Consortium of Plant Growth – Promoting Rhizobacteria Enhances Oilseed Rape (*Brassica Napus* L.) Growth Under Normal And Saline Conditions

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Abstract

Development of a preparation, 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 grimontii*), B16 (*Sphingobacterium kitahiroshimense*), and B19 (*Microbacterium oxydans*) showed the best *in vitro* plant growth – promoting (PGP) characteristics. B14 strain inhibited the growth of *B. cinerea*, *C. acutatum*, and *P. lingam* and B14 - inoculated plants had the best ability to grow in salt concentrations of 100 mM NaCl. 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 chlorophyll content index, shoot length and number of live leaves, compared to the untreated control and single inoculant treatments. Consortium also induced the 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.

Introduction

Chemicalization of agriculture frequently rely on pesticides and synthetic fertilizers which adversely affect the natural microflora 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 (Kannojia et al. 2019). Among these options, the use 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 plant growth – promoting rhizobacteria 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

1.1. 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 hours. Fifty colonies were selected and used for further study. The selection was made based on their color, shape and size.

1.2. Determination of PGPR traits

1.2.1. 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 (10 000 rpm/ 10 min). The supernatant was reserved and 1 ml was mixed with 2 ml of Salkowski's reagent (2 % 0.5 $FeCl_3$ in 35 % $HCLO_4$ solution), then kept in the dark. After 30 minutes of incubation, IAA concentration was measured using Hitachi U-2500 spectrophotometer at a wavelength of 530 nm, relative to a standard curve.

1.2.2. 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 hours with shaking. The samples centrifuged (6000 rpm / 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 (0.5M). The tests were incubated under the same conditions as above. The samples were centrifuged twice (6000 rpm / 10 min / 4 °C) washed with 5 ml of 0.1M Tris-HCl (pH 7.6). Then, the pellet was suspended in 1 ml of 0.1M Tris-HCl (pH 7.6) and placed in eppendorff (1.5 ml). Samples were centrifuged (10 000 rpm / 5 min) once again and after separation of the supernatant, the bacteria were suspended in 600 µl 0.1M 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 (0.56M) was added to the tubes and the samples were centrifuged (10 000 rpm / 5 min). After separation of the remaining lysed cells, 1 ml of supernatant and 800 µL of HCl (0.56M) and 300 µl of 2,4-dinitrophenylhydrazine reagent (0.2 %). Samples were incubated again at 30 °C for 30 min in a water bath. In the last step of the reaction, 2 ml NaCl (2M) 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 λ = 540 nm). The ACC deaminase activity was reported as the amount of α-ketobutyrate (mM) per hour per milligram of bacterial protein. Protein was determined by the Bradford method (1976).

1.2.3. Determination of phosphate solubilization

Phosphate (P) solubilization was determined on Pikovaskaya's agar medium (g/l: glucose 10; $Ca_3(PO_4)_2$ 5; $(NH_4)_2SO_4$ 0.5; NaCl 0.2; $MgSO_4$ x $7H_2O$ 0.1; KCl 0.1; yeast extract 0.5; $MnSO_4$ and $FeSO_4$ trace; agar 15; 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).

1.2.4. Determination of siderophores production

Siderophores were assayed according to Schwyn 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.

1.2.5. 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_2HPO_4 6.0; KH_2PO_4 3.0; $(NH_4)_2SO_4$ 1.0; $MgSO_4$ x $7H_2O$ 0.2. Incubation was performed at 26 °C for 48 hours on a rotary shaker (120 rpm). After incubation, the culture was centrifuged (6000 rpm/5 min) and 4 ml of the supernatant was acidified with 1N 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 2M $FeCl_3$ 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.

1.2.6. 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 hours, the Nessler's reagent (0.5 ml) was added to each tube. The orange color of the suspension indicated the presence of ammonia. All analyses were made in triplicate.

1.2.7. 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_2CO_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.

