Consortium of plant growth-promoting rhizobacteria enhances oilseed rape (*Brassica napus* L.) growth under normal and saline conditions

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Abstract

A preparation development, which stimulates plant growth under normal and saline conditions, and protects against fungal infections, would increase crop yields and reduce damage in agriculture. This study was conducted using bacterial isolates from rape rhizosphere as a plant growth promoter and an alternative to chemical fertilizers. Three from fifty bacterial isolates: B14 (*Pseudomonas* sp.), B16 (*Sphingobacterium* sp.), and B19 (*Microbacterium* sp.) showed the best in vitro plant growth-promoting (PGP) characteristics. B14 strain had the best antifungal activity against phytopathogens inhibiting growth of *B. cinerea*, *C. acutatum*, and *P. lingam*. Moreover, B14, B16 and B19 isolates coded for several genes involved in PGP activities, aimed at improving nutrient availability, resistance to abiotic stress, and fungal pathogen suppression. Microbial consortium (B14, B16, and B19) had the best effect on rape growth, significantly increasing number of live leaves, compared to the untreated control and single inoculant treatments. Moreover, the consortium induced significant increase in shoots length and chlorophyll content in comparison to *Pseudomonas* sp. B14 and *Microbacterium* sp. B19. The consortium also induced plants tolerance to salt stress. The genomic information as well as the observed traits, and beneficial attributes towards rape, make the rhizobacterial consortium an ideal candidate for further development as biofertilizers.

Keywords Rhizobacteria · Beneficial microorganisms · Oilseed rape · Plant growth promotion · Plant pathogens inhibition · Salt stress tolerance

Introduction

Chemicalization of agriculture frequently relies on pesticides and synthetic fertilizers which adversely affect the natural microbial communities in the rhizosphere and balance in the natural ecosystem (Singh et al. 2017; Dash et al. 2018). In response to this fact, scientists are searching for environmentally friendly alternative improving crop production and controlling plant pathogens (Kannoja et al. 2019). Among these options, the use of microorganisms can reduce environmental problems caused by chemicals and pesticides (Mekonnen and Fenta 2020).

These microorganisms inhabiting the rhizosphere and exerting beneficial effect on plant development are termed as plant growth-promoting rhizobacteria (PGPR) (Adedeji et al. 2020). PGPR have a positive effect on plant vegetation by stimulating their growth by various mechanisms. PGPR can directly affect plant growth facilitating their uptake of nutrients, such as nitrogen (N) and phosphorus (P). The other beneficial characteristics related to plant growth promotion are synthesis of indol-3-acetic acid (IAA), production of siderophores and the ability to decrease plant ethylene levels through the 1-aminocyclopropane-1-carboxylate (ACC) deaminase. PGPR can also support plant growth indirectly by inhibiting soil-borne plant pathogens due to antimicrobial compounds and
extracellular enzymes production (Chouyia et al. 2020). These beneficial microorganisms are also capable of protecting plants from abiotic and biotic stress (Huang et al. 2017).

Oilseed rape (Brassica napus L.) is an important plantation crop which is grown across different continents including Europe, Canada, South Asia, China and Australia (Neik et al. 2017). B. napus is the world’s second most popular crop cultivated for its edible oil (Carré and Pouzet 2014). However, organic production of oilseed rape is difficult due to its very high nutrient requirements and its susceptibility to pests and diseases (Robson et al. 2002; Sanogo et al. 2015). Moreover, among various biotic and abiotic stresses, salinity is the major factor limiting successful cultivation of rape (Gyawali et al. 2019).

Lally et al. (2017) demonstrated that application of PGPR increased Brassica napus height and biomass under greenhouse and field conditions. Whereas, Saber et al. (2013) showed the positive effect of PGPR on rapeseed yield under saline conditions. However, it is not known whether PGPR can have a positive effect on Brassica napus, under both normal and saline conditions and at the same time protect the plant against pathogens. Thereby, we aimed to find bacteria stimulating rape growth under normal and saline conditions, and protecting against fungal pathogens, which would increase crop yields and reduce damage in rapeseed cultivation. Furthermore, we examined genes that contribute to plant growth promotion and biocontrol potential. According to Bloemberg and Lugtenberg (2001), identification of genes involving in plant growth promotion and biocontrol activities is important to develop successful biofertilizers.

**Materials and methods**

**Collection of plant material and isolation of bacteria from rhizosphere**

Five rape plants (variety of Arazzo) were collected from a field cultivated with rape in Górsk (Poland 53°01′46.1″N 18°26′59.4″E). Firstly, the soil surrounding the root was removed, then the separated roots of the plant were washed with sterile distilled water to remove the remaining soil. The cleared roots were cut with a sterile scalpel into 1–2 cm pieces and prepared in the appropriate decimal dilutions. Subsequently, surface spreading was carried out on nutrient agar medium (Biomaxima, Poland) supplemented with (40 μg/ml) antifungal agent amphotericin B to prevent the growth of mold and fungi. The plates were incubated at 28 °C for 72 h. Fifty colonies were selected and used for further study. The selection was made based on their color, shape and size.

**Determination of PGPR traits**

All analyses were made in triplicate.

**Determination of IAA production**

To determine the amounts of indole-3-acetic acid (IAA), a colorimetric technique was performed with Salkowski’s method using Van Urk Salkowski reagent (Ehmann 1977). The isolates were grown in medium containing (g/l): peptone 5.0; yeast extract 3.0; l-tryptophan 1.0, and were incubated at 28 °C for 4 days. After incubation, the cultures were centrifuged (10,080g/10 min). The supernatant was reserved and 1 ml was mixed with 2 ml of Salkowski’s reagent (2% 0.5 FeCl₃ in 35% HClO₄ solution), then kept in the dark. After 30 min of incubation, IAA concentration was measured using Hitachi U-2500 spectrophotometer at a wavelength of 530 nm, relative to a standard curve.

