


## Article

# Effects of Mineral-Solubilizing Microorganisms on Root Growth, Soil Nutrient Content, and Enzyme Activities in the Rhizosphere Soil of *Robinia pseudoacacia*

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**Abstract:** Background: Abandoned mining sites are becoming increasingly common due to anthropogenic activities. Consequently, external-soil spray seeding technology has attracted increasing attention as a strategy to remediate them. However, significant challenges remain that greatly inhibit the efficacy of such technologies, such as insufficient nutrients available for plants. Methods: For this study, we designed an experiment, which involved the addition of mineral-solubilizing microorganisms and *R. pseudoacacia* seedlings to the external-soil spray seeding (ESSS) substrate, and measured the soil nutrients, enzyme activities, and root growth of *R. pseudoacacia*. Results: First, the combination of certain mineral-solubilizing microorganisms with ESSS advanced its efficiency by increasing the availability of soil nutrients and soil enzymatic activities in association with *R. pseudoacacia*. Furthermore, the improvement of root growth of *R. pseudoacacia* was intimately related to soil nutrients, particularly for soil total nitrogen (TN) and total sulfur (TS). In general, the effects of the J2 (combined *Bacillus thuringiensis* and *Gongronella butleri*) treatment for soil nutrients, enzyme activities, and plant growth were the strongest. Conclusion: In summary, the results of our experiment revealed that these mineral-solubilizing microorganisms conveyed a promotional effect on *R. pseudoacacia* seedlings by increasing the soil nutrient content. These results provide basic data and microbial resources for the development and applications of mineral-solubilizing microorganisms for abandoned mine remediation.

**Keywords:** mineral-solubilizing microorganisms; ecological restoration technique; root growth; soil nutrient contents; soil enzyme activities



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## 1. Introduction

The emergence of steep rocky slopes is increased rapidly in China due to the construction of highways and railways, as well as the over-exploitation of rock mineral resources [1,2]. These activities have severely damaged ecosystems [3,4]. At present, multiple strategies are employed for restoring the abandoned mine sites [5–7], where the external-soil spray seeding (ESSS) technique has become a common technique for their ecological restoration [8,9]. The ESSS (Figure 1) technique contains the spraying of artificial mixed soil materials (soil, water-retaining agents, fertilizer, and plant seeds) onto rocky slopes toward the ecological restoration of abandoned mine sites [10]. Soil comprises the core element of this technique, which provides plants with water and nutrient sources [11]. However, major challenges remain, as the soils on steep slopes are inadequate and the available nutrients absorbed by plants are unsatisfactory [12]; therefore, this technique has been difficult to establish toward the promotion of long-term ecological restoration maintenance in abandoned mining areas [13].



**Figure 1.** External-soil spray seeding (ESSS) technique.

The addition of roots can strengthen the soil, as the root growth is intimately related to the soil [14]. Soil nutrient and soil enzyme activity can affect root growth [15–17]. Therefore, it is very important to study the changes in root growth, soil nutrients, and soil enzyme activity for the promotion of long-term ecological restoration maintenance in abandoned mining areas. The application of mineral-solubilizing microorganisms is a novel attempt for abandoned mine remediation, which can accelerate mineral weathering [18–20]. In recent years, the research into mineral-solubilizing microorganisms have been more in the scope of Plant Growth-Promoting Rhizobacteria (PGPR); the functions of mineral-solubilizing microorganisms also have many similarities with PGPR. Firstly, increased essential minerals solubilized by microorganisms (e.g., P, K, S, Mg, Cu, Fe, and Zn) contribute to the promotion of plant growth [21]. Secondly, these microorganisms can also promote plant development through the generation of plant-growth-promoting substances and siderophore production [22,23]. Moreover, several microorganisms have the capacity to improve plant growth through the stimulation of plant hormones and root elongation (including the formation of root hairs and lateral roots) [24–27].

Previous studies have reported that root and plant growth are intimately correlated with the availability of soil nutrients [28–30] and soil enzyme activities [31–33]. Rhizospheric microorganisms are crucial in nutrient cycling, they can increase soil enzyme activities and the mineralization of available nutrients in the soil; thus, accelerating root growth [21,23,26,34]. For instance, the combination of *Advenella incenata* P4, *Serratia plymuthica* P35, *Providencia rettgeri* P2, and *Acinetobacter calcoaceticus* P19 microorganisms increased soil urease, phosphatase, catalase, invertase activity, available phosphorus (AP), available potassium (AK), and available nitrogen in the rhizosphere of *Avena sativa* [35]. The addition of *Glomus etunicatum* also increased the invertase, urease, proteinase, catalase, and phosphatase activities in the rhizosphere soil of maize [36]. Moreover, the application of *Bacillus sp.* strain UM15 improved soil available phosphorus and total nitrogen [37]. Therefore, microorganisms were observed to be closely correlated with soil nutrients and enzyme activities.