1.2.8. Determination of fungal cell lytic enzymes production

The studies examined the production of enzymes associated with their antifungal activity. Chitinase production of all bacterial isolates was studied using the medium composed (g/l): peptone 1.0, $FeSO_4$ × $7H_2O$ 0.1, iron gluconate 0.1, yeast extract 0.1, colloidal chitin – 7.0 g dry mass, agar 15.0 g. 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-b-glucanase activity. Chitinase activity was studied using the medium composed of (g/l): peptone 1.0, yeast extract 0.1, colloidal chitin – 2.0 g. 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 hour, 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-b-glucanase was measured by mixing 100 µl of sample, 50 µl of 50 mM phosphate buffer (pH 5.9), containing 1 % laminarin (Sigma). After incubation (1 hour, 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}.

1.3. Determination of antifungal activity

Bacteria 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, *Colletrotrichum acutatum* 2153, *Sclerotina sclerotium* 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 PDA at 26 °C for 96 hours. Then the cultures were stored at 4°C. The tested bacterial strains were grown 24 hours 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.

1.4. 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). The matrix for the PCR was the genomic DNA separated 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/ Yoon et al. 2017).

1.5. Whole genome sequencing and analysis

Genomic DNA extracted from the three PGPR's 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 platforms. 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 Trimmomatic 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.

1.6. Genome data availability

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.

1.7. Compatibility 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 (10^7 CFU/ml) and 2 µl inoculum of each strain was placed on a Petri dish containing nutrient agar medium (Biomaxima, 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.

1.8. 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 10^9 cfu per ml) for 30 min. A control was incubated with 10 ml of sterile distilled water. Seeds for sowing were prepared according to the modified method of Piernik et al. (2017). Three replicates of 10 seeds for each strain and consortium, were germinated in the palette containing a sterile mixture of sand and vermiculite (1:1). All variants were put in a growth chamber at 22 °C and 16-h light period under a sodium lighting system [100 µmol/m^2/s PAR (photosynthetically active radiation)]. The palettes were watered by equal amount of Hoagland's solution each day to ensure homogeneity of nutrient supply. Germinated seeds were counted after 48 h and then each day for 2 weeks. Plants were grown for 6 weeks. Then plants were washed up from the substrate, and the content of chlorophyll in rape leaves was measured (as the CCI - chlorophyll content index). The measurements were carried out using a chlorophyll meter CCM-200plus. Then, live (LL) and dead leaves (DL) were counted. Each plant was divided into parts: leaves, petioles, shoots, epicotyls, roots and the lengths of shoots (shoot), epicotyls (epic) and roots (root) were measured. The leaves were scanned and their photosynthetic surface (Al) was measured using the digiShape 1.3 program (Moraczewski 2005). All parts of the plants were dried at 85 °C for 48 hours. After this time, they were weighed to obtain weights of leaves (Wl), petioles (Wpet), epicotyls (Wep) and roots (Wroot), respectively. Based on the obtained dry masses, two indexes for plant growth analysis were calculated (Kvét et al. 1971). These were SLA (specific leaf area, Al/Wl) and LWR (leaf weight ratio, Wl/W), where W is the plant's total biomass.

1.9. 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 H_2O. Then, the seeds were incubated by dipping in bacterial suspensions (10 ml of 10^9 cfu per 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/m^2/s). After 12 days of seedlings transfer, the plant material was dried at 85°C for 48 hours and dry weight of shoots and roots were measured. Each experiment was performed at least in three biological replicates.

1.10. Statistical analysis

Statistical analysis of the data from the effect of rhizobacteria on rape growth were performed using canonical analysis (CCA). The indirect ordination analysis CCA was performed in the Canoco 5.0 program (ter Braak 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 were performed in Past3, v 3.25. To determine significant differences in response variables, ANOVA 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

2.1. 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 NH_3 production (Table S1). Three strains B14, B16 and B19 showed the best *in vitro* plant growth – promoting characteristics: ACC deaminase activity (> 5.9 mM α-ketobutyrate/mg protein/h), IAA production (> 15 µg/ml), salicylic acid production (> 1.9 µg/ml), siderophores production (> 1.8), and chitinases 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 have 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 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.

