in IAA production. Isolates of genera *Aneurinibacillus*, *Lysobacter* and *Kosakonia* produced IAA (1, 1and 3) and siderophore (1, 1 and 2 no.), respectively but did not showed nitrogen fixing ability. In total, 85.71% isolates produced IAA (10–66 µg/mL) and 92.85% were siderophore producers (35.71%-301.48% SU). 66.66% strains had ability to fix nitrogen on Jensen's nitrogen-free medium (Fig. 2).

All the bacterial strains were able to produce one or more cell wall degrading enzymes. The isolates belonging to several genera *Paenibacillus*, *Aneurinibacillus, Bacillus*, *Beijerinckia, Cedecea, Kosakonia, Lysobacter, Oxynema, Pseudomonas, Pantoea, Pesudoxanthomonas* and *Serratia* were positive for chitinase (1, 1, 10, 1, 1, 1, 1, 1, 7, 2, 2 and 1 no. of strains), protease (1, 1, 12, 1, 1, 1, 1, 1, 10, 2, 2 and 1 no. of strains) and amylase production (1, 1, 12, 1, 1, 1, 2, 1, 1, 8, 1 and 1 no. of strains), respectively. Genus *Lactobacillus* was unable to produce chitinase and protease but was positive for amylase (1 strain) production. Similarly, strains of genera *Ensifer* and *Enterobacter* produced chitinase (1, 1 no. of strains) and amylase (1, 1 no. of strains), respectively but lacked protease activity. In total, 73.81% strains exhibited chitinase activity, and 80.95% were protease and amylase producers. Majority of bacterial strains (88.09%) displayed ammonia production [*Aneurinibacillus* (1 strain), *Bacillus* (11 strains)*, Beijerinckia* (1 strain),

Cedecea (1strain), *Ensifer* (1strain), *Enterobacter* (1strain), *Kosakonia* (3 strain), *Lactobacillus* (1strain), *Lysobacter* (1strain), *Oxynema* (1strain), *Pseudomonas* (10 strain), *Pantoea* (1strain), *Paenibacillus* (1strain), *Pseudoxanthomonas* (2 strains) and *Serratia* (1strain)], whereas; only 40.47% were HCN producers [*Aneurinibacillus* (1 strain), *Bacillus* (7 strains), *Cedecea* (1 strain), *Pseudomonas* (6 strain) and *Paenibacillus* (1 strain) and *Serratia* (1 strain)] (Fig. 2).

The antagonistic activity was observed for endophytic strains against phytopathogenic fungi (*F. oxysporum*, *F. graminearum* and *R. solani*). 40.47% strains inhibited the growth of *F. oxysporum* (44.44%–77.77% GI), 33.33% and 26.19% strains were antagonist against *F. graminearum* (48.88%–71.42% GI) and *R. solani* (44.44%–77.77% GI), respectively. Only six strains (KA2, KA14, KU14, KU21, SI12 and SI13) showed antagonism against all fungal pathogens, out of which SI12 strain was most effective against all the three tested fungal phytopathogens (Table 1).

Effect of endophytic bacteria on plant growth promotion

A pot experiment was conducted to validate the in vitro PGP activities of selected strains (*Pseudomonas mediterranea* KA7, *Pseudomonas oryzihabitans* KU5, *Bacillus flexux* KA10, *Pseudomonas aeruginosa* SI12, *Pseudoxanthomonas japonensis* KU13, *Pseudomonas putida* KU2, *Cedecea lapagei* KU14, *Pantoea agglomerans* KA14, *Pseudomonas koreensis* KA11, *Bacillus simplex* KA2, *Bacillus subtilis* KU21) on the growth of *R. officinalis*. The in vitro PGP traits of selected strains have been depicted in Table 1.

Effect on physical characteristics

Most of the isolates significantly ($P \leq 0.05$) increased growth parameters of *R. officinalis* over uninoculated control. Treatments receiving *P. aeruginosa* SI12 (T5) inoculation showed maximum stimulatory effects for overall plant growth parameters over uninoculated control, which was statistically at par with the treatments receiving *C. lapagei* KU14 (T8) and *B. subtilis* KU21 (T12) inoculation (Table 2). These incremental effects were 43.95%, 45.61%, and 46.39% for number of lateral branches, 38.30%, 29.04% and 38.57% on stem height, 34.76%, 37.21% and 40.91% for shoot biomass, 62.89%, 70.70% and 63.29% for root biomass, respectively over untreated control.

