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# Isolation and Characterization of Plant Growth Promoting Bacteria from the Rhizosphere of *Chamaecytisus ruthenicus* (Russian Broom) Growing on Chalky Soil †

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**Abstract:** Bacteria (PGPR) 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 study was done on the bacteria present in the rhizosphere of the wild legume plant *Chamacytisus ruthenicus* growing on chalky soil. The purpose of the study was to evaluate the abundance of culturable bacteria, assess the morphology of the bacterial cells, profile the chalky soil bacterial community, and characterize their 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 top soil, the second sample from the soil 15 cm beneath the surface and the third sample was from the rhizosphere. The result of the study revealed that the abundance of bacteria in the first, second and third soil sample were $4.25 \times 10^8$ cfu/g, $3.58 \times 10^8$ cfu/g and $10.1 \times 10^7$ 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 belongs to *Proteobacteria, Actinobacteria*, *Gemmatimonadetes*, *Acidobacteria*, and *Bacteroidetes* were the most dominant group in the community. Six bacterial isolates (Z10, Z11, Z12, Z15, Z26, and Z44) were chosen to examine their inhibition effect 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 stimulation effect on the length and fresh weight of the shoots and roots of wheat, maize and oats seeds. As a 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 rhizosperic bacteria (PGPR); chalky soil; phytopathogenic microbes; *Chamacytisus 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 shoot and root. On the other hand, some PGPR affects 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 rhizosphere of different plant species. However, no research has been done 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 chalky soil bacterial community, and characterize their ability to stimulate plant growth.

## 2. Methods

### *2.1. Soil Sample Collection*

Soil samples were collected from non-agricultural soil of Belgorod region of the Russian Federation. The location of the site is 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 soil, the second from 15 cm beneath the surface, and the third from the rhizosphere. Each soil sample was placed in sterile polythene bags and brought to the laboratory for immediate analysis, and the rest was stored at 4 °C for further studies.

### *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 that contain reduced concentration 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, 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 the phase contrast mode was carried out using a Nikon Eclipse Ci microscope (Nikon, Tokyo, Japan) equipped with ProgRes SpeedXT camera (Jenoptic, Jena, Germany).

### *2.4. Soils 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 is used, where all raw reads are 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 statistics methods (principal component analysis).

### *2.5. Bacterial Inhibition against Phytopathogenic Microbes*

Bacteria inhibition test 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 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 bacteria were added to plates containing LB and distributed with sterile glass beads. Then, 5 µL of each bacterial isolate was distributed evenly throughout the plates. Similar to that, a little piece of fungal body was put in the middle of a plate containing Sabouraud dextrose agar, and 5 µL of bacterial isolates were 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 of seed 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. During a week, 15 mL of water each day was added to the plates. The growth stimulation experiment was performed in triplicate. The length and fresh weight of the shoot and root of the seedlings were measured and compared after the seeds had germinated. The data was statistically evaluated using *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 bacteria concentration ($4.25 \times 10^8$ cfu/g) was recorded in the initial soil sample. While bacteria concentration in the second soil sample was $3.58 \times 10^8$ cfu/g. Nevertheless, the bacteria concentration in the third soil sample was $10.1 \times 10^7$ cfu/g, which was 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 was shown. The representative bacterial cells were shaped like rods, spheres, and filaments. Also, a few of the bacterial cells contained spore.

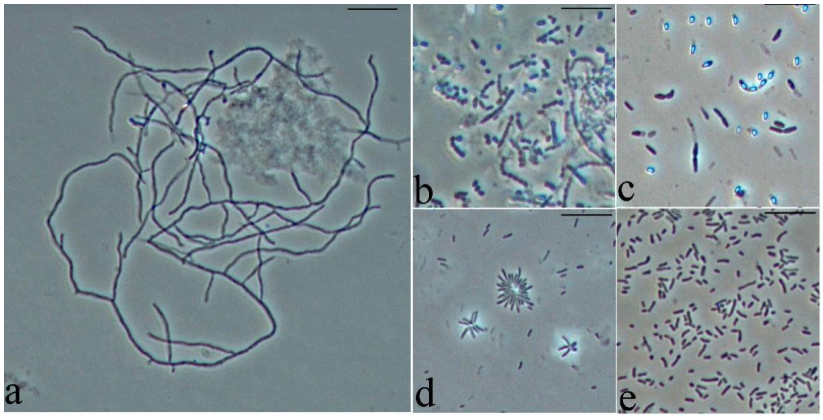

**Figure 1.** The morphological diversity of chalky soil bacterial cells: (**a**) branching aerial mycelium of actinomycete—producer of bright orange pigment; (**b**) streptococci; (**c**) diplobacilli with spores; (**d**) thin long rod-shaped cells, forming rosettes; (**e**) unevenly dividing rod-shaped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