2.2. Identification of strains and analysis of their genomes

Based on the analysis of the sequence of the gene encoding 16S rRNA, the strains were classified as *Pseudomonas grimontii* B14 (NCBI accession number MN589843), *Sphingobacterium kitahiroshimense* B16 (MN589844), and *Microbacterium oxydans* B19 (MN589846). Furthermore, B14, B16 and B19 PGPR strains were used for the whole genome sequencing. The general genomic features of sequenced strains are summarized in Table S2 and Table S3. Annotation using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) 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, 1676437, 1008784, and 612023 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 were presented in Table 3. All of the three B14, B16, and B19 genomes contained genes associated with phosphate transport (including *pstA*, *C, S)*, IAA biosynthesis (*trpA, B, D, C*), acetoin & 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, genes which were present in *P. grimontii* B14 genome, were not detected in other genomes. These were involved in ACC deaminase production (*acdS* and *dcyD*), nitrogen fixation (*narG* and *nirS*), and 4-hydroxybenzoate synthesis (*ubiC*). Moreover, only in the *S. kitahiroshimense* B16 genome we were able to identify genes responsible for chitinase production (*chiA*) and pyrrolnitrin production (*gacA*). Whereas, the *M. oxydans* 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_2S 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: *asbF* gene).

2.3. 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. *P. grimontii* B14, and *S. kitahiroshimense* B16 significantly increased number of live leaves (4.06, and 3.87, respectively), compared to the untreated control (3.33). Moreover, B16 strain induced significant increase in shoots length (13.6 mm), compared to the control (12.7 mm) (Table S4). Interestingly, the consortium was more effective in promoting rape growth (Fig. 1 and Fig. 2), significantly increasing chlorophyll index (41.8 mm), number of live leaves (5.05), and length of shoots (16.9 mm), compared to the untreated control and to single inoculant treatments.

Three high producing ACC deaminase and IAA rhizobacteria B14, B16, B19 and their consortium were further used to determine whether they confer stress tolerance to plants (Fig. 3). First, our experiment demonstrated the capacity of B14, B16, B19 and consortium to affect the early stages of rape development under normal conditions. When compared to the control-inoculated plants, B14, B16, B19 and consortium significantly increased dry weight of roots and shoots. The consortium of these strains had the best influence on the growth of rape, which confirmed our results above in pot experiment. On the other hand, B14, B16, B19 and consortium- inoculated plants showed stress tolerance activity on salt stress: roots and shoots were more developed than those of control-inoculated plants. However, B14- inoculated seedlings had the best ability to grow in salt concentrations of 100 mM NaCl: differences in roots and shoots weight between B14 with NaCl and B14 without NaCl were the least visible, compared to the other strains and the untreated control.

Discussion

PGPR inoculation have 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 (*P. grimontii* B14, *S. kitahiroshimense* B16, and *M. oxydans* 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 Otieno et al. (2015) *pqq* 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 *pqq* genes. However, they contained several *pst* genes involved in phosphate transport. According to Hudek et al. (2016) the *pst* phosphate up-take system is responsible for the phosphate accumulation when cells are in phosphate-limited environments.

Plant growth – promoting rhizobacteria by producing salicylic acid can induce systemic resistance (ISR) in plants, 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 *P. grimontii* B14 showing the highest activity of SA. What is more, *P. grimontii* carries *pchA* gene involved in salicylic acid biosynthesis, and thus had the best antifungal effect against *C. acutatum*.

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 in order 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 *P. grimontii* B14, and *M. oxydans* 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 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 (H_2S) 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 chorismatelyase, 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 *ivlC* 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 QS systems (Jung et al. 2017; Zuniga et al. 2017). Our results showed that genome of B19 possessed *lsr B,C,D,F* genes involved in internalizing, phosphorylating and processing of the AI-2, a small signaling molecule in quorum-sensing (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 (*P. grimontii* B14, *S. kitahiroshimense* B16, and *M. oxydans* B19) was first examined. When the antagonism between strains was not observed, consortium of these strains was used to determine its effect on rape (*Brassica napus* L. var. *napus*) growth. Interestingly, the consortium was more effective in promoting rape growth than single inoculant treatments, significantly increased chlorophyll content, number of live leaves and length of shoots. Our results are in agreement with other studies. For example, Emami et al. (2019) demonstrated that co-inoculation of eight bacteria from different taxa (*Bacillus*, *Microbacterium*, *Nocardia*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas*) increased plant growth compared to single bacterial inoculation. In another experiment, when PGPR *Pseudomonas fluorescens* FAP2 and *Bacillus licheniformis* B642 were co-inoculated, plant growth parameters increased compare 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). According to Latif Khan et al. (2016) IAA and ACC deaminase producing bacteria can protect plants against various a 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). In our study, consortium of rhizobacteria induced the plants tolerance to salt stress significantly increasing dry weight of roots and shoots. Although B14, B16 and B19 strains were able to produce high concentrations of IAA, we did not find *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 *trp* cluster genes (*trpA*, *B*, *D*, *C*), which are known as precursors of IAA synthesis in tryptophan – independent pathways (Lo et al. 2018). Moreover, B14, B16 and B19 strains were able to produce high concentration of ACC deaminase enzyme. However, *P. grimontii* B14 showing the highest activity of IAA and ACC deaminase and containing genes responsible for ACC production (*acdS* and *dcyD)*, had the best ability to induce plant tolerance to salt stress. Previous study has shown that *Leclercia adecarboxylata* exhibiting high ACC deaminase activity and significant amount of IAA improved *Solanum lycopersicum* L. growth and salinity stress tolerance (Kang et al. 2019). In other study, *Aneurinibacillus aneurinilyticus* and *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.