Effect on physiological characteristics

Photosynthetic pigments of *R. officinalis* leaves were determined to evaluate the impact of endophytic strains on photosynthetic efficiency of host plant. Data corresponding to biochemical parameters revealed that treatment T5
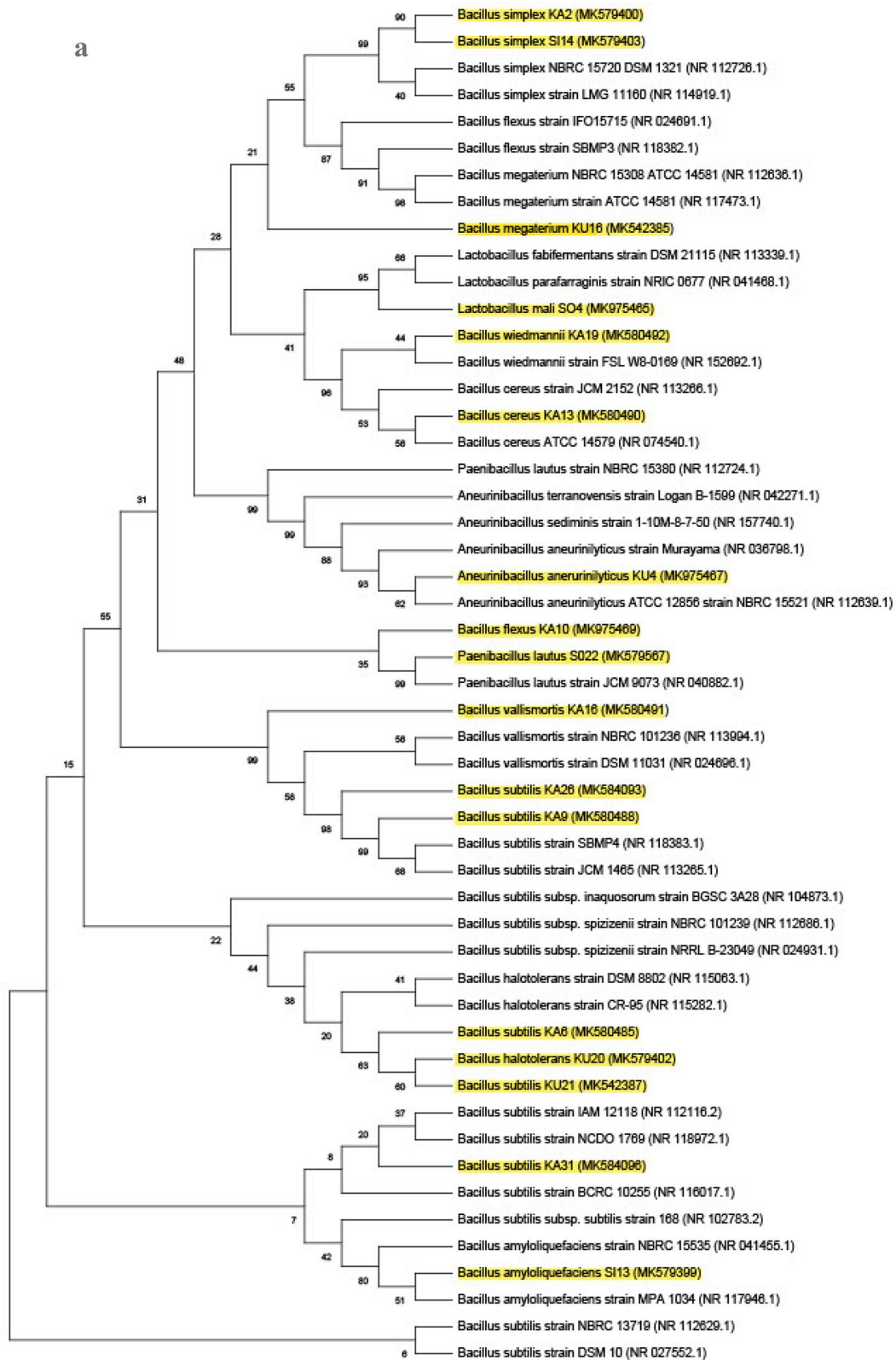
Fig. 1 Phylogenetic tree of gram positive endophytic isolates (highlighted) and their closely associated taxa obtained from GenBank based on 16S rDNA sequence (**a**); Phylogenetic tree of gram negative endophytic isolates (highlighted) and their closely associated taxa obtained from GenBank based on 16S rDNA sequence (**b**)
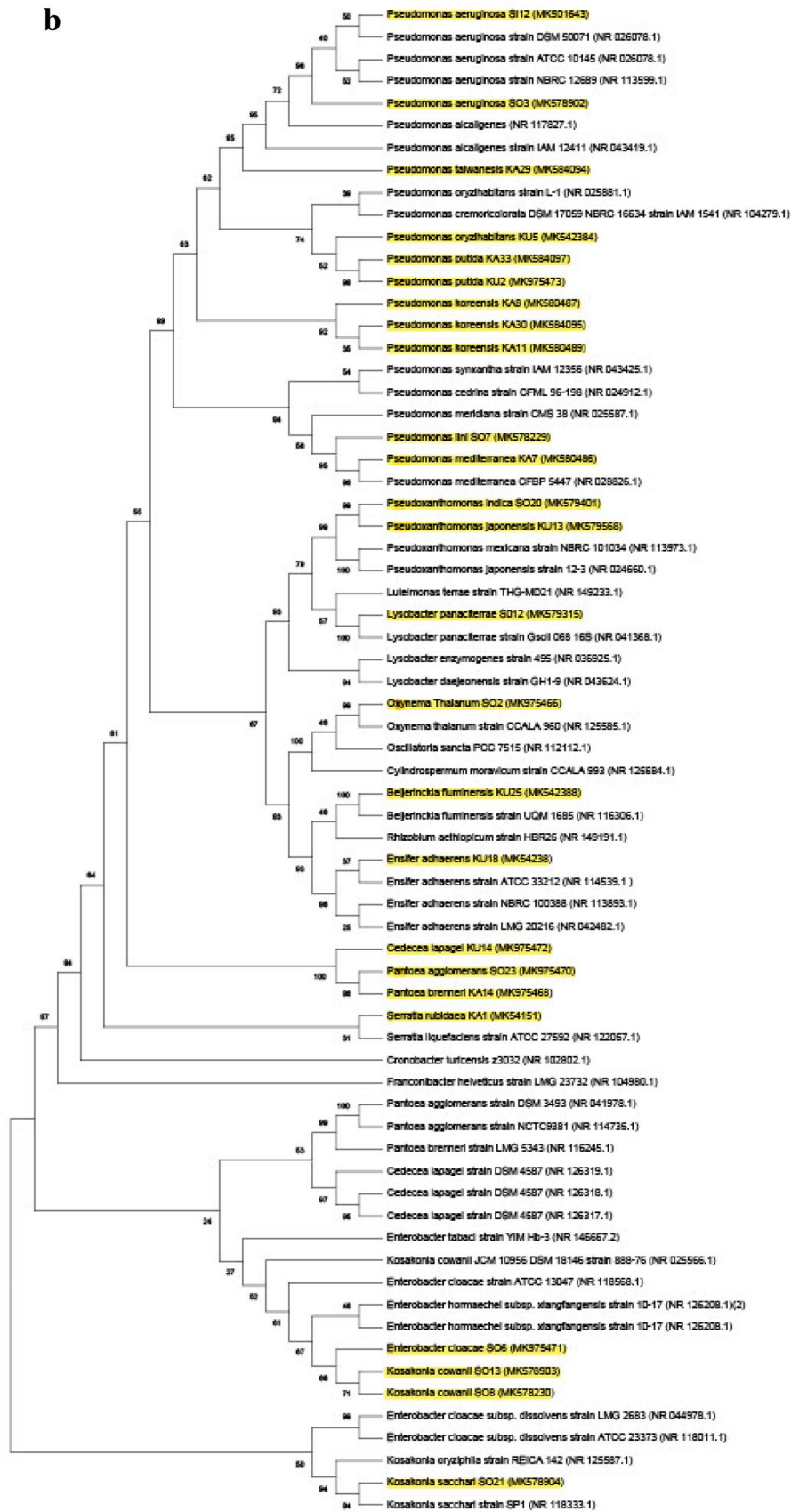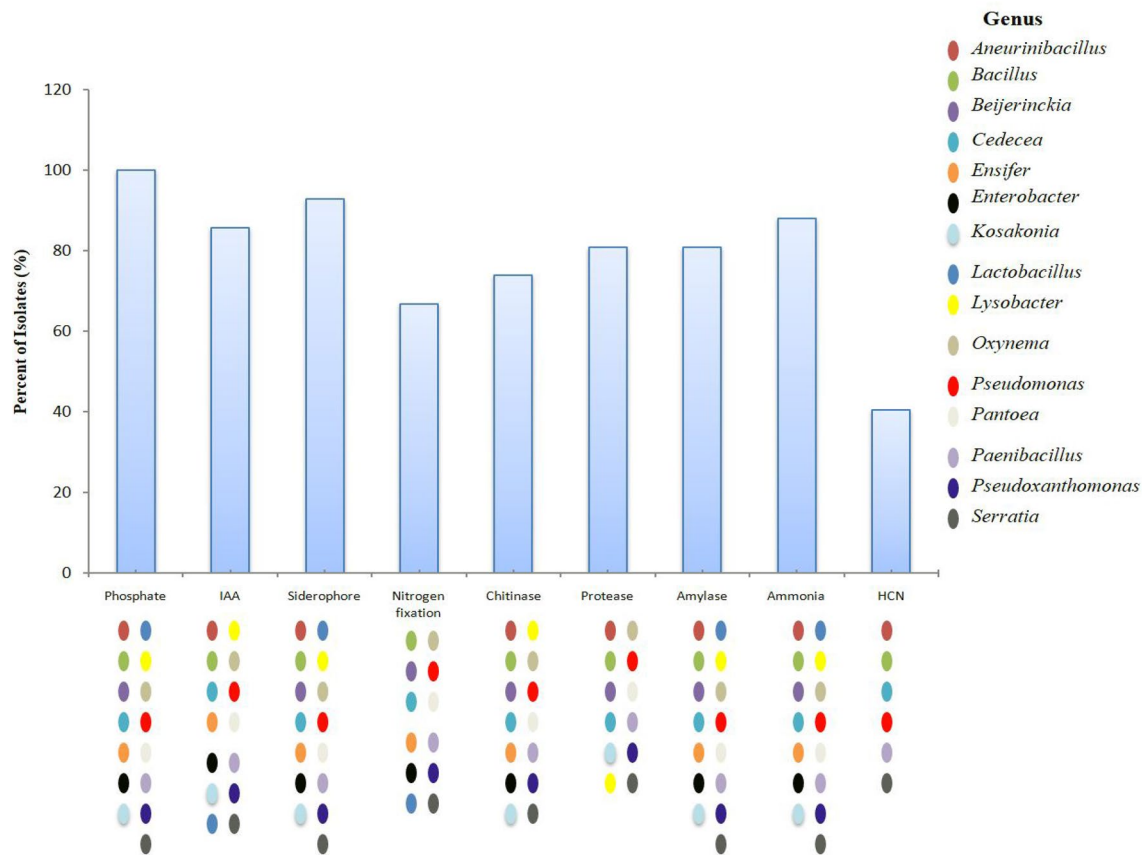
b