**Determination of ACC deaminase activity**

The ACC deaminase activity was determined by the modified Honma and Shimomura (1978) method. In the first step, 10 ml of liquid medium (nutrient broth) was inoculated with isolated bacteria and incubated at 30 °C for 24 h with shaking. The samples centrifuged (4032g/10 min/4 °C). The pellet was suspended in 5 ml of DF fluid medium (Dworkin and Foster 1958) and centrifuged. Then, the pellets were resuspended in 5 ml DF with 30 μl ACC (0.5 M). The tests were incubated under the same conditions as above. The samples were centrifuged twice (4032g/10 min/4 °C) washed with 5 ml of 0.1 M Tris–HCl (pH 7.6). Then, the pellet was suspended in 1 ml of 0.1 M Tris–HCl (pH 7.6) and placed in microcentrifuge tube (1.5 ml). Samples were centrifuged (10,080g/5 min) once again and after separation of the supernatant, the bacteria were suspended in 600 μl 0.1 M Tris–HCl (pH 8.5). Then, 30 μl of toluene was added to destroy the cells and release the enzyme. The resulting slurry (200 μl) was taken for further stages. The remaining volume was used to determine the content of the bacterial protein. 20 μl substrate-ACC was added to the mixture and the samples were incubated at 30 °C for 30 min. After incubation, 1 ml HCl (0.56 M) was added to the tubes and the samples were centrifuged (10,080g/5 min). After separation of the remaining lysed cells, 1 ml of supernatant and 800 μL of HCl (0.56 M) and 300 μl of 2,4-dinitrophenylhydrazine reagent (0.2%) were added. Samples were incubated again at 30 °C for 30 min in a water bath. In the last step of the reaction, 2 ml NaCl (2 M) was added. Positive samples that acquire a red color, which intensity is proportional to the amount of product (α-ketobutyrate), were measured.
spectrophotometrically (absorbance at $\lambda = 540$ nm). The ACC deaminase activity was reported as the amount of $\alpha$-ketobutyrate (mM) per hour per milligram of bacterial protein. Protein was determined by the Bradford method (1976).

**Determination of phosphate solubilization**

Phosphate (P) solubilization was determined on Pikovskaya’s agar medium (g/l: glucose 10.0; Ca$_3$(PO$_4$)$_2$ 5.0; KH$_2$PO$_4$ 3.0; (NH$_4$)$_2$SO$_4$ 1.0; MgSO$_4 \times$ 7H$_2$O 0.5; NaCl 0.2; MgSO$_4 \times$ 7H$_2$O 0.1; KCl 0.1; yeast extract 0.5; MnSO$_4 \times$ 2H$_2$O 0.002; FeSO$_4 \times$ 7H$_2$O 0.002; agar 15.0; pH 7.0). Isolates were transferred on the medium and incubated for 7 days at 26 °C. Production of clear halos around the colonies indicated a positive result for phosphate solubilization (Ahmad et al. 2008).

**Determination of siderophores production**

Siderophores were assayed according to Schynw and Neilands (1987) method. Tested strains were transferred on Chrome Azurol S agar medium (Alexander and Zuberer 1991), and incubated at 26 °C for 4 days. The bacteria, which produce siderophores, gave orange zones around the colonies. Siderophores production index was evaluated as the ratio between the halo diameter and the diameter of the colony growth.

**Determination of salicylic acid**

Production of salicylic acid (SA) was determined according to Meyer et al. (1992). The tested bacteria were grown in 100 ml of medium containing (g/l): succinic acid 4.0; K$_2$HPO$_4$ 6.0; KH$_2$PO$_4$ 3.0; (NH$_4$)$_2$SO$_4$ 1.0; MgSO$_4 \times$ 7H$_2$O 0.2. Incubation was performed at 26 °C for 48 h. After incubation, the culture was centrifuged (4032 g/5 min) and 4 ml of the supernatant was acidified with 1 N HCl to pH 2.0 and SA was extracted in CHCl$_3$ (2 × 2 ml). To the pooled CHCl$_3$ phases, 4 ml of distilled water and 5 µl of 2 M FeCl$_3$ were added. The absorbance of the purple iron-SA complex, in the aqueous phase was measured at 527 nm in a Hitachi F 2500 spectrophotometer. A standard curve was prepared with SA dissolved in succinate medium. The quantity of SA in the culture was expressed as µg/ml.

**Determination of ammonia production**

The production of ammonia (NH$_3$) was detected in the nutrient broth (Biomaxima, Poland). After incubation at 26 °C for 72 h, the Nessler’s reagent (0.5 ml) was added to each tube. The orange color of the suspension indicated the presence of ammonia.

**Determination of HCN production**

Hydrogen cyanide (HCN) production was detected according to Lorck (1948) method. The bacterial isolates were streaked on nutrient agar amended with glycine (4.4 g/l). Filter paper discs soaked in picric acid (0.5%) prepared in Na$_2$CO$_3$ solution (2%) were kept inside the lid of the Petri plates. Petri dishes were sealed with parafilm and incubated in the dark at 28 °C for 3–4 days. The paper discs changed color from yellow to brown indicating HCN production.

**Determination of fungal cell lytic enzymes production**

Chitinase production of all bacterial isolates was studied using the medium composed (g/l): peptone 1.0; FeSO$_4 \times$ 7H$_2$O 0.1; iron gluconate 0.1; yeast extract 0.1; colloidal chitin 7.0 g dry mass; agar 15.0. After 14 days of incubation at a temperature of 22 °C, the bright halo diameter around the colonies was measured, as the ability of the bacteria to produce chitinase (Swiontek Brzezinska et al. 2013). The colloidal chitin was prepared according to Lingappa and Lockwood (1962).

Moreover, three bacterial isolates B14, B16 and B19 were further used to determine quantitative assessment of chitinase and 1,3-β-glucanase activity. Chitinase activity was studied using the medium composed of (g/l): peptone 1.0, yeast extract 0.1, colloidal chitin 2.0. pH of medium was 6.8–7.2. After 4 days of incubation at a temperature of 26 °C, the chitinase activity was measured (Swiontek Brzezinska et al. 2013). The fluorogenic substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide [4-MU-GlcNAc] was used to detect chitinase activity (Sigma-Aldrich). The reaction mixture contained: 1 ml crude enzymes, 0.125 ml substrate 4MU- GlcNAc solution (the final concentration in a sample was 50 µM/l and 0.125 ml of phosphate buffer (50 mM, pH 7). The control sample, prior to addition of the substrate, was treated with 0.1 ml solution of HgCl$_2$ in order to deactivate the enzymes present in the sample (final concentration: 4 mM/l). After incubation (1 h, 40 °C), enzymatic reactions were stopped by adding HgCl$_2$. The released 4-methylumbelliferone (MU) was measured fluorimetrically (318 nm excitation and 445 nm emission) using the Hitachi F 2500 spectrofluorometer. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 nM MU/ml.