It should be noted that *Pseudomonas grimontii* was found as pathogen of bacterial rot disease, in turnip (*Brassica rapa* var. *rapa*) plants (Sawada et al. 2019). The strains isolated from turnip, caused diseases symptoms on leaves and the main roots. However, the identical strain *P. grimontii* isolated from mineral water in France, was not pathogenic on turnip (Sawada et al. 2019). Also, in our results, any diseases symptoms was observed. It suggest that *P. grimontii* might be a pathogenic and nonpathogenic strain. Also, *Microbacterium oxydans* is well known as a soil microorganism but like many other environmental bacterial species, it has been found in several samples of human clinical specimens, e.g. throat swab, superficial wound, endophthalmitis, urine, sinus aspirate, dialysis fluid, blood culture, and lymph node. However, it is still not known, whether these strains originated from the environment or has habitat in humans (Gneiding et al. 2008).

Conclusions

The results obtained in the current study demonstrated that growth of *Brassica napus* was stimulated after seeds were inoculated with consortium of rhizobacteria. Co-inoculation of three PGPR (*P. grimontii* B14, *S. kitahiroshimense* B16 and *M. oxydans* B19) significantly enhanced the number of live leaves, length of shoots and chlorophyll content of rape compared to single inoculant treatments. 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.

Declarations

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

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Tables

Table 1 PGP traits of three the best rhizobacterial strains

Strains	IAA (µg/ml)	ACC (mmol a-ketobutyrate/mg protein/h)	P-solubilization (in mm)	Siderophore (d_{halo}/d_{colony})	SA (µg/ml)	Chitinase (in mm)	HCN	Ammonia
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

Table 2 Fungal cell lytic enzymes production, antibiotic resistance, and *in vitro* mycelial growth inhibition of plant pathogens by bacterial isolates.

Characteristic	Isolates		
	B14	B16	B19
ACTIVITY (U/ml)			
Chitinase	0.39±0.02	10.80±0.16	0.32±0.10
1,3-b-glucanase	0.01±0.02	0.12±0.09	0.05±0.04
ANTAGONISM (%)			
Fusarium solani	0	0	0
Fusarium oxysporum	0	5	10
Fusarium culmorum	2	16	0
Botrytis cinerea	30	0	30
Alternaria alternata	0	10	8
Phytophthora megasperma	12	6	6
Phytophthora cactorum	8	0	0
Penicillium verrucosum	17	19	0
Colletotrichum acutatum	30	25	0
Sclerotina sclerotium	0	0	0
Phoma lingam	40	0	0

Means ± SE are presented; (+): resistance; (−): sensitive

Table 3 List of genes contributing to plant growth promotion traits in the B14, B16, and B19 genomes