*R. pseudoacacia* is the dominant tree species in abandoned mine restoration due to its capacity for tolerating dry environments and nitrogen-fixing abilities [38,39]. The microorganisms in this study were mineral-solubilizing microorganisms isolated from the soil surrounding weathered dolostones, which secreted organic acid to enhance the release of Ca and Mg [40,41]. In our previous study, these mineral solubilizing microorganisms

were found to enhance root growth of *Lespedeza bicolor*, *L. bicolor* root-reinforced soil shear strength, as well as the tensile force and strength of *L. bicolor* roots [16,17]. However, the response of soil nutrient content, and enzyme activities in the rhizosphere soil of *R. pseudoacacia* remain unknown.

The main objectives of this study were to: (1) verify the impacts of mineral-solubilizing microorganisms on soil nutrients and enzyme activities, (2) demonstrate the relationships between root growth, soil nutrients, and soil enzyme activities under mineral-solubilizing microorganism treatments. Based on the previous researches [17,35], we hypothesized that: (1) the application of mineral-solubilizing microorganisms promotes soil nutrients and enzyme activities and (2) the root growth is intimately related to the soil nutrient and soil enzyme activity. These results highlighted the roles of mineral-solubilizing microorganisms for the restoration of abandoned mine sites while providing fundamental data and microbial resources toward the development of additional applications for mineral-solubilizing microorganisms.

## 2. Materials and Methods

### 2.1. Study Site

The pot experiments were finished in a greenhouse of Nanjing Forestry University (31°7' N, 119°12' E). The experiment lasted from December 2018 to November 2019.

### 2.2. Plant Seeds, Microbial Strains, and Soil

*R. pseudoacacia* seeds were purchased from the Tianhe Nursery Garden Company (Jiangsu, China). The germinated seeds were transferred to a seedling-raising disk and cultivated using nursery substrates, whereas the germination process referred to a previous study [16].

NL-1 (*Streptomyces thermocarboxydus*), NL-11 (*Bacillus thuringiensis*), and NL-15 (*Gongronella butleri*) were isolated from the soil that surrounded weathered dolostones in Mufu mountain, Nanjing, China, where the specific isolation process, screening, and identification referred to a previous study [40,42]. In our previous research, the effects of *S. thermocarboxydus* treatment, a combination of *B. thuringiensis* and *G. butleri* treatment, and a combination of *S. thermocarboxydus*, *B. thuringiensis* and *G. butleri* treatment were significant for the root growth and photosynthesis system of *Amorpha fruticosa* [43]. Consequently, we selected these three treatments to conduct this experiment.

Topsoil (5–30 cm) was collected from the Xiashu Forest Farm and mixed with some extra materials as substrates, and the specific ratio was following in: soil, wood fiber, organic fertilizer, peat soil, rock powder was 92, 0.7, 5, 2, 0.3) [16]. The soil type was yellow brown, whereas the topsoil properties included available phosphorus, 10.00 mg kg<sup>-1</sup>, available potassium, 101.39 mg kg<sup>-1</sup>, at a pH of 7.15.

### 2.3. Experimental Treatments

The experiments included four treatments: NL-11 (J1), NL-11 + NL-15 (J2), NL-1 + NL-11 + NL-15 (J3), and control treatment (CK), which were employed in each group, which consisted of three replicates, totaling 12 pots (diameter was 29.5 cm and height was 19.5 cm), respectively. Each pot was filled with 5 kg of nursery substrates, 15 g of a water-retaining agent, and 60 mL of microorganisms (the total amount of mixed microorganisms was 60 mL). The concentration of the microbial application was  $2.9 \times 10^9$  CFU/mL, and the microorganisms were added to the soil prior to sowing.

There were three germinated seeds planted in each pot, and after a month, two of these were removed to ensure the health of only one plant. The plants were grown under greenhouse conditions, and maintained at temperatures from 18–35 °C, with a relative humidity of 40 to 80%, and a daily photoperiod that ranged from 10 to 14 h [16]. During the growth period, the plants were watered every two weeks. We collected rhizospheric soil and fresh root samples in November 2019 transferred them to a refrigerator and quickly



transported it to the laboratory. The root samples were washed in a basin to avoid the loss of fine roots, after which plant root growth parameter analysis was immediately performed.