1,3-β-glucanase was studied using the medium composed of (g/l): laminarin 5.0; K$_2$HPO$_4$ 1.0; NaNO$_3$ 3.0; KCl 0.5; MgSO$_4 \times$ 7H$_2$O 0.5; FeSO$_4 \times$ 7H$_2$O 0.5. After 72 h of incubation at a temperature of 30 °C, the culture was centrifuged (10 000g/5 min) and 100 µl of the supernatant was mixed with 50 µl of 50 mM phosphate buffer (pH 5.9), containing 1% laminarin (Sigma). After incubation (1 h, 40 °C), the reducing sugar produced was determined by Miller (1959).
One unit of enzyme activity \((U)\) was defined as the amount of enzyme that produced 1 µmol of reducing sugar min\(^{-1}\).

**Determination of antifungal activity**

Bacterial cultures were tested for growth inhibitory effect on the mycelium growth of *Fusarium solani* 25, *Fusarium oxysporum* 872, *Fusarium culmorum* 2333, *Botrytis cinerea* 873, *Alternaria alternata* 783, *Phytophthora megasperma* 404, *Phytophthora cactorum* 1925, *Penicillium verrucosum* 1681, *Colletotrichum acutatum* 2153, *Sclerotinia sclerotiorum* 2242, and *Phoma lingam* 2284. Molds were obtained from the Pathogens Bank of the Institute of Plant Protection in Poznań (Poland). Molds were grown on Potato Dextrose Agar (PDA) at 26 °C for 96 h. Then, the cultures were stored at 4 °C. The tested bacterial strains were grown 24 h at 28 °C in PDA. In the next step, agar disc of each molds (with diameter of 5 mm) was placed in the agar discs containing the spread bacteria. Cultures were incubated at 26 °C for 7 days. After incubation, the diameter of the fungal mycelium was measured. At the same time, for each molds, species control experiments were made (without bacteria). Inhibition rate was calculated from the following formula (Oldal et al. 2002):

Inhibition (\(\%\)) = \(C - B/C\), where \(C\) is the diameter (mm) of the control molds and \(B\) is the diameter of the molds grown in the presence of the bacteria. Each assay was performed in triplicate.

**Identification of the rhizobacterial strains**

The three rhizobacterial strains, B14, B16, and B19, were identified based on the analysis of the gene sequence encoding 16S rRNA. Amplification of the 16S rRNA gene was carried out with the 27F and 1401R primers (Watanabe et al. 1990). For PCR, genomic DNA was extracted from bacterial cells harvested during logarithmic growth stage by the method according to Kutchma et al. (1998). After purification, the PCR product was sequenced by the DNA Sequencing and Oligonucleotides Synthesis Laboratory at IBB (PAN—Polish Academy of Sciences, Poland). The obtained nucleotide sequence was identified using the EzBioCloud (https://www.ezbiocloud.net/). The sequences were deposited to Genbank under the following accession numbers: *Pseudomonas grimonii* B14 (NCBI accession number MN589843), *Sphingobacterium kitahiroshimense* B16 (MN589844), and *Microbacterium oxydan* B19 (MN589846).

**Whole-genome sequencing and analysis**

Genomic DNA extracted from the three PGPR isolates, B14, B16, and B19 was sent for whole-genome sequencing at the University of Birmingham, United Kingdom. Whole-genome libraries were prepared using a Nextera XT Library Prep kit (Illumina, San Diego, USA) according to the manufacturer's protocol with the following modifications: 2 ng of DNA instead of 1 ng was used as input, and PCR elongation time was increased to 1 min from 30 s. DNA quantification and library preparation were carried out on a Hamilton Microlab automated liquid handling platform. Pooled libraries were quantified using the KAPA Library Quantification Kits for Illumina platforms on a Roche LightCycler 96 real-time PCR machine.

Libraries were sequenced on the Illumina HiSeq 250 bp paired-end HiSeq protocol. Reads were adapter trimmed using Trimmmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger et al. 2014). De novo assembly was carried out with SPAdes version 3.7 (Bankevich et al. 2012), and contigs were annotated using Prokka 1.11 (Seemann 2014). Further annotation was made using NCBI’s Prokaryotic Genome Annotation Pipeline (PGAP).

To get insight into the molecular mechanisms encoded in the genomes of rhizobacterial strains B14, B16, and B19 amino acid sequences predicted by Prokka were used as input to GenBank Trans Extractor (http://www.bioinformatics.org/sms2/genbank_trans.html) for prediction of protein translations of the DNA sequence. These sequences were assigned KO identifiers (KEGG Orthology) via “annotate sequence” (https://www.kegg.jp/kegg/tool/annotate_sequence.html), and were used to perform KEGG pathway analysis (https://www.kegg.jp/kegg/tool/map_pathway.html). The annotated genes of three PGPRs were inspected for identifying those involved in PGP functions, improvement nutrient availability, pathogenic fungi suppression, abiotic stress tolerance, quorum sensing and other important relevant functions.

The strains were also identified based on their genome sequences. First, LINbase (http://www.LINbase.org, Tian et al. 2020) was used to find bacteria that are similar to the uploaded genomes. Then, B14, B16, and B19 genomes were compared to the most similar genomes using ANI calculator, available from EzBioCloud (Yoon et al. 2017) and the genome-to-genome distance calculator v 2.0 and formula 2 (Meier-Kolthoff et al. 2013) at DSMZ (https://ggdc.dsmz.de).

Sequencing data were deposited in the Sequence Read Archive at NCBI under BioProject accession PRJNA614621. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAAUVM000000000, JAAUVN000000000, JAAUVO000000000, and the version described in this paper in version JAAUVM010000000, JAAUVN010000000, JAAUVO010000000, respectively, for B14, B16, and B19 strains.
Compatbility assay

Three strains (B14, B16 and B19) were tested for their antagonistic effect for consortium development. Two colonies of three different strains were resuspended with sterile water (10⁷ CFU/ml) and 2 µl inoculum of each strain was placed on a Petri dish containing nutrient agar medium (Bio-maxima, Poland). Each strain was inoculated 1 cm apart from other strains and then the plates were incubated at 28 °C for 48 h (Hashmi et al. 2019; Kumar et al. 2011). The presence of the inhibition was tested for each strain. Each assay was performed in triplicate.