Fig. 1 (continued)

Fig. 2 In vitro screening for beneficial plant traits of bacterial strains. Colored dots represent different genus in which activities were observed

(*P. aeruginosa* SI12) had maximum significant ($P \leq 0.05$) increase in total chlorophyll (33.09%), carotenoids (39.87%) and total phenol content (24.55%) of *R. officinalis* leaves over uninoculated control, which was at par with T8, T11 and T12 for chlorophyll and treatments T3, T7, T8, T11 and T12 in case of carotenoids (Fig. 3).

Principal component analysis of growth characteristics of *R. officinalis* in response to inoculation with endophytic bacteria

Principal component analysis of growth parameters of *R. officinalis* revealed that PC1 and PC2 accounted for 94.67% and 3.93% of the total data variation, respectively (Fig. 4). PC1 comprised treatments with T3 (*P. oryzihabitans* KU5), T6 (*P. japonensis* KU13), T7 (*P. putida* KU2), and T9 (*P. agglomerans* KA14) showed a strong relationship with several lateral branches and stem height. While in PC2, treatments with T4 (*Bacillus flexus* KA10), T7 (*P. putida* KU2), and T9 (*P. agglomerans* KA14) reported more influence on root biomass, total chlorophyll, and phenol content. This analysis showed that inoculation with potential plant

growth-promoting bacterial endophytes had a significant effect on growth and productivity of *R. officinalis*.

Effect on plant nutrient concentration

The data appended in Fig. 5 illustrate that treatment (T12) receiving *B. subtilis* KU21 inoculation had maximum significant increase in N, P and K content by 33.33%, 61.54% and 54.54%, respectively over untreated control and was at par with T5 (*P. aeruginosa* SI12), T8 (*C. lapagei* KU14) and T11 (*B. simplex* KA2) for N content and with T5 (*P. aeruginosa* SI12), T7 (*P. putida* KU2), T8 (*C. lapagei* KU14), T9 (*P. agglomerans* KA14) and T11 (*B. simplex* KA2) for P content.

Effect on soil properties and endophytic population

None of the treatments influenced the soil pH, E.C. and O.C. significantly in comparison with the initial soil test values (data not shown) recorded before the trial whereas the contents of available nutrients (NPK) increased significantly by the sole application of endophytic strains (Fig. 6). Maximum significant increase in available NPK content (16.79%,

Table 1 Beneficial plant traits of endophytic bacteria 624 under in vitro conditions