Plant growth – promoting traits	B14	B16	B19	Genes
IAA production	-	-	-	*ipdC*
	+	+	+	*trpA, trpB, trpD, trpC*
ACC deaminase production	+	-	-	*acdS*
	-	-	-	*rimM*
	+	+	-	*dcyD*
Phosphate solubilization	-	-	-	*pqq*
	+	+	+	*pstA, pstC, pstS*
	-	+	+	*pstB*
Siderophores production	-	-	-	*pvd, fpvA, mbtH, acrA,B, fhu*
	+	-	-	*entD*
	-	-	+	*asbF*
Salicylic acid production	+	-	-	*pchA*
	-	+	-	*pchB*
HCN production	-	-	-	*hcnA, hcnC*
	-	-	+	*hcnB*
Chitinase production	-	+	-	*chiA*
	-	-	-	*chiB, chiC, chiD*
	-	-	-	*GluNAcaseA, GluNAcaseB, GluNAcaseC*
1,3-β-glucanase production	-	-	-	*gluA, gluB, gluC*
Acetoin & butanediol synthesis	+	+	+	*poxB*
	-	-	-	*budA, B, C*
	-	+	-	*als*
H_2S production	+	-	-	*cysC*
	+	+	-	*cysN, cysJ*
	-	-	-	*cysI*
Nitrogen cycle	-	-	-	*amoA, nifH, nosZ*
	+	-	-	*narG*
	+	-	-	*nirS*
4-hydroxybenzoate production	+	-	-	*ubiC*
GABA production	+	-	+	*gabD, gabT*
Acetohydroxyacid synthesis	+	-	+	*ilvH, ilvI*
Ketol-acid reductoisomerase synthesis	+	+	+	*ivlC*
Riboflavin synthesis	+	+	+	*ribFHBED*

DAPG synthesis	-	-	-	*phlABCDEFGH*
Pyrrolnitrin production	-	+	-	*gacA*
Heat shock proteins	+	+	+	*dnaK*
	-	-	-	*dnaJ, groE*
Glycine-betaine production	+	-	+	*soxB*
	+	+	+	*opu*
	+	+	+	*proX*
Quorum sensing	-	-	-	*luxS*
	-	-	+	*lsr B, C, D, F*