Effect of rhizobacteria inoculation on rape growth

The plant growth promotion ability of the isolates on rape growth was conducted in pot experiment. Rape cultivar seeds were sterilized (2 min) in a 1:1 mixture of 30% hydrogen peroxide and 96% ethanol, and washed three times with sterile distilled water (Piernik et al. 2017). A sterility test of water after the last washing was done on Petri dishes with R2A medium (DifcoTM) in three replicates and incubated at 20 °C for 5 days. Bacterial and fungal colonies were checked. Before sowing, sterilized seeds were incubated with strains B14, B16, B19 and consortium of these bacteria by dipping in bacterial suspensions (10 ml of CFU/ml) with strains B14, B16, B19 and consortium of these bacteria for 30 min. A control was incubated with 10 ml of sterile distilled water. Then, seeds were sown on ½ MS plates (Murashige and Skoog basal salts, Sigma) with or without 100 mM NaCl, stratified in the dark for 2 days at 4 °C, and placed vertically in growth chambers at 22 °C with a photoperiod of 16/8 h (light/dark, 100 µmol/ m²/s PAR). After 12 days of seedlings transfer, the plant material was dried at 85 °C for 48 h and dry weight of shoots and roots were measured. Each experiment was performed at least in three biological replicates.

Soil salinity is often measured by electrical conductivity (EC) (Hardie and Doyle 2012). The EC of 100 mM NaCl amounted to 10 deciSiemens per meter (dS/m). According to Yanagawa and Fujimaki (2013), B. napus L. was found to have threshold values of soil salinity at about 10 dS/m. Therefore, in this study, 100 mM NaCl solution was applied.

Salt stress tolerance assay

The effect of the rhizobacterial strains on rape growth under salt stress condition was tested by the modified Eida et al. (2020) method. Rape seeds were sterilized by shaking for 10 min in 70% ethanol + 0.05% SDS, washed two times in 96% ethanol, and one time with sterilized H2O. Then, the seeds were incubated by dipping in bacterial suspensions (10 ml of CFU/ml) with strains B14, B16, B19 and consortium of these bacteria for 30 min. A control was incubated with 10 ml of sterile distilled water. Then, seeds were sown on ½ MS plates (Murashige and Skoog basal salts, Sigma) with or without 100 mM NaCl, stratified in the dark for 2 days at 4 °C, and placed vertically in growth chambers at 22 °C with a photoperiod of 16/8 h (light/dark, 100 µmol/ m²/s PAR). After 12 days of seedlings transfer, the plant material was dried at 85 °C for 48 h and dry weight of shoots and roots were measured. Each experiment was performed at least in three biological replicates.

Statistical analysis

Statistical analysis of the data from the effect of rhizobacteria on rape growth was performed using canonical correspondence analysis (CCA). The indirect ordination analysis CCA was performed in the Canoco 5.0 program (terBraak and Šmilauer 2012). The result of the analysis is a diagram in which the cultivars are marked with the triangle symbols and the parameters are vectors. Variable designations near the vector arrowhead are analogous to those indicated in parentheses above. At the same time, forward selection and Monte Carlo permutation test were performed. That indicated which variables were statistically significant for the variability of our data.

Statistical analysis of the data from salt stress tolerance assay was performed in Past3, v 3.25. To determine significant differences in response variables, ANOVA tests were performed. The assumptions for ANOVA were checked using Shapiro–Wilk test for normality, and Levene’s test for homogeneity of variances. Tukey’s multiple comparison test was performed to find means that are significantly different from each other.
Results

Selection of strains based on their PGPR traits

Fifty bacterial isolates were isolated from the rhizosphere of rape. The isolates were screened for different PGPR traits such as IAA, SA, ACC deaminase, phosphate solubilization, siderophores, chitinase, HCN, and NH₃ production (Table S1). Three strains B14, B16 and B19 showed the best in vitro plant growth-promoting characteristics: deaminase activity (> 5.9 mMα-ketobutyrate/mg protein/h), IAA production (> 15 µg/ml), salicylic acid production (> 1.9 µg/ml), siderophores production (> 2 mm). Moreover, two of the three strains (B16 and B19) were able to produce ammonia, while only one strain (B19) was positive for hydrogen cyanide production (Table 1). Therefore, these three strains were used for further study.

The quantitative assessment of chitinases and 1,3-β-glucanases, and in vitro mycelial growth inhibition of plant pathogens by these three bacterial isolates were investigated (Table 2). The strain B14 had the best antifungal activity against phytopathogens. It inhibited growth of P. lingam, B. cinerea and C. acutatum, in the rate of 40, 30, and 30%, respectively. The strain B16 has induced the mycelial growth inhibition of C. acutatum and the antagonistic effect expressed in inhibition rate of 25%, while strain B19 strongly inhibited the growth of B. cinerea in the rate of 30%. It was found that all tested strains had the ability to produce chitinases and 1,3-β-glucanases; however, strain B16 showed the highest enzymes activity of 10.8, and 0.12 U/ml, respectively.

Identification of strains and analysis of their genomes

Based on the gene encoding sequence analysis 16S rRNA, the strains were classified as Pseudomonas grimonii B14, Sphingobacterium kitahiroshimense B16, and Microbacterium oxydans B19. 16S rRNA gene sequence similarity values were 99.86% for P. grimonii B14, 99.35% for S. kitahiroshimense B16 and 99.78% for Microbacterium oxydans B19. Furthermore, B14, B16 and B19 PGPR strains were used for the whole-genome sequencing. Based on the genome sequences, the strains were classified as Pseudomonas sp. B14 (2022) B14, Sphingobacterium sp. B16 (2022) B16, and Microbacterium sp. B19 (2022) B19. Based on LINbase, Pseudomonas sp. MIACH was related the most to the strain B14, Sphingobacterium sp. IITKGP-BTPF85 strain to the B16, and Microbacterium sp. ZOR0019 L978_5, Microbacterium maritypicum MF109, Microbacterium oxydans BEL163 RN51 strains to the B19. For B14 versus Pseudomonas sp. MIACH, OrthoANIu value was 94.67%, confirming being closely related genomes. Value in silico DNA–DNA hybridization (DDH) was 42.1%.