Endophytic strains	Biofertilizer activities				Antagonistic properties							
	Phosphate solubilization (µg/mL)	IAA production (µg/mL)	Nitrogen fixing ability*	Siderophore production (% SU)	Lytic enzymes**			HCN Production#	Ammonia production##	Biocontrol activity (% GI)		
					Chitinase	Protease	Amylase			F.oxysporum	F. graminearum	R. solani
P. mediterranea KA7	170.00±2.88 fghi	35.00±1.73 f	++	74.32±1.16 gh	+	−	++	+	+	76.66 (61.10) b	55.55 (48.17) cd	0.00
P. oryzihabitans KU5	200.00±1.15 e	65.00±2.88 a	++	48.56±0.72 j	++	+	++	++	++	55.55 (48.16) d	0.00	77.77 (61.86) a
B. flexux KA10	145.00±4.04 j	15.00±0.58 j	++	78.23±1.59 fg	−	+++	+	++	+	0.00	0.00	72.22 (58.18) b
P. aeruginosa SI12	176.00±1.73 fg	48.00±2.89 bcd	+++	102.34±2.06 de	++	++	+++	+++	+++	77.77 (61.83) a	71.42 (57.66) a	77.77 (61.88) a
P. japonensis KU13	175.00±2.88 fgh	28.00±1.73 gh	+++	166.67±2.43 c	++	++	++	++	++	54.76 (47.72) de	0.00	0.00
P. putida KU2	215.00±5.77 d	24.00±1.73 ghi	++	220.31±0.78 b	+++	+++	+	++	++	44.44 (41.78) f	48.88 (44.34) de	0.00
C. lapagei KU14	250.00±2.31 c	30.00±2.89 fg	++	35.71±2.45 k		++	+	+++	++	77.77 (61.90) a	66.66 (54.72) b	55.55 (48.17) e
P. agglomerans KA14	320.00±6.92 b	30.00±1.73 fg	++	103.23±1.29 d	+++	+++	−	+++	+	76.66 (61.14) b	56.7 (48.83) c	66.66 (54.72) c
P. koreensis KA11	180.00±2.31 f	49.00±3.46 bc	+	81.90±1.87 f	++	+	−	++	+	77.77 (61.90) a	55.55 (48.17) cd	0.00
B. simplex KA2	115.00±1.15 k	42.00±1.16 de	+	71.43±2.19 hi	++	++	+	++	++	55.55 (48.17) d	55.55 (48.17) cd	55.55 (48.17) e
B. subtilis KU21	375.00±4.04 a	52.00±2.31 b	+++	301.48±1.62 a	+++	++	+++	+++	++	60.00 (50.75) c	66.66 (54.73) b	62.00 (51.93) d
LSD (P≤0.05)	10.79	6.66	−	5.17	−	−	−	−	−	3.21	2.37	2.52

*Values ranging from <3 mm (+) colony diameter; 3–6 mm (++) colony diameter; >6 mm (+++) colony diameter; **Values ranging from zone diameter of <1.5 mm (+); 1.5–3 mm (++); >3 mm (+++); no activity (-); #light brown color (+); dark brown colour (++); orange brown colour (+++); no activity (-); ##(+) fair; (++) good; (+++) very good; ammonia producers; no activity (-); Values in parentheses are arc sine-transformed; With in a column, means followed by the same letter are not significantly different based on LSD$_{0.05}$

Each value within columns represents mean of three replicates

Table 2 Effect of inoculation with endophytic bacteria on physical growth parameters of *R. officinalis*

Treatment	Number of lateral branches	Stem height (cm)	Root length (cm)	Root biomass (g/plant)	Shoot biomass (g/plant)
T1 (Control)	12.33 ± 0.19^{jkl}	29.80 ± 0.46^{l}	13.20 ± 0.12^{kl}	0.58 ± 0.05^{ijk}	3.35 ± 0.20^{hijkl}
T2 (*P. mediterranea* KA7)	15.88 ± 0.51^{de}	35.53 ± 0.71^{efg}	15.84 ± 0.49^{defg}	0.74 ± 0.02^{efgh}	3.90 ± 0.52^{cdefgh}
T3 (*P. oryzihabitans* KU5)	15.53 ± 0.31^{defg}	36.11 ± 0.49^{def}	15.96 ± 0.55^{def}	0.76 ± 0.03^{defg}	3.88 ± 0.58^{cdefghi}
T4 (*B. flexux* KA10)	13.41 ± 0.62^{ij}	31.48 ± 0.66^{k}	14.89 ± 0.28^{efghij}	0.69 ± 0.04^{ghijk}	3.59 ± 0.29^{fghijk}
T5 (*P. aeruginosa* SI12)	17.74 ± 0.43^{abc}	41.21 ± 0.43^{ab}	18.72 ± 0.42^{a}	0.99 ± 0.05^{a}	4.51 ± 0.59^{abc}
T6 (*P. japonensis* KU13)	12.55 ± 0.56^{jk}	33.51 ± 0.63^{j}	14.01 ± 0.58^{jk}	0.72 ± 0.03^{ghi}	3.69 ± 0.17^{fghij}
T7 (*P. putida* KU2)	14.52 ± 0.30^{ghi}	36.77 ± 1.01^{de}	16.08 ± 0.05^{de}	0.87 ± 0.04^{abcdef}	4.37 ± 0.21^{abcd}
T8 (*C. lapagei* KU14)	17.95 ± 0.55^{ab}	38.45 ± 0.91^{c}	17.46 ± 0.26^{bc}	0.94 ± 0.02^{ab}	4.59 ± 0.42^{ab}
T9 (*P. agglomerans* KA14)	15.82 ± 0.47^{def}	35.13 ± 0.97^{ghi}	15.30 ± 0.17^{efgh}	0.90 ± 0.12^{abcd}	4.08 ± 0.05^{bcdefg}
T10 (*P. koreensis* KA11)	14.95 ± 0.54^{efgh}	35.46 ± 1.19^{efgh}	15.23 ± 0.49^{efghi}	0.88 ± 0.06^{abcde}	4.09 ± 0.21^{abcdef}
T11 (*B. simplex* KA2)	16.51 ± 0.29^{cd}	37.51 ± 0.79^{cd}	16.61 ± 0.35^{cd}	0.92 ± 0.01^{abc}	4.35 ± 0.20^{abcde}
T12 (*B. subtilis* KU21)	18.05 ± 0.58^{a}	41.29 ± 0.745^{a}	18.10 ± 0.58^{ab}	0.94 ± 0.03^{ab}	4.72 ± 0.58^{a}
LSD ($P \leq 0.05$)	1.27	1.49	1.19	0.14	0.63

