

Proceeding Paper

Isolation and Characterization of Plant Growth-Promoting Bacteria from the Rhizosphere of *Chamaecytisus ruthenicus* (Russian Broom) Growing in Chalky Soil [†]

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Abstract: Plant growth-promoting (PGPR) bacteria are beneficial soil bacteria that enhance plant growth against biotic and abiotic stress. Numerous studies have been carried out over the past three decades on the isolation and characterization of rhizospheric bacteria. However, no studies have been conducted on the bacteria present in the rhizosphere of the wild legume plant *Chamaecytisus ruthenicus* when growing in chalky soil. The purpose of this study was to evaluate the abundance of culturable bacteria, assess the morphology of the bacterial cells, profile the chalky soil bacterial community, and characterize its ability to stimulate plant growth. Three soil samples were collected in January at a temperature of 2–4 °C. The first sample was taken from the topsoil, the second sample from the soil 15 cm beneath the surface, and the third sample from the rhizosphere. The results of the study revealed that the abundances of bacteria in the first, second, and third soil samples were 4.25×10^8 cfu/g, 3.58×10^8 cfu/g, and 10.1×10^7 cfu/g, respectively. Furthermore, a total of 23 rhizospheric bacteria were isolated based on differences in their morphological characteristics. The 16S rRNA soil profiling result showed that bacteria belonging to *Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Acidobacteria*, and *Bacteroidetes* were the most dominant groups in the community. Six bacterial isolates (Z10, Z11, Z12, Z15, Z26, and Z44) were chosen to examine the inhibition effects on phytopathogenic microbes and their ability to promote plant growth. The bacterial isolates Z11 and Z15 showed good inhibition against all tested phytopathogenic fungi, while bacterial isolates Z10, Z12, Z15, Z26, and Z44 showed stimulatory effects on the length and fresh weight of the shoots and roots of wheat, maize, and oat seeds. In conclusion, the present study is the first report of chalky soil-associated bacteria found in the rhizosphere of the wild legume plant in the Belgorod region of Russia.

Keywords: plant growth-promoting rhizospheric bacteria (PGPR); chalky soil; phytopathogenic microbes; *Chamaecytisus ruthenicus*; colony-forming unit (CFU); soil profile



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1. Introduction

Agro-chemical usage in contemporary agriculture has raised public concern because of its negative effects on the environment and animal health [1]. According to several studies [2,3], plant growth-promoting rhizobacteria (PGPR) have often been utilized as a potential substitute for agro-chemicals. PGPR is a naturally occurring soil bacterium that lives in a plant rhizosphere and promotes plant growth, either directly or indirectly [4,5]. Nevertheless, the mode and mechanism of PGPR activity differ depending on the host plant species, soil type, and nutritional status of the soil [6]. The majority of PGPR isolates

cause a notable increase in plant height, root length, and dry matter production in the plant's shoots and roots. On the other hand, some PGPR affect plant health by inhibiting the growth of phytopathogens [6,7]. Since the last few decades, several PGPR species from the genera *Azospirillum*, *Pseudomonas*, *Klebsiella*, *Azotobacter*, *Enterobacter*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Serratia*, *Herbaspirillum*, *Acinetobacter*, *Aeromonas*, *Agrobacterium*, *Bradyrhizobium*, and *Xanthomonas* have been reported as effective plant growth promoters [4,6,8,9]. Today, researchers are still looking for potential PGPR in the rhizospheres of different plant species. However, no research has been conducted on chalky soil bacteria present in the rhizosphere of *Chamaecytisus ruthenicus*. Therefore, the aim of the present study was to evaluate the abundance of culturable bacteria, assess the morphology of the bacterial cells, profile the bacterial community in chalky soil, and characterize its ability to stimulate plant growth.

2. Methods

2.1. Soil Sample Collection

Soil samples were collected from non-agricultural soil of the Belgorod region of the Russian Federation. The location of the site was at 36.484050° N latitude and 50.579872° E longitude. Three chalky soil samples were randomly collected in January 2023 at a temperature range of 2–4 °C. The first sample was taken from the top layer of the soil, the second from 15 cm beneath the surface, and the third from the rhizosphere. Each soil sample was placed in a sterile polythene bag and brought to the laboratory for immediate analysis, and the remainder was stored at 4 °C for further study.