Figures

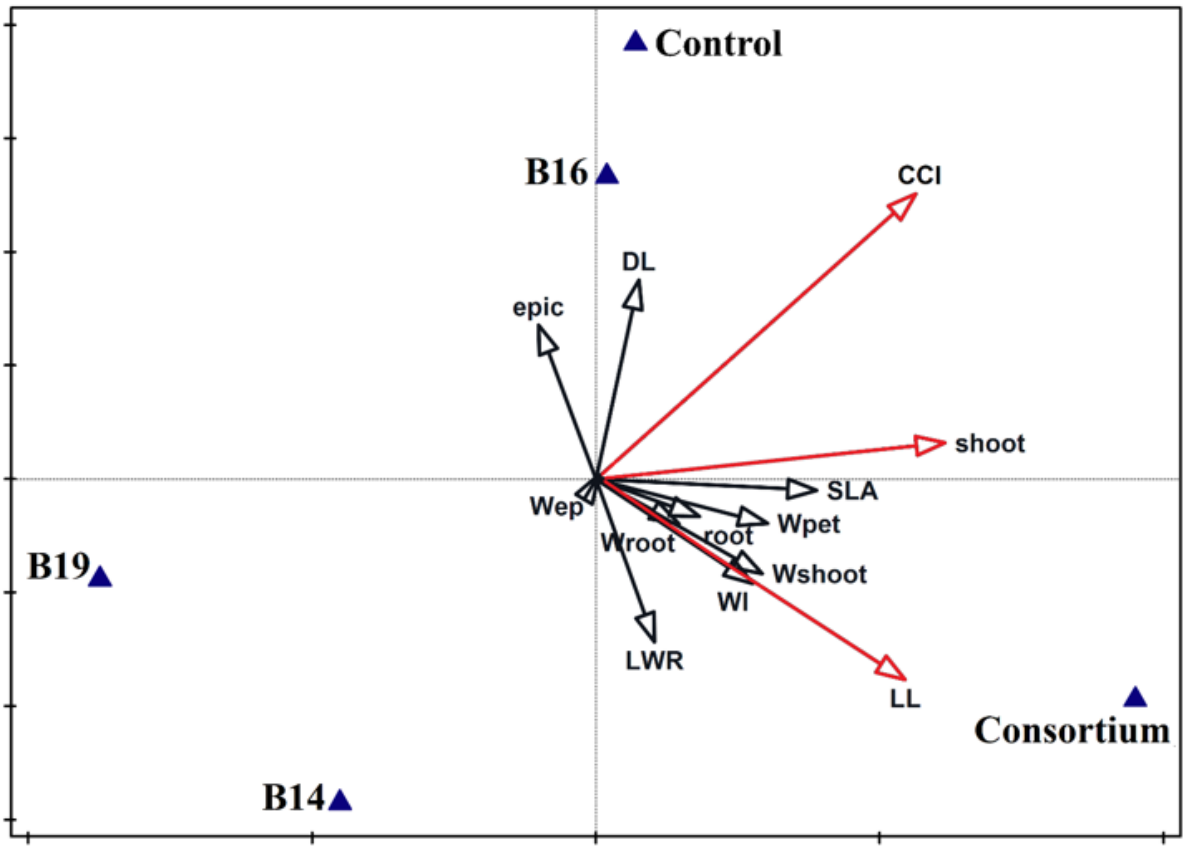


Figure 1

Canonical correspondence analysis for data on the functional traits of different cultivars of rape. Statistically significant variables in the data set were marked with red vectors. Together they account for 22.4% of the total variability.

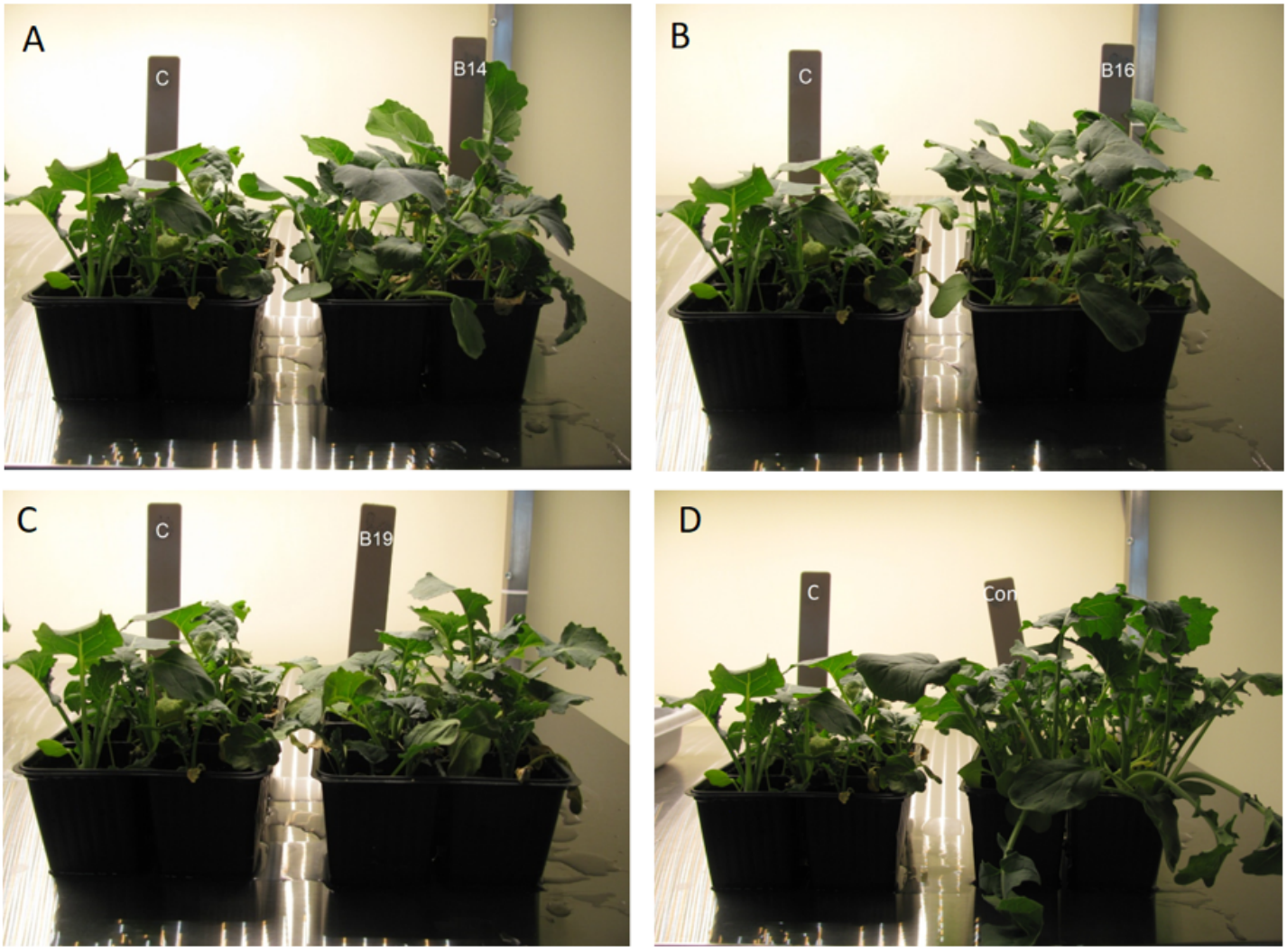
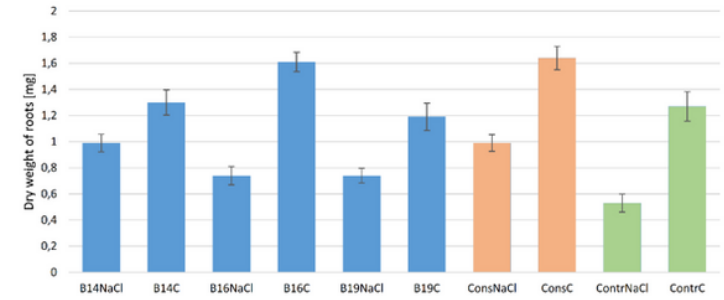
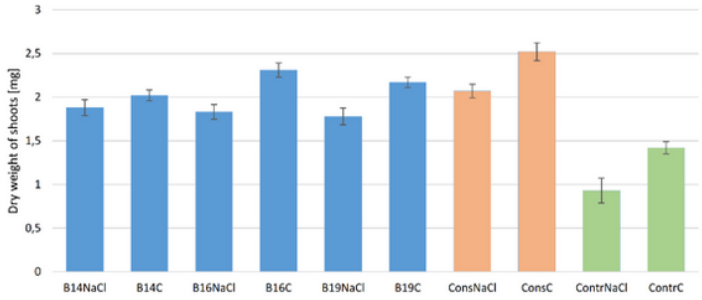