The data represent mean of 3 replicates ± standard error (SE). Within columns, means followed by same letter are not significantly different ($P \leq 0.05$)
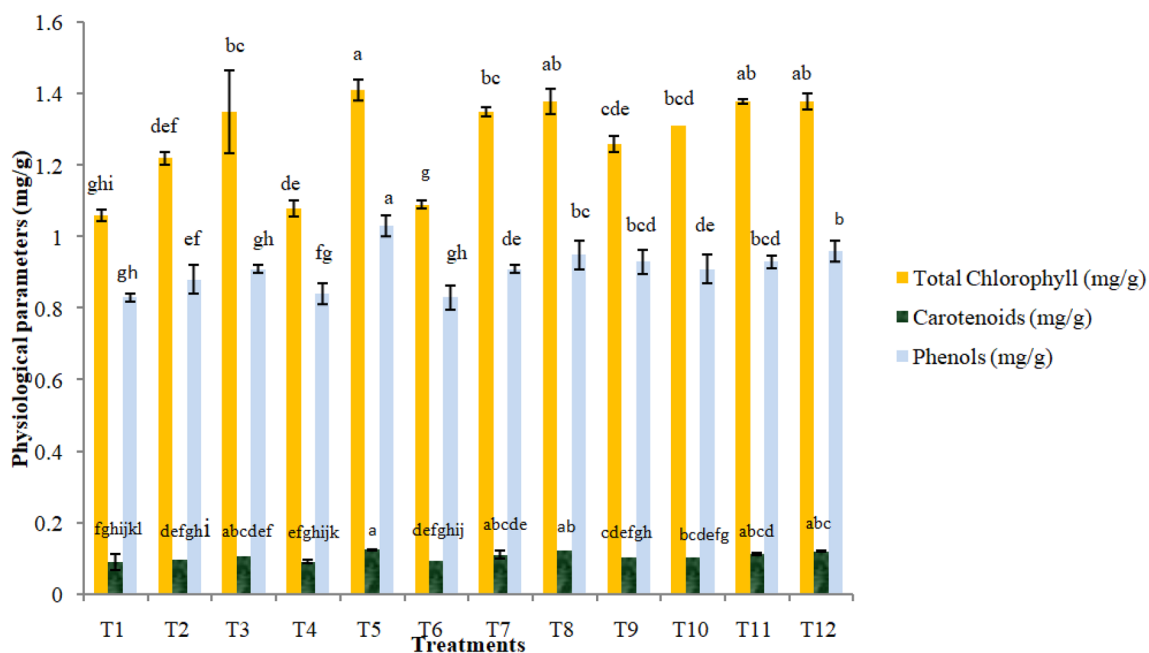

Fig. 3 Effect of inoculation with endophytic bacteria on physiological characteristics of *R. officinalis*. Bars marked by different letters indicate significant differences based on LSD at $P \leq 0.05$; error bars indicate means ± SE by LSD test (n = 3). T1 (Control); T2 (*P. mediterranea* KA7); T3 (*P. oryzihabitans* KU5); T4 (*B. flexux* KA10); T5 (*P. aeruginosa* SI12); T6 (*P. japonensis* KU13); T7 (*P. putida* KU2); T8 (*C. lapagei* KU14); T9 (*P. agglomerans* KA14); T10 (*P. koreensis* KA11); T11 (*B. simplex* KA2); T12 (*B. subtilis* KU21)

36.26% and 7.56%) were recorded in treatment T12 (*B. subtilis* KU21) which was statistically at par with T8 (*C. lapagei* KU14) for N, T5 (*P. aeruginosa* SI12) and T8 (*C. lapagei* KU14) for P and T11 (*B. simplex* KA2) for K content. None of the treatments influenced the soil pH, E.C., O.C. significantly over soil initial test value (data not shown).

Total endophytic bacterial count varied from 31.00 to 54×10^2 cfu/g root with the maximum count (54×10^2 cfu/g) in *B. subtilis* KU21 (T12) inoculated plants (Table 3).
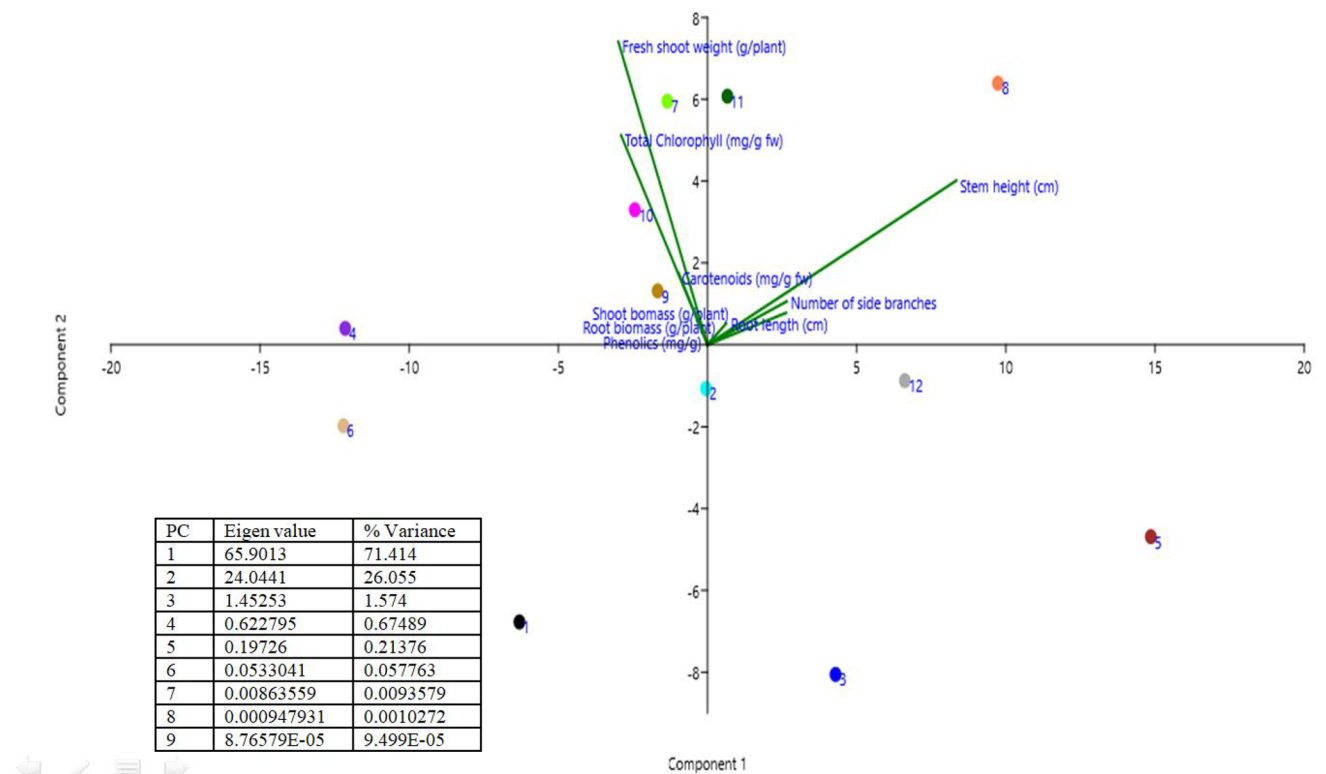
Fig. 4 PCA of growth characteristics of *R. officinalis* in response to endophytic bacterial isolates inoculation.1-T1 (Control), 2-T2 (*P. mediterranea* KA7), 3-T3(*P. oryzihabitans* KU5), 4-T4 (*B. flexux* KA10), 5-T5 (*P. aeruginosa* SI12), 6-T6 (*P. japonensis* KU13), 7-T7 (*P. putida* KU2), 8-T8 (*C. lapagei* KU14), 9-T9 (*P. agglomerans* KA14), 10-T10 (*P. koreensis* KA11), 11-T11 (*B.simplex* KA2), 12-T12 (*B.subtilis* KU21)