2.2. Bacterial Abundance and Isolation

The colony-forming units (CFU) of soil bacteria were estimated using the serial dilution technique, as described in [10], with a few modifications. Five grams from each soil sample were suspended in 50 mL of sterilized water. After being serially diluted, 100 µL from each (10^3 – 10^5) dilution was transferred onto plates containing reduced concentrations of Luria-Bertani (LB) growth medium (composition: yeast extract 1 g/L, peptone 2 g/L, sodium chloride 5 g/L, and agar 20 g/L). Then, the plates were incubated at 29 °C for 48 h. The colonies that appeared on the plates after 48 h of incubation were counted. After purification, the bacterial colonies were isolated on the basis of their color, shape, size, and pigment. Then, the purified colonies were reserved in the refrigerator for further study.

2.3. Microscopy Examination of Bacterial Isolates

Light microscopy of samples in phase contrast mode was carried out using a Nikon Eclipse Ci microscope (Nikon, Tokyo, Japan) equipped with a ProgRes SpeedXT camera (Jenoptic, Jena, Germany).

2.4. Soil Profile by 16S rRNA Gene Amplicon Sequencing

Sequence analysis was performed using the QIIME2 v.2022.2 software [11] and the MicrobiomeAnalyst 2.0 web service [12]. Sequence quality control was carried out using the deblur plugin [13] in positive mode. Then, SortMeRNA was used, where all raw reads were compared to the GreenGenes reference database [14]. The remaining sequences were assigned a taxonomy using a pretrained classifier (Naive Bayes classifiers) that was assembled from the complete 16S rRNA gene sequence using the GreenGenes reference database [14]. To assess biodiversity, alpha (Shannon, Pielow index, observed OTUs) and beta diversity indices (uniFrac “unweighted” and “weighted” methods) were calculated. The results are presented using PCoA multivariate statistical methods (principal component analysis).

2.5. Bacterial Inhibition against Phytopathogenic Microbes

Bacterial inhibition tests were examined using six bacterial isolates (Z10, Z11, Z12, Z15, Z26, and Z44) against three phytopathogenic bacteria (*Erwinia herbicola* ATCC 27155, *Pectobacterium carotovorum* B15, and *Micrococcus roseus* B1236) and five phytopathogenic

fungi (*Fusarium avenaceum* F-132, *Rhizoctonia solani* F-895, *Alternaria brassicicola* F-1864, *Bipolaris sorokiniana* F-4006, and *Pythium ultimum* F-4782). The phytopathogenic microbes were obtained from the Microbiology Regional Center, Belgorod State University, Belgorod Region, Russia. The bacterial isolates and the phytopathogenic microbes were cultured and incubated at 29 °C for five and three days, respectively. To examine the inhibition effect, 100 µL of each phytopathogenic bacterium was added to plates containing LB and distributed with sterile glass beads. Then, 5 µL of each bacterial isolate was distributed evenly throughout the plates. Similarly, a small piece of a fungal body was put in the middle of a plate containing Sabouraud dextrose agar, and 5 µL of bacterial isolate was sprinkled around the fungal body. All plates were incubated at 29 °C for 48 h. The occurrence of inhibition zones around the bacterial strains indicated the inhibition effect.

2.6. Bacterial Growth Stimulation Effect on Seed Germination

In a well-controlled experiment, seed germination was performed to evaluate the effects of bacterial stimulation on the development of four seeds (wheat, maize, oats, and lentils). In 10 mL of LB liquid medium, six bacterial strands were cultured and incubated at 29 °C for 72 h. On a total of 28 plates, 25 seeds from each variety were distributed. Thereafter, except for the control group, 15 mL of bacterial solution that had been diluted to an OD600 of 0.1 was applied to each plate. The control group, however, received merely water as an addition. For a week, 15 mL of water was added to the plates each day. The growth stimulation experiment was performed in triplicate. The lengths and fresh weights of the shoots and roots of the seedlings were measured and compared after the seeds had germinated. The data were statistically evaluated using a *t*-test at *p* = 5%.