Table 1  PGPR traits of three the best rhizobacterial strains

| Strains | IAA (µg/ml) | ACC (mmol-ketobutyrate/mg protein/h) | P- solubilization (in mm) | Siderophore (d₅₀/d₅₀ colony) | SA (µg/ml) | Chitinase (in mm) | HCN (µg/ml) | Ammonia (µg/ml) |
|---------|-------------|-----------------------------------|--------------------------|-------------------------------|------------|-------------------|-------------|-----------------|
| B14     | 17.59 ± 0.24 | 7.67 ± 0.13                        | –                        | 3.8                           | 2.99 ± 0.10| 3                 | –           | –               |
| B16     | 15.57 ± 0.15 | 5.98 ± 0.11                        | –                        | 1.8                           | 2.29 ± 0.15| 15                | –           | +               |
| B19     | 16.29 ± 0.35 | 6.84 ± 0.15                        | –                        | 2.57                          | 1.99 ± 0.10| 2                 | +           | +               |

Means ± SE are presented
(+): Positive result
(−): Negative result

Microbacterium oxydans B19. 16S rRNA gene sequence similarity values were 99.86% for P. grimonii B14, 99.35% for S. kitahiroshimense B16 and 99.78% for Microbacterium oxydans B19. Furthermore, B14, B16 and B19 PGPR strains were used for the whole-genome sequencing. Based on the genome sequences, the strains were classified as Pseudomonas sp. B14 (2022) B14, Sphingobacterium sp. B16 (2022) B16, and Microbacterium sp. B19 (2022) B19. Based on LINbase, Pseudomonas sp. MIACH was related the most to the strain B14, Sphingobacterium sp. IITKGP-BTPF85 strain to the B16, and Microbacterium sp. ZOR0019 L978_5, Microbacterium maritypicum MF109, Microbacterium oxydans BEL163 RN51 strains to the B19. For B14 versus Pseudomonas sp. MIACH, OrthoANIu value was 94.67%, confirming being closely related genomes. Value in silico DNA–DNA hybridization (DDH) was 42.1%.
B16 vs *Sphingobacterium* sp. IITKGP-BTPF85, ANI value was 96.24%, confirming being closely related genomes and dDDH was 72.54%, confirming that both strains belonged to the same species. In case of B19, the highest ANI value was 85.34%, and the highest dDDH—0.06%—was for *Microbacterium* sp. ZOR0019 L978_5, confirming that the two strains are moderately related close relatives. The general genomic features of sequenced strains are summarized in Table S2 and Table S3. Annotation identified 6,211 coding genes, and 5,978 protein-coding genes in the B14 genome. The genome of strain B16 contained 6.02 Mb chromosome with a GC content of 36.3%, including 5,064 genes, and 4,916 protein-coding genes. Strain B19 contained a 4.06 Mb chromosome with GC content of 68.6%, including 3,937 genes, and 3,839 protein-coding genes (Table S2). In total, 1,676,437, 1,008,784, and 612,023 reads were obtained from the whole-genome sequencing of the B14, B16, and B19 strains, respectively (Table S3).

The identified genes which are involved in PGPR ability to improve nutrient availability, resistance to abiotic stress and fungi pathogen suppression are presented in Table 3. All of the three B14, B16, and B19 genomes contained genes associated with phosphate transport (including *psbA, C, S*), IAA biosynthesis (*trpA, B, D, C*), acetoin and butanediol synthesis (*poxB*), glycine-betaine production (*opu* and *proX*), riboflavin biosynthesis (*ribF, H, B, E, D*), and heat-shock proteins production (*dnaK*). On the other hand, some genes which were present in *Pseudomonas* sp. B14 genome, were not detected in other genomes. These were involved in ACC deaminase production (*acds* and *dcyD*), nitrogen fixation (*nurG* and *nirS*), and 4-hydroxybenzoate synthesis (*ubiC*). Moreover, only in the *Sphingobacterium* sp. B16 genome, we were able to identify genes responsible for chitinase production (*chiA*) and pyroloitrin production (*gcaA*). Whereas, the *Microbacterium* sp. B19 genome contained genes involved in HCN production (*hcnB*), and quorum sensing (*lsrB, C, D, F*). B14 and B16 also coded for genes contributing to salicylic acid production (B14: *pcha* gene and B16: *pchB* gene), and *H_2*S biosynthesis (B14: *cysC, N, J* genes and B16: *cysN, J* genes). In addition, both B14, and B19 genomes coded for genes involved in GABA production (*gabD, T*), and siderophores sequestration (B14: *entD* gene and B16: *ashF* gene).

### Effect of rhizobacteria inoculation on rape growth under normal and salt condition

Based on individual PGP properties supported by the genome data, we selected three strains B14, B16, B19 for greenhouse experiment. The consortium was most effective in promoting rape growth (Fig. 1 and Fig. S1), significantly increasing number of live leaves (5.05) compared to the untreated control (3.33) and to single inoculant treatments: *Pseudomonas* sp. B14, *Sphingobacterium* sp. B16, and *Microbacterium* sp. B19 (4.06, 3.87, and 2.87, respectively). Interestingly, the consortium induced significant increase in shoots length (16.9 mm), compared to B14 (9.81 mm) and B19 (9.13 mm) but not to the control. Moreover, the consortium significantly increased chlorophyll content index (41.8), compared to B14 (24.4) and B19 (28.9) but also not to the control (Table 4).

Three ACC deaminase and IAA-producing rhizobacteria B14, B16, B19 and their consortium were further used to determine whether they confer stress tolerance to plants (Fig. 2). B14, B16, B19 and consortium-inoculated plants showed a considerable salt stress tolerance, significantly increasing dry weight of roots compared to the control.