Colonization studies with endophytic bacteria

In order to determine the ability to colonize and survive inoculated strains within plant roots, isolation of root endophytic bacteria from each treatment was done at termination of experiment. Control (uninoculated) was also used in colonization studies to ensure that the inoculated bacteria were recovered. In general, the representative control plant roots yielded no indigenous strain. Furthermore, the colony morphologies of the endophytes retrieved from inoculated plants were indistinguishable from the morphologies of the inoculated strains. The isolated strains with similar colony morphology were further identified biochemically and showed similar trends as that of inoculated ones (Table S3). The root endosphere of *B. subtilis* KU21 (T12) inoculated plants accounted for a maximum number (48×10^2 cfu/g) of bacterial colonies matching *B. subtilis* KU21 with maximum Simpson's index of dominance (0.79).

Intrinsic antibiotic resistance (IAR) of potential endophytic bacteria

Assessment of the IAR pattern of *B. subtilis* KU21, *C. lapagei* KU14 and *P. aeruginosa* SI12 showed considerable variation in terms of zone of clearance around the antibiotic discs (Table 4). It was observed that the tested strains were resistant to most of the antibiotics. But polymyxin B and colistin was lethal to *P. aeruginosa* SI12. Similarly, *C. lapagei* KU21 was susceptible to neomycin and Co-trimoxazole. Amikacin inhibited the growth of all three tested strains.

Discussion

Despite the great interest in plants used for the purpose of traditional medicine, little is known about the symbiotic associations of these plants with endophytic microorganisms (Silva et al. 2019). The present study evaluated the multifunctional potential of bacterial strains isolated from the roots of *R. officinalis* native to the North-Western Himalayan region of Himachal Pradesh, India. In this study, a collection of 42 root endophytic bacteria of *R. officinalis* were obtained from 4 different rosemary cultivating locations of Himachal Pradesh. These strains were identified using 16S rDNA sequencing and phylogenetic analysis. The isolates belonged to 15 genera and 32 species, mainly belonging to Proteobacteria and Firmicutes. These results confirmed
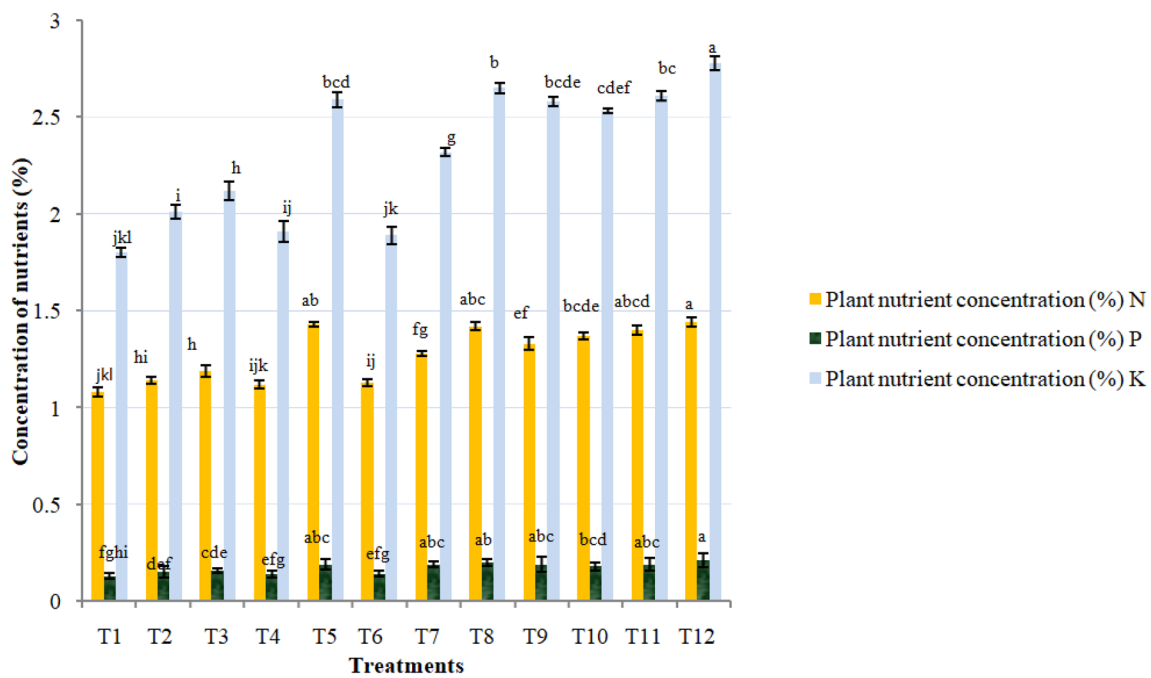
Fig. 5 Effect of liquid bacterial inoculum on nutrient concentration of *R. officinalis*. Bars marked by different letters indicate significant differences based on LSD at $P \leq 0.05$; error bars indicate means $\pm$ SE by LSD test (n = 3). T1 (Control); T2 (*P. mediterranea* KA7); T3 (*P. oryzihabitans* KU5); T4 (*B. flexux* KA10); T5 (*P. aeruginosa* SI12); T6 (*P. japonensis* KU13); T7 (*P. putida* KU2); T8 (*C. lapagei* KU14); T9 (*P. agglomerans* KA14); T10 (*P. koreensis* KA11); T11 (*B. simplex* KA2); T12 (*B. subtilis* KU21)
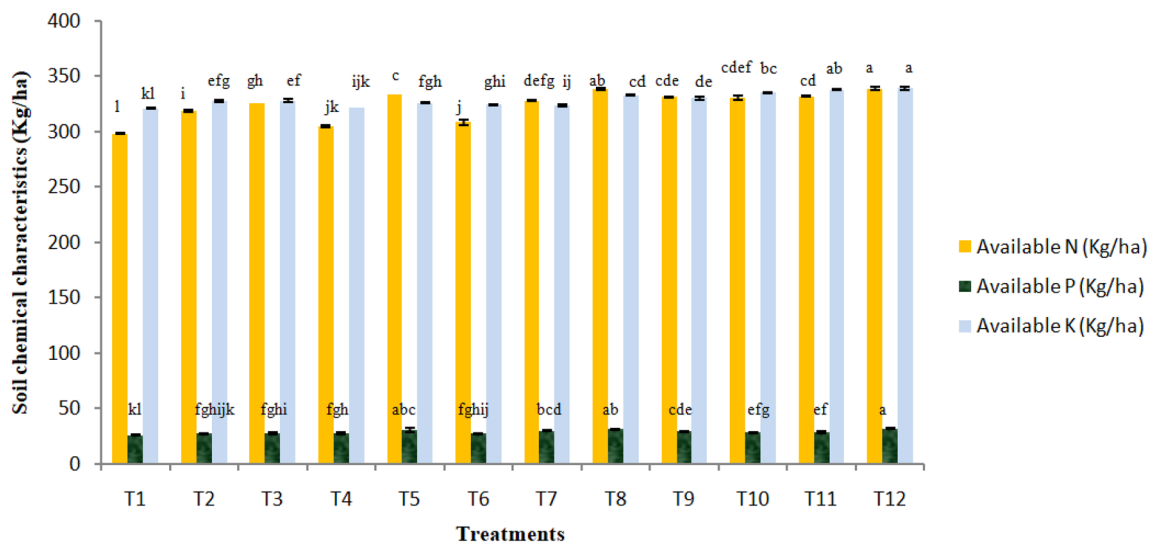