3. Results

3.1. Abundance and Isolation of Chalky Soil Bacteria

The abundance of bacteria isolated from the three soil samples was computed. The highest bacterial concentration (4.25×10^8 cfu/g) was recorded in the initial soil sample, while the bacterial concentration in the second soil sample was 3.58×10^8 cfu/g. Nevertheless, the bacterial concentration in the third soil sample was 10.1×10^7 cfu/g, which is relatively low. Following repeated purification of the bacterial colonies, a total of 23 chalky soil bacteria were isolated based on morphological differences.

3.2. Morphology of the Isolated Strains

Bacteria with a wide range of morphologies are found in chalky soil. In Figure 1, the morphology of bacterial cells is shown. The representative bacterial cells are shaped like either rods, spheres, or filaments. Also, a few of the bacterial cells contained spores.

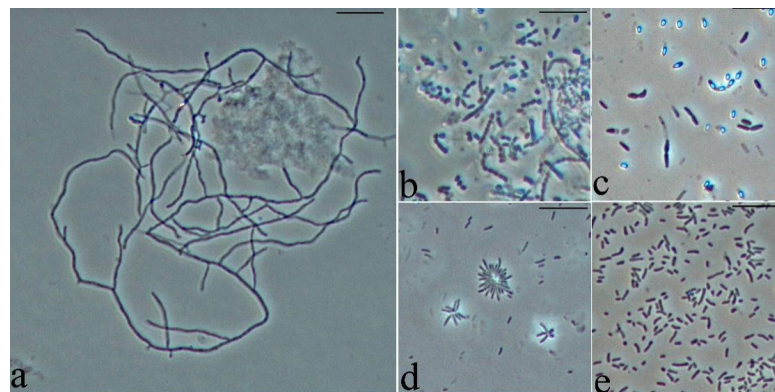


Figure 1. The morphological diversity of chalky soil bacterial cells: (a) branching aerial mycelium of actinomycete—producer of bright orange pigment; (b) streptococci; (c) diplobacilli with spores; (d) thin, long, rod-shaped cells forming rosettes; (e) unevenly dividing rod-shaped cells. Scale bar is 10 µm.

3.3. Profile of Soil Bacteria Community

The results of the soil profile presented in Figure 2 revealed that the chalky soil bacterial community comprised 19 phyla of bacteria. Based on the comparison of the average relative abundance, the bacterial community was clustered into three groups (major, medium, and minor phyla). The group *Proteobacteria* was the most dominant and cosmopolitan among the listed chalky soil bacterial community. Furthermore, the genus *Arenimonas* (belonging to *Proteobacteria*) was the most numerous genus in all soil samples. However, *Flavobacterium* (which belongs to *Bacteroidetes*) was the most dominant genus in the rhizosphere. The results of the bacterial alpha diversity analysis showed a significant abundance of species with an uneven distribution of taxa in the samples. The results of the PCoA analysis of the community structures of bacteria showed that there was a difference between samples, but according to the results of the PERMANOVA analyses, there were no significant differences in β diversity among the groups.

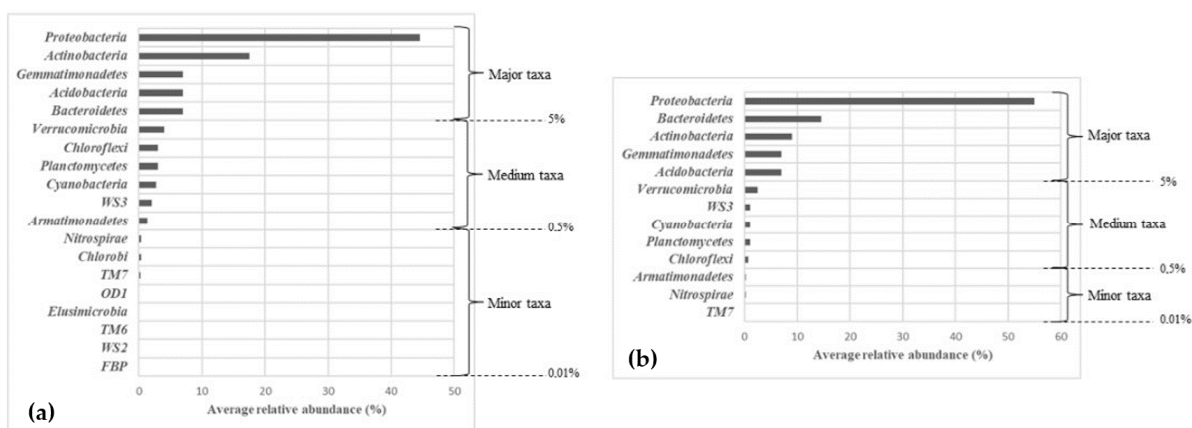


Figure 2. The average relative abundance of the phyla of the chalky soil bacterial community in soil samples 1 and 2 (a) and in the rhizosphere (b).