Figure 2

Effect of rhizospheric bacteria on the rape growth: A) Pseudomonas grimontii B14, B) Sphingobacterium kitahiroshimense B16, C) Microbacterium oxydans B19, and D) consortium of B14, B16, and B19 compared to the control.



	B14NaCl	B14C	B16NaCl	B16C	B19NaCl	B19C	ConsNaCl	ConsC	ContrNaCl	ContrC
B14NaCl		0.983	1.000	0.034	0.999	0.409	0.890	0.000	0.000	0.017
B14C			0.890	0.409	0.675	0.973	1.000	0.006	0.000	0.000
B16NaCl				0.010	1.000	0.199	0.675	0.000	0.000	0.052
B16C					0.003	0.983	0.675	0.818	0.000	0.000
B19NaCl						0.079	0.409	0.000	0.000	0.141
B19C							0.999	0.168	0.000	0.000
ConsNaCl								0.021	0.000	0.000
ConsC									0.000	0.000
ContrNaCl										0.008
ContrC										



	B14NaCl	B14C	B16NaCl	B16C	B19NaCl	B19C	ConsNaCl	ConsC	ContrNaCl	ContrC
B14NaCl		0.206	0.501	0.000	0.501	0.783	1.000	0.000	0.006	0.337
B14C			0.000	0.206	0.000	0.995	0.206	0.116	0.000	1.000
B16NaCl				0.000	1.000	0.008	0.501	0.000	0.732	0.001
B16C					0.000	0.018	0.000	1.000	0.000	0.116
B19NaCl						0.008	0.501	0.000	0.732	0.001
B19C							0.783	0.008	0.000	1.000
ConsNaCl								0.000	0.006	0.337
ConsC									0.000	0.060
ContrNaCl										0.000
ContrC										

Figure 3

Dry weight of roots and shoots of 12-day-old-seedlings inoculated by B14, B16, B19 and consortium of B14, B16, B19 with or without 100mM NaCl. All plots represent the mean of 3 biological replicates (n > 10). Error bars represent SE. Based on ANOVA, $P < 0.05$. Tables under the plots indicate which treatments were significantly different from others.

Supplementary Files

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