Fig. 6 Effect of liquid bacterial inoculum on soil available nutrients (NPK). Bars marked by different letters indicate significant differences based on LSD at $P \leq 0.05$; error bars indicate means $\pm$ SE by LSD test (n = 3). T1 (Control); T2 (*P. mediterranea* KA7); T3 (*P. oryzihabitans* KU5); T4 (*B. flexux* KA10); T5 (*P. aeruginosa* SI12); T6 (*P. japonensis* KU13); T7 (*P. putida* KU2); T8 (*C. lapagei* KU14); T9 (*P. agglomerans* KA14); T10 (*P. koreensis* KA11); T11 (*B. simplex* KA2); T12 (*B. subtilis* KU21)

rich endophytic pool in medicinal plants, in agreement with the previous studies (Elmagzob et al. 2019; Silva et al. 2019; Abdelshafy Mohamad et al. 2020). The predominant reported genera were *Pseudomonas* and *Bacillus*. Our findings are in line with the earlier studies which confirmed that *Bacillus* and *Pseudomonas* as PGPBEs have been widely found in medicinal plants such as *Pinellia ternate, Lycium Chinese, Digitalis pupurae,* (Miller et al. 2012); *Ginkgo biloba* (Yuan et al. 2012); *Lonicera japonica* (Zhao et al. 2015); *Clerodendrum colebrookianum* Walp. (Passari et al.

Table 3 Effect of inoculation with endophytic bacteria on microbiological parameters (post trial)

Treatment	Total viable endophytic bacterial population ($10^2 \times$cfu/g root)	Population of inoculated endophytic bacteria ($10^2 \times$cfu/g root)	Population of inoculated endophytic bacteria ($10^2 \times$cfu/g root)
T1 (Control)	31.00 ± 1.16^{ij}	0.00	0.00
T2 (*P. mediterranea* KA7)	49.00 ± 4.04^{bc}	35.00 ± 1.15^{cd}	0.51 ± 0.06^{cdefgh}
T3 (*P. oryzihabitans* KU5)	41.00 ± 2.31^{ef}	32.00 ± 1.21^{def}	0.61 ± 0.04^{abcd}
T4 (*B. flexux* KA10)	35.00 ± 1.73^{hi}	25.00 ± 2.31^{hi}	0.51 ± 0.07^{cdefgh}
T5 (*P. aeruginosa* SI12)	51.00 ± 1.16^{ab}	43.00 ± 1.73^{b}	0.71 ± 0.05^{ab}
T6 (*P. japonensis* KU13)	37.00 ± 3.46^{fgh}	26.00 ± 0.58^{h}	0.49 ± 0.06^{cdefghi}
T7 (*P. putida* KU2)	43.00 ± 1.73^{de}	33.00 ± 0.57^{de}	0.59 ± 0.10^{bcdef}
T8 (*C. lapagei* KU14)	49.00 ± 2.88^{bc}	38.00 ± 1.73^{c}	0.60 ± 0.46^{bcde}
T9 (*P. agglomerans* KA14)	43.00 ± 1.15^{de}	32.00 ± 0.58^{def}	0.55 ± 0.07^{bcdefg}
T10 (*P. koreensis* KA11)	39.00 ± 2.02^{efg}	31.00 ± 1.73^{efg}	0.63 ± 0.06^{abc}
T11 (*B. simplex* KA2)	47.00 ± 3.46^{bcd}	35.00 ± 2.31^{cd}	0.55 ± 0.05^{bcdefg}
T12 (*B. subtilis* KU21)	54.00 ± 2.31^{a}	48.00 ± 2.89^{a}	0.79 ± 0.07^{a}
LSD ($P \leq 0.05$)	4.24	3.77	0.18

The data represent mean of 3 replicates $\pm$ standard error (SE). Within columns, means followed by same letter are not significantly different ($P \leq 0.05$)

2016); *Thyme vulgaris* (Abdelshafy Mohamad et al. 2020)**;** *Fagonia mollis* (ALKahtani et al. 2020); *Pulicaria incisa* (Fouda et al. 2021)**.**

The main purpose of the current study was to understand the interaction of endophytes with the host plant which involves mobilization of nutrients, production of phytohormones, siderophores, and antagonistic compounds. That is why bacterial strains were screened for in vitro traits of plant growth promotion with an aim to obtain potential candidates. These strains exhibited multifaceted PGP traits. Similar investigations reported that endophytic bacteria exhibited multiple traits of PGP (Ahmed et al. 2019; Fouda et al. 2021). Among all, the strains of *Bacillus, Pseudomonas* and *Cedecea* exhibited the highest amounts of P-solubilization, siderophore, IAA, HCN, ammonia and lytic enzymes (chitinase, protease, and amylase). Earlier studies have also indicated that several *Bacillus* species isolated from medicinal plants produced phytohormones, solubilized P and improved growth of tomato (Abdelshafy Mohamad et al. 2020) and *Pseudowintera colorata* (Purushotham et al. 2020). Similarly, *Pseudomonas* species produced IAA and increased plant biomass of medicinal plant *Astragalus mongholicus* (Sun et al. 2019). In contrast, few studies had reported PGP potential of *Cedecea*. For example Beniassa et al. (2019) reported the potential of *Cedecea* as P-solubilizer, nitrogen fixer and HCN producers under in vitro conditions.