3.4. Bacterial Inhibition against Phytopathogenic Microbes

The results of the bacterial inhibition test presented in Table 1 revealed that most of the tested bacterial isolates showed inhibition effects against *Micrococcus roseus* B1236. Nevertheless, no bacterial isolates exhibited inhibition effects against either *Erwinia herbicola* ATCC 27155 or *Pectobacterium carotovora* B15. In contrast, bacterial isolates Z11 and Z15 showed inhibitory effects against all tested phytopathogenic fungi. The inhibition zone presented in Figure 3 indicates that these two bacterial strains exhibited a potent inhibitory impact on *Pythium ultimum* F-4782 and *Bipolaris sorokiniana* F-4006.

Table 1. Antagonistic test against phytopathogenic microbes.

Phytopathogenic Bacteria and Fungi	Bacterial Isolates					
	Z10	Z11	Z12	Z15	Z26	Z44
<i>Erwinia herbicola</i> ATCC 27155	–	–	–	–	–	–
<i>Micrococcus roseus</i> B1236	–	+	+	+	+	–
<i>Pectobacterium carotovorum</i> B15	–	–	–	–	–	–
<i>Fusarium avenaceum</i> F-132	+	+	+	+	–	+
<i>Rhizoctonia solani</i> F-895	–	+	–	+	–	–
<i>Alternaria brassicicola</i> F-1864	–	+	+	+	–	–
<i>Bipolaris sorokiniana</i> F-4006	+	+	–	+	+	–
<i>Pythium ultimum</i> F-4782	–	+	–	+	–	–

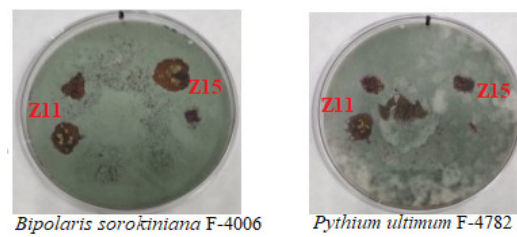


Figure 3. Inhibition zone shown by bacterial isolates against phytopathogenic fungi.

3.5. Bacterial Growth Stimulation Effect on Germinated Seeds

The growth stimulation effect of bacterial isolates on the lengths and fresh weights of the shoots and roots of the germinated seeds is presented in Figure 4. In Figure 4a, it is shown that the bacterial isolate Z10 caused a significant increase in the shoot length of wheat. Whereas bacterial isolates Z11, Z12, Z15, Z26, and Z44 led to significantly increased shoot length in oats. Moreover, bacterial isolates Z10 and Z11 induced a significant increase in the shoot length of lentils ($p = 0.05$). On the other hand, the stimulation effect on root length shown in Figure 4b revealed that no bacterial isolates showed significant changes in the root lengths of wheat, maize, or lentils. However, bacterial isolate Z11 significantly decreased the root length of oats ($p = 0.05$). In the case of stimulation effects on the fresh weights of shoots and roots represented in Figure 4c,d, bacterial isolates Z10 and Z12 led to significant increases in the fresh weights of the shoots and roots of lentils, respectively. Furthermore, bacterial isolates Z15 and Z26 also caused significant increases in the fresh weights of the shoots and roots of maize, respectively ($p = 0.05$).