### Discussion

PGPR inoculation has a positive influence on various plant growth parameters, such as root and shoot length, dry biomass, thereby increasing crop yields (Khan et al. 2020). According to Ansari and Ahmad (2019), the application of bacterial strains with multi plant growth-promoting traits is more beneficial than with a single plant growth-promoting trait. In our study, three rhizobacterial strains (*Pseudomonas* sp. B14, *Sphingobacterium* sp. B16, and *Microbacterium* sp. B19) showed multiple PGP traits. Moreover, the whole-genome sequencing of these strains helped to verify almost all the plant growth-promoting abilities in vitro. We found several genes involved in salicylic acid, ACC deaminase, and IAA function, and production of other metabolites as hydrogen cyanide, siderophores, fungal cell lytic enzymes, which can enhance plant growth indirectly by inhibiting pathogens. Previously, genes with similar functions in other PGPRs were reported (Bruto et al. 2014; Gupta et al. 2014).

Phosphate-solubilizing bacteria are beneficial microorganisms, which can hydrolyze organic and inorganic insoluble phosphorus to soluble form that can easily be assimilated by plants (Kalayu 2019). According to Otiono et al. (2015) *pqg* gene cluster is involved in solubilization of mineral phosphates. Our results showed that B14, B16 and B19 strains were unable to solubilize phosphates and lacked the required *pqg* genes. However, they contained several *pst* genes involved in phosphate transport. According to Hudek et al. (2016), the *pst* phosphate uptake system is responsible for the phosphate accumulation when cells are in phosphate-limited environments.

PGPR by producing salicylic acid can induce systemic resistance (ISR) in plants, and hence protect the plants against pathogens and parasites (Tripathi et al. 2019). *Bacillus amyloliquefaciens* strain induced salicylic acid-dependent resistance in tomato plants against *Tomato spotted wilt virus* and *Potato virus Y* (Beris et al. 2018).
Li et al. (2015) observed that *B. amyloliquefaciens* could stimulate production of salicylic acid in cucumber leaves, suggesting that SA-mediated defense response was stimulated and in doing so exerted its biocontrol activity against a broad range of pathogens. Furthermore, *P. aeruginosa* produced pyoverdine and salicylic acid that are implicated in protecting tomatoes against *Alternaria solani* and tobacco plants against *Peronospora tabacina* (Fallahzadeh et al. 2010). In our study, all of the rhizobacterial strains could produce salicylic acid with *Pseudomonas* sp. B14 showing the highest activity of SA. What is more, *Pseudomonas* sp. carries *pchA* gene involved in salicylic acid biosynthesis, and thus had the best antifungal effect against *C. acutatum*.

| Plant growth – promoting traits | B14 | B16 | B19 | Genes |
|---------------------------------|-----|-----|-----|-------|
| IAA production                  | −   | −   | −   | *ipdC* |
| +                               | *trpA, trpB, trpD, trpC* |
| ACC deaminase production        | +   | −   | −   | *acdS* |
| −                               | *rinM* |
| +                               | *dcyD* |
| Phosphate solubilization        | −   | −   | −   | *pqg* |
| +                               | *pstA, pstC, pstS* |
| −                               | *pstB* |
| Siderophores production         | −   | −   | −   | *pvd, fpvA, mbtH, acrA, acrB, fhu* |
| +                               | *entD* |
| −                               | *ashF* |
| Salicylic acid production       | +   | −   | −   | *pchA* |
| −                               | *pchB* |
| HCN production                  | −   | −   | −   | *hcnA, hcnC* |
| −                               | *hcnB* |
| Chitinase production            | −   | +   | −   | *chiA* |
| −                               | *chiB, chiC, chiD* |
| −                               | *GluNCaseA, GluNCaseB, GluNCaseC* |
| 1,3-β-glucanase production      | −   | −   | −   | *gluA, gluB, gluC* |
| Acetoin and butanediol synthesis| +   | +   | +   | *posB* |
| −                               | *budA, budB, budC* |
| −                               | *als* |
| H₂S production                  | +   | −   | −   | *cysC* |
| +                               | *cysN, cysJ* |
| −                               | *cysI* |
| Nitrogen cycle                  | −   | −   | −   | *amoA, nifH, nosZ* |
| +                               | *narG* |
| +                               | *nirS* |
| 4-hydroxybenzoate production    | +   | −   | −   | *ubiC* |
| GABA production                 | +   | +   | +   | *gabD, gabT* |
| Acetohydroxyacid synthesis      | +   | −   | +   | *iivH, iivI* |
| Ketol-acid reductoisomerase synthesis | +   | +   | +   | *ivlC* |
| Riboflavin synthesis            | +   | +   | +   | *ribFHBED* |
| DAPG synthesis                  | +   | +   | +   | *phlABCDEFGH* |
| Pyrrolnitrin production         | −   | −   | −   | *gacA* |
| Heat shock proteins             | +   | +   | +   | *dnaK* |
| −                               | *dnaI, groE* |
| Glycine-betaine production      | +   | −   | +   | *soxB* |
| +                               | *opu* |
| +                               | *proX* |
| Quorum sensing                  | −   | −   | +   | *lsr B, C, D, F* |

Table 3 List of genes contributing to plant growth promotion traits in the B14, B16, and B19 genomes
Inhibition of the mycelial growth of phytopathogens may happen due to siderophores released by rhizobacteria (Islam et al. 2018). This mechanism depends on the role of siderophores as competitors for Fe to reduce the Fe availability for plant pathogens (Ahmed and Holmström 2014). According to previous reports, siderophore-producing P. fluorescens strains inhibited Fusarium wilt disease in tomatoes (Arya et al. 2018). Our results showed that strains Pseudomonas sp. B14, and Microbacterium sp. B19 that produced the highest siderophores activity, had the best antifungal activity against B. cinerea. We found that B14 strain carried entD gene which encodes a product necessary for the synthesis of the iron-chelating and transport siderophore enterobactin (Khan et al. 2018), while the genome of B19 contained asbF gene involved in 3,4-dihydroxybenzoic acid synthase that is necessary for petrobactin production (Hotta et al. 2010). Previous studies indicated that Brevibacillus brevis showed strong antagonistic effect against F. oxysporum and siderophore synthesis gene cluster with 83% similarity to petrobactin was found in the genome of this strain (Sheng et al. 2018).