### *3.3. Profile of Soil Bacteria Community*

The result 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* (belongs to the *Proteobacteria*) was the most numerous genus in all soil samples. However, *Flavobacterium* (which belongs to the *Bacteroidetes*) was the most dominant genus in the rhizosphere. The results of the bacterial alpha diversity analysis showed a large 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 is a difference between samples, but according to the results of the PERMANOVA analyses, there were no significant differences in β diversity among the groups.

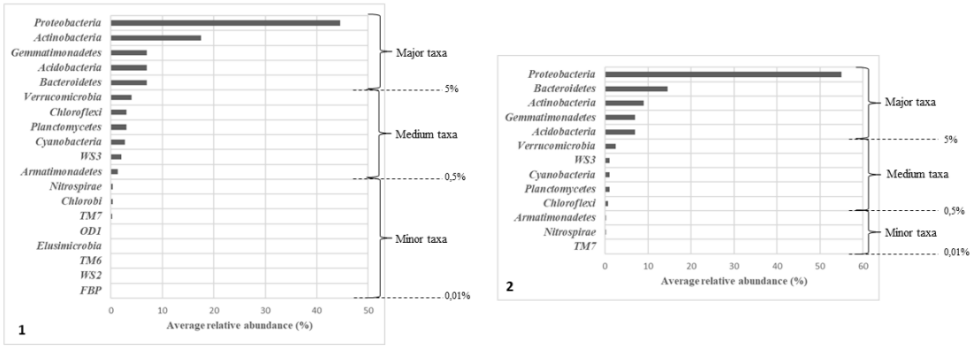


**Figure 2.** The average relative abundance of the phyla of chalky soil bacterial community in soil sample 1&2 (**1**) and in rhizosphere (**2**).

### *3.4. Bacterial Inhibition against Phytopathogenic Microbes*

The result of the bacterial inhibition test presented in Table 1 revealed that most tested bacterial isolates showed inhibition effects against *Micrococcus roseus* B1236. Nevertheless, no bacterial isolates exhibited inhibition effects against both *Erwinia herbicola* ATCC 27155 and *Pectobacterium carotovora* B15. In contrast, bacterial isolates Z11 and Z15 showed an inhibitory effect against all tested phytopathogenic fungi. The inhibition zone presented in Figure 3 indicated 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** |
| *Erwinia herbicola* ATCC 27155 | - | - | - | - | - | - |
| *Micrococcus roseus* B1236 | - | + | + | + | + | - |
| *Pectobacterium carotovorum* B15 | - | - | - | - | - | - |
| *Fusarium avenaceum* F-132 | + | + | + | + | - | + |
| *Rhizoctonia solani* F-895 | - | + | - | + | - | - |
| *Alternaria brassicicola* F-1864 | - | + | + | + | - | - |
| *Bipolaris sorokiniana* F-4006 | + | + | - | + | + | - |
| *Pythium ultimum* F-4782 | - | + | - | + | - | - |