In vitro screening for antifungal activity showed that six strains inhibited the growth of all tested fungal pathogens (*F. oxysporum, F. graminearum,* and *R. solani*). These antagonistic strains exhibited various antifungal properties viz*.,* siderophore, chitinase, protease, amylase, HCN, and

ammonia production, etc. Thus, these endophytes can protect the plant from phytopathogenic fungi either by degrading the cell wall or by stimulating systemic resistance in plants (Mohamad 2018). Similar work has been carried out by Egamberdieva et al. (2017), and Fouda et al. (2021) who have reported that endophytic strains associated with medicinal plants *Ziziphora capital* and *Pulicaria incisa* respectively, exhibited antifungal activity by producing several antimicrobial compounds. As per phytopathogenic data, the biocontrol ability of these strains can be further assessed on wide range of host plant to protect against fungal pathogens.

To authenticate the results of in vitro studies, we selected endophytic isolates possessing multifarious PGPTs for in vivo experiments. In pot experiment, the application of endophytic strains significantly increased the physical growth parameters of *R. officinalis* seedlings over untreated control, especially *P. aeruginosa* SI12, *C. lapagei* KU14 and *B. subtilis* KU21. The increased shoot/ root parameters in the inoculated plants is attributed to the release of a variety of plant growth regulators in the rhizosphere, resulting in an altered root architecture that may have prompted an expansion in the total root surface area and consequently, improved the water and nutrient uptake, especially N and P, with positive effects on plant growth as a whole (Montano et al. 2014; ALKahtani et al. 2020). Similar results were documented by Fouda et al. (2021) with the isolates *B. cereus* BI-8 and *B. subtilis* BI-10 isolated from medicinal plant *Pulicaria incisa* which showed an increase in various physical growth parameters of maize seedlings.

The inoculation of *R. officinalis* seedlings with indigenous endophytes was also reported to have affected several

Table 4 Antibiotic sensitivity test of endophytic bacteria

Antibiotics	Concentration (µg/disc)	Zone of clearance (mm)		
		B. subtilis KU21	P. aeruginosa S112	C. lapagei KU14
Hexa universal-1				
Bacitracin	10	–	–	–
Chloramphenicol	30	–	–	–
Penicillin G	10	–	–	–
Polymyxin B	300	–	3.20	–
Gentamicin	10	–	–	–
Neomycin	30	–	–	2.40
Hexa universal -2				
Cefotaxime	30	–	–	–
Augmentin	30	–	–	–
Erythromycin	10	–	–	–
Chloramphenicol	30	–	–	–
Ofloxacin	5	–	–	–
Co-trimoxazole	25	–	–	2.80
Hexa Pseudo 1				
Cefoperazone	75	–	–	–
Piperacillin	100	–	–	–
Levofloxacin	5	–	–	–
Gentamicin	10	–	–	–
Amikacin	30	2.30	1.60	2.80
Colistin	10	–	3.10	–
Hexa Pseudo 2				
Imipenem	10	–	–	–
Aztreonam	30	–	–	–
Sulbactam	10	–	–	–
Tazobactam	10	–	–	–
Ceftazidime	30	–	–	–
Netillin	30	–	–	–

–= Resistant

physiological properties of plants. The significantly increased contents of total chlorophyll were observed in plants inoculated with *B. subtilis* KU21, followed by *P. aeruginosa* SI12, *C. lapagei* KU14. The results of the present study corresponded with those of Zhang et al. (2008) and Jang et al. (2018), who reported that PGPR *B. subtilis* GB03 and *B. subtilis* JS improved photosynthetic capability by augmenting photosynthetic efficiency and chlorophyll levels in *Arabidopsis* and Poplar, respectively. Plants possess a variety of antioxidant molecules predominantly phenols that alleviate the reactive oxygen species and defend host cells against adverse conditions. The results of the present study suggested that there were certain elicitors in the microbial cultures which played a vital role in enhancing the phenolic content of *R. officinalis* leaves. Several studies have likewise reported the promising effect of endophytic bacteria in boosting phenolics and flavanoid contents in *Withania somnifera* (L.), Dunal, and sweet basil (Gupta and Pandey 2015; Singh et al. 2016).

In case of nutrient acquisition, an improved NPK concentration in plants inoculated with *B. subtilis* KU21 followed by *P. aeruginosa* SI12, *C. lapagei* KU14 was observed. This enhanced capacity of the plant to attain and utilize more nutrients could be attributed to the bioinoculation effect on the stimulated root system (Egamberdieva et al. 2017). Moreover, these microbes are also capable of solubilizing mineral nutrients, resulting in increased levels of available NPK in the soil, thereby facilitating their availability to plants (Setiawati and Mutmainnah 2016; Adhikari and Pandey 2020). For example, *Bacillus* possessing mineral solubilizing and nitrogen-fixing ability, significantly increased NP content in maize (ALKahtani et al. 2020). The endophytic strains of the current investigation were found to be capable of solubilizing P and fixing N under in vitro conditions, thus providing more NP to *R. officinalis* seedlings.

In a nutshell, among the eleven selected bacterial endophytes for plant growth promotion experiment, *B. subtilis* KU21, *P. aeruginosa* SI12, and *C. lapagei* KU14 improved the growth parameters of *R. officinalis* significantly. The PGP bacteria resistant to antibiotics may have survival and competitive qualities required for a good bioinoculant to be used as biofertilizer (Kloepper et al. 1980). Further field trials for exploring the future application of these strains in enhancing *R. officinalis* productivity are well under way. The current study has also reported the first time occurrence of *C. lapagei* as an endophytic bacterium from *R. officinalis*. Although, some studies have reported *C. lapagei* as an inhabitant of rhizo and endorhizosphere possessing multiple plant growth-promoting traits (Zhang et al. 2008; Benaissa et al. 2019). The in vivo growth promotion studies for this strain have not been evaluated so far. The current findings point out the first ever evidence of the growth promoting potential of *C. lapagei* on *R. officinalis* under in vivo conditions.

Code availability Not applicable.

Declarations

Conflict of interest The authors have declared no conflict of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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