Moreover, some studies reported hydrogen cyanide and ammonia-producing PGPR in biological control to enhance crop production (Agbodjato et al. 2015; Kumar et al. 2016). According to Rijavec and Lapanje (2016), HCN is involved in chelation of metals leading to an indirect increase in nutrient availability for the rhizospheric bacteria and their host plant. In our study, B19 strain was found as a HCN and ammonia-producer. Furthermore, the analysis of B19 genome revealed the presence of hcnB gene along with two other biosynthetic genes hcbA and hcnC involved in HCN production. Abd El-Rahman et al. (2019) found positive correlations between the ability of strains to produce HCN and their plant protection ability in controlling the crown gall disease caused by Agrobacterium tumefaciens and root-knot nematode, Meloidogyne incognita.

Microbial enzymes like 1,3-β-glucanase, and chitinase have been used to inhibit the growth of plant pathogens by degrading fungal cell wall (Aktuganov et al. 2008). Moreover, these authors indicated synergism between the antifungal action mechanisms of these enzymes in which 1,3-β-glucanase is the initiator of the fungal cell wall

### Table 4 Effect of bacterial inoculation on rape growth

| Parameter                  | Control      | B14          | B16          | B19          | Consortium (B14, B19, B16) | Significant level | p |
|----------------------------|--------------|--------------|--------------|--------------|---------------------------|-------------------|---|
| Number of live leaves      | 3.33 ± 0.29b | 4.06 ± 0.31b | 3.87 ± 0.40b | 2.87 ± 0.19b | 5.05 ± 0.29a              | *                 |   |
| Number of death leaves     | 1.13 ± 0.24  | 0.56 ± 0.20  | 0.53 ± 0.13  | 0.33 ± 0.13  | 0.50 ± 0.15               |                   |   |
| Length of shoots (mm)      | 12.7 ± 1.3a  | 9.81 ± 0.77b | 13.6 ± 1.4a  | 9.13 ± 0.74b | 16.9 ± 1.5a               | *                 |   |
| Length of roots (mm)       | 133.1 ± 11.7 | 149.5 ± 10.7 | 147.1 ± 13.4 | 115.1 ± 11.4 | 145.4 ± 8.57              |                   |   |
| Length of epicotyls (mm)   | 27.3 ± 3.08  | 25.6 ± 1.59  | 25.4 ± 2.27  | 21.3 ± 1.96  | 20.5 ± 1.67               |                   |   |
| Dry weight of leaves (g)   | 0.148 ± 0.034 | 0.234 ± 0.055 | 0.220 ± 0.054 | 0.101 ± 0.015 | 0.282 ± 0.067             |                   |   |
| Dry weight of petioles (g) | 0.048 ± 0.011 | 0.067 ± 0.019 | 0.081 ± 0.022 | 0.025 ± 0.006 | 0.088 ± 0.020             |                   |   |
| Dry weight of roots (g)    | 0.032 ± 0.006 | 0.053 ± 0.011 | 0.068 ± 0.015 | 0.036 ± 0.005 | 0.057 ± 0.012             |                   |   |
| Dry weight of epicotyls (g) | 0.019 ± 0.003 | 0.023 ± 0.003 | 0.023 ± 0.004 | 0.016 ± 0.002 | 0.019 ± 0.003             |                   |   |
| Dry weight of shoots (g)   | 0.020 ± 0.004 | 0.029 ± 0.006 | 0.033 ± 0.007 | 0.018 ± 0.003 | 0.038 ± 0.006             |                   |   |
| Specific leaf area SLA (cm²/g) | 342.6 ± 29.6 | 324.0 ± 28.4 | 312.2 ± 19.9 | 251.9 ± 24.0 | 371.8 ± 26.9              |                   |   |
| Leaf weight ratio LWR (g/g) | 0.52 ± 0.02  | 0.55 ± 0.02  | 0.48 ± 0.03  | 0.51 ± 0.02  | 0.54 ± 0.02               |                   |   |
| Chlorophyll Content Index   | 41.5 ± 2.15a | 24.4 ± 1.30b | 39.9 ± 3.74a | 28.9 ± 2.83b | 41.8 ± 1.87a              |                   |   |

Significance levels (p) symbols: ns not significant differences

*p < 0.05 (value obtained by permutation Monte Carlo test with Bonferroni correction in CCA. If there are statistically significant differences, the letters (a,b) indicate groups that differ from each other.
hydrolysis, whereas the degradation process is regulated by chitinases. Our results showed that B16 had strong antifungal activity against *C. acutatum* and produced the highest activity of chitinases, and 1,3-β-glucanases. While the chitinolytic activity was present in all the three strains, only B16 contained gene involved in chitin degradation (*chiA*). It could be explained by much higher B16 activity of chitinases than the other strains. Neither *gluA*, *gluB*, *gluC* genes encoding enzymes associated with 1,3-β-glucanase were present in the B16 genome, but it could be explained by a low activity of these enzymes in the culture. Chen et al. (2004) reported that *Bacillus subtilis* expressing *chiA* gene exhibited a greater antifungal activity against *Botrytis elliptica* than *B. subtilis* control strain. We believe that antifungal effect of B16 strain could be caused by the expression of *chiA* gene.

The annotated genomes of B14, B16 and B19 strains were also inspected for identifying other important genes involved in relevant functions. For example, *poxB* and *als* genes which are involved in the production of acetoin and 2,3-butanediol, known to directly influence the plant growth promotion (Sharifi and Ryu 2018). In addition, *cysC*, and *cysN*, *cysJ* genes involved in biosynthesis of hydrogen sulfide (H₂S) which according to Zhou et al. (2018), plays role in increasing seed germination and plant growth. Moreover, all of three strains encoded riboflavin synthase (*ribFHBED*), which catalyzes biosynthesis of riboflavin—one of the B vitamins known as stimulator of plant growth and protectant of plant defense (Dakora et al. 2015). In addition to these, 4-hydroxybenzoate produced by the PGPRs act as antibiotics and suppress plant pathogens (Grossi et al. 2020). In B14 genome, we were able to identify the *ubiC* that codes for chorismate lyase, an enzyme which allows for 4-hydroxybenzoate synthesis. In B14 and B19 genomes we also found *gabD* and *gabT* genes contributing to synthesis of γ-aminobutyric acid (GABA), which is responsible for pest and disease inhibition (Shariati et al. 2017). A further determinant for secondary metabolites production is acetohydroxyacid synthase (AHAS) and ketol-acid reductoisomerase (KARI). Both of these enzymes belong to the KEGG pathways ‘Biosynthesis of antibiotics’ and ‘Biosynthesis of secondary metabolites’ and are capable of forming precursors for secondary metabolites, e.g., cyanogenic glycosides, glucosinolates, and acyl-sugars (Nelkner et al. 2019). In the genome B14 and B19, the genes *ilvH* and *ilvI* coding for the small and large AHAS subunit, were annotated. Also, the gene *ilvC* was predicted, coding for KARI.