*(+) represented growth inhibition* *(-) represented no growth inhibition*

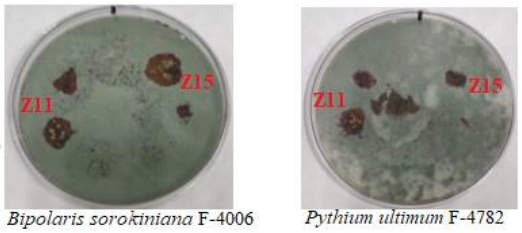


**Figure 3.** Inhibition zone showed by bacterial isolates against phytopathogenic fungi.

### *3.5. Bacterial Growth Stimulation Effect on Germinated Seeds*

The growth stimulation effect of bacterial isolates on the length and fresh weight of the shoot and root of the germinated seeds is presented in Figure 4. In Figure 4a, the bacterial isolate Z10 caused a significant increase in the shoot length of wheat. Whereas bacterial isolates Z11, Z12, Z15, Z26, and Z44 showed a singificantly increased shoot length in oats. Moreover, bacterial isolates Z10 and Z11 showed a significant increase in the shoot length of lentils ($p = 0.05$). On the other hand, the stimulation effect on root length in Figure 4b revealed that all bacterial isolates showed no significant change in the root length 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 weight of shoot and root that were represented in Figure 4c,d, bacterial isolates Z10 and Z12 showed significant increases in the fresh weight of shoot and root of lentils, respectively. Further more, bacterial isolates Z15 and Z26 also showed a significant increase in the fresh weight of the shoot and root of maize, respectively ($p = 0.05$).

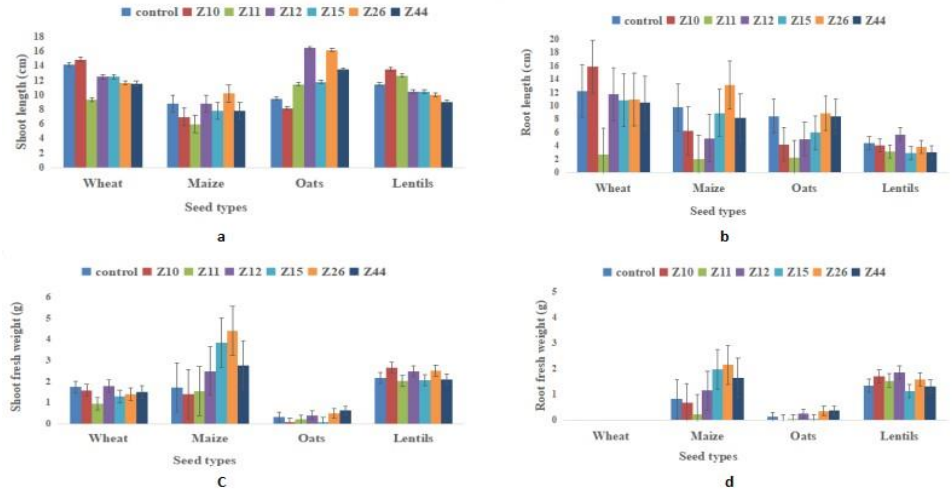


**Figure 4.** The growth stimulation effect on the shoot length (**a**), root length (**b**), shot fresh weight (**c**) and root fresh weight (**d**) by chalky soil bacterial isolates on the germinated seeds of wheat, maize, oats, and lentils.

## 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 that encourages plant development and inhibits phytopathogenic microorganisms mostly comes 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 plant. As compared to other soil types, the average chalky soil bacterial abundance in the current study was $2.95 \times 10^8$ cfu/g, which is low. In sandy, clay, and loamy soil, for instance, the bacterial populations are $1.9 \times 10^9$ cfu/g, $1.8 \times 10^{10}$ cfu/g, and $2.3 \times 10^{10}$

cfu/g, respectively [15]. Based on the result of 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 inhibiton effect on the tested phytopatogenic microbes. In particular, bacterial isolates Z11 and Z15 showed strong inhibition effect against *Pythium ultimum* and *Bipolaris sorokiniana*. Moreover, bacterial isolates (Z10, Z12, Z15, Z26, and Z44) had better growth stimulating effect on the length and fresh weight of shoot and root of the germinated seeds of wheat, maize, oats, and lentils.

## 5. Conclusions

The current study was done on the isolation and characterization of soil bacteria found in the rhizosphere of *Chamaecytisus ruthenicus* growing on chalky soil. In the present study, the average abundance of chalky soil bacteria was varied from $10.1 \times 10^7$ cfu/g in the rhizosphere up to $4.25 \times 10^8$ cfu/g in chalky soil. On the other hand, chalky soil contains 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 stimulation activities on the germinated seeds of wheat, maize, and oats. In the future, further investigation will be carried out on those potent chalky soil bacterial isolates. Finally, 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.

**Institutional Review Board Statement:** This article does not contain descriptions of studies with human participants or animals performed by any of the authors.

**Conflicts of Interest:** This authors declare no conflict of interest in the finanical or any other sphere.

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