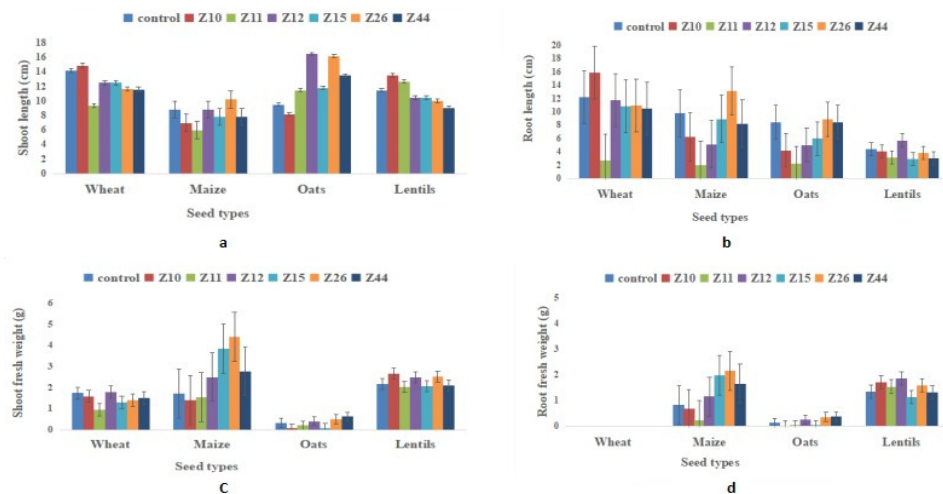


Figure 4. The growth stimulation effect on the shoot length (a), root length (b), shoot fresh weight (c), and root fresh weight (d) by chalky soil bacterial isolates on the germinated seeds of wheat, maize, oats, and lentils.

4. Discussion

The usage of PGPR as a biofertilizer and/or biocontrol agent is a crucial tactic for environmentally friendly and sustainable farming methods. The most often documented PGPR bacteria that encourage plant development and inhibit phytopathogenic microorganisms mostly come from the genera *Bacillus* and *Pseudomonas* [5]. In the present investigation, a total of 23 different kinds of chalky soil bacteria were isolated from the rhizosphere of wild legume plants. As compared to other soil types, the average chalky soil bacterial abundance in the current study was 2.95×10^8 cfu/g, which is low. In sandy, clay, and loamy soil, for instance, the bacterial populations are 1.9×10^9 cfu/g, 1.8×10^{10} cfu/g, and 2.3×10^{10} cfu/g, respectively [15]. Based on the results of the soil profile using 16S rRNA gene sequencing, around 19 different chalky soil bacterial groups were described.

The predominant bacterial phyla in all soil samples were *Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Acidobacteria*, and *Bacteroidetes*. Many crop plants were damaged by phytopathogenic fungi such *Alternaria* sp., *Rhizoctonia solani*, *Pythium ultimum*, and *Fusarium solani* [16]. In the present study, bacterial isolates Z10, Z11, Z12, Z15, Z26, and Z44 showed good inhibition effects on the tested phytopathogenic microbes. In particular, bacterial isolates Z11 and Z15 showed strong inhibition effects against *Pythium ultimum* and *Bipolaris sorokiniana*. Moreover, bacterial isolates Z10, Z12, Z15, Z26, and Z44 had better growth-stimulating effects on the lengths and fresh weights of shoots and roots of the germinated seeds of wheat, maize, oats, and lentils.

5. Conclusions

The current study was conducted to assess the isolation and characterization of soil bacteria found in the rhizosphere of *Chamaecytisus ruthenicus* growing in chalky soil. In the present study, the average abundance of chalky soil bacteria varied from 10.1×10^7 cfu/g in the rhizosphere to 4.25×10^8 cfu/g in chalky soil. On the other hand, the chalky soil contained bacteria with a wide range of morphologies. From the listed bacterial community, *Proteobacteria* was the most dominant and cosmopolitan group of bacteria. Furthermore, the chalky soil bacterial isolates Z10, Z11, Z12, Z15, Z26, and Z44 showed inhibition activity against the phytopathogenic bacteria *Micrococcus roseus* B1236 and all the tested phytopathogenic fungi. In addition, bacterial isolates Z10, Z12, Z15, Z26, and Z44 showed plant growth-stimulating effects on the germinated seeds of wheat, maize, and oats. In the future, further investigations will be carried out on these potent chalky soil bacterial isolates. Finally, the present study is the first report of chalky soil-associated bacteria found in the rhizosphere of a wild legume plant in the Belgorod region of Russia.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ECP2023-14709/s1>.

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