Recent studies have indicated that genes involved in plant growth-promoting effects, biofilm formation, plant...
colonization, and in triggering induced systemic resistance are regulated by quorum-sensing (QS) systems (Jung et al. 2017; Zuniga et al. 2017). Our results showed that genome of B19 possessed \textit{lsr B,C,D,F} genes involved in internalizing, phosphorylating and processing of the AI-2, a small signaling molecule in QS (Torres Cerna et al. 2019). These observations suggest that B19 may have quorum-sensing ability that can contribute to its symbiotic relationship with the host plant.

Based on individual PGP properties supported by the genome data, three strains B14, B16, B19 were used for greenhouse experiment. It has been reported that bacterial strains with different PGP properties can be used as consortium which can work synergistically enhancing each other’s beneficial effect (Meena et al. 2017). According to Thomloudi et al. (2019), compatibility among the strains should be first considered in the process of designing a microbial consortium applied to plants. It is important to minimize their antagonism, since they will not interfere with each other’s growth and colonization capacity (Thomloudi et al. 2019). For this reason, compatibility among the three isolates (\textit{Pseudomonas} sp. B14, \textit{Sphingobacterium} sp. B16, and \textit{Microbacterium} sp. B19) was first examined. When the antagonism between strains was not observed, consortium of these strains was used to determine its effect on rape (\textit{Brassica napus} L. var. \textit{napus}) growth. Interestingly, the consortium was more effective in promoting rape growth than single inoculant treatments, significantly increasing number of live leaves. Moreover, the consortium induced significant increase of shoots length and chlorophyll content, compared to \textit{Pseudomonas} sp. B14 and \textit{Microbacterium} sp. B19. Our results are in agreement with other studies. For example, Emami et al. (2019) demonstrated that co-inoculation of eight bacteria from different taxa (\textit{Bacillus}, \textit{Microbacterium}, \textit{Nocardia}, \textit{Pseudomonas}, \textit{Serratia}, and \textit{Stenotrophomonas}) increased plant growth compared to single bacterial inoculation. In another experiment, when PGPR \textit{Pseudomonas fluorescens} FAP2 and \textit{Bacillus licheniformis} B642 were co-inoculated, plant growth parameters increased compared to control (Ansari and Ahmad 2019).

Inoculation of PGPR has become also a promising alternative to alleviate plant stress caused by salinity conditions (Pandey and Gupta 2019). The salinity is one of the major abiotic factors that reduce cultivation of many plants, including \textit{B. napus} L. (Gyawali et al. 2019). Therefore, there is a need to find salt-tolerant bacteria that can be used to enhance salt tolerance in rape plants. According to Latif Khan et al. (2016) IAA and ACC deaminase-producing bacteria can protect plants against various stresses. PGPR use small molecules in root exudates and convert them into indole acetic acid, which is utilized by the plant roots. The process results in the activation of plant’s endogenous auxin signaling pathway, leading to plant growth promotion and plant cells proliferation (Dakora and Phillips 2002). IAA accumulation induces transcription of ACC synthase genes, which increases amounts of ACC, leading to the ethylene production. ACC deaminase-expressing PGPR may break down some of the excess ACC and decrease plant ethylene levels under abiotic stress (Gamalero and Glick 2015). Although B14, B16 and B19 strains were able to produce high concentrations of IAA, we did not find \textit{ipdc} gene encoding for indolepyruvate decarboxylase—an enzyme producing indole acetic acid from tryptophan via IPyA pathway. Nevertheless IAA biosynthesis in bacteria can be divided into tryptophan-dependent and tryptophan-independent pathways (Li et al. 2018). In all of three genomes, we identified some of the \textit{trp} cluster genes (\textit{trpA}, \textit{B}, \textit{D}, \textit{C}), which are known as precursors of IAA synthesis in tryptophan-independent pathways (Lo et al. 2018). B14, B16 and B19 strains were also able to produce high concentration of ACC deaminase enzyme. Interestingly, B14, B16 and B19 strains and their consortium induced the plants tolerance to salt stress, significantly increasing dry weight of roots. Previous study has shown that \textit{Leclercia adecarboxylata} exhibiting high ACC deaminase activity and significant amount of IAA improved \textit{Solanum lycopersicum} L. growth and salinity stress tolerance (Kang et al. 2019). In other study, \textit{Aneurinibacillus aneurinilyticus} and \textit{Paenibacillus} sp. exhibiting high ACC deaminase activity promoted the growth attributes of French Bean under normal as well as saline conditions (Pandey and Gupta 2019). Moreover, it has been reported that bacteria production of osmoprotectants, cold-shock and heat-shock proteins can help their survival under harsh environmental conditions (Gupta et al. 2014). Interestingly, the genomes of our three rhizobacteria coded for several osmoregulant glycine-betaine synthesis genes, and heat-shock proteins genes.

**Conclusions**

The results obtained in the current study demonstrated that growth of \textit{Brassica napus} was stimulated after seeds were inoculated with consortium of rhizobacteria. Co-inoculation of three PGPR (\textit{Pseudomonas} sp. B14, \textit{Sphingobacterium} sp. B16 and \textit{Microbacterium} sp. B19) significantly enhanced number of live leaves compared to the control and single inoculant treatments. Moreover, the consortium significantly increased shoots length and chlorophyll content of rape compared to \textit{Pseudomonas} sp. B14 and \textit{Microbacterium} sp. B19. The PGPR in consortium not only promoted growth of rape, but also induced tolerance in plants to salt stress. These results indicated that this rhizobacterial consortium could be used to facilitate effective plant growth promotion in rape plants under normal and salt conditions.
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Declarations

Conflict of interest
The authors declare that they have no conflict of interest.

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