#### 2.4. Plant Root Growth Parameter Analysis

The root systems were scanned using a LA2400 Scanner (Expression 12000XL, EPSON, Long Beach, CA, USA). The scanned images were analyzed using WINRHIZO software to obtain the average root diameter (RD), total root length (RL), total root surface area (RS), and total root volume (RV).

#### 2.5. Analysis of Soil Properties

The soil pH was determined using a PB-10 pH meter [44]. The total carbon, total sulfur, and total nitrogen were quantified using an elemental analyzer. The AP was measured using the molybdate-blue colorimetric method [45]. The AK was determined using a flame photometer [44]. The soil urease activity was revealed by measuring the quantity of ammonium released from the soil [46], whereas the soil phosphatase activity was measured using the phenyl phosphate disodium colorimetric method [47]. The soil sucrase activity was measured via 3,5-diyl salicylic acid colorimetry [48], and the soil catalase activity was discovered using the KMnO<sub>4</sub> titrimetric method [49]. An analysis of the soil microstructures on the root surfaces was performed using scanning electron microscopy (SEM).

#### 2.6. Statistical Analyses

The Duncan test was employed when one-way ANOVA (SPSS 26) revealed the effects of the microbial treatment on soil nutrients, enzyme activities, and root growth parameters. The Pearson test was performed using R software (R 3.4.3) to reveal the relationships between root growth of *R. pseudoacacia*, soil nutrients, and enzyme activities. Other charts were created by Origin 2015 (OriginLab Corporation, Northampton, MA, USA).

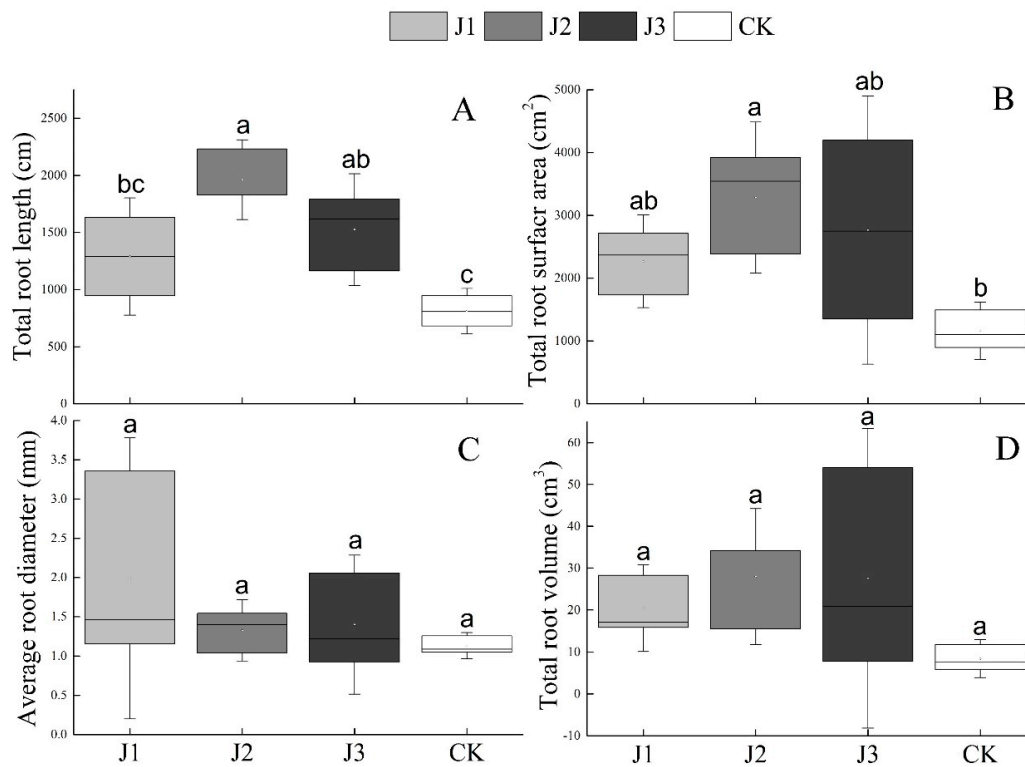
### 3. Results

#### 3.1. Plant Root Growth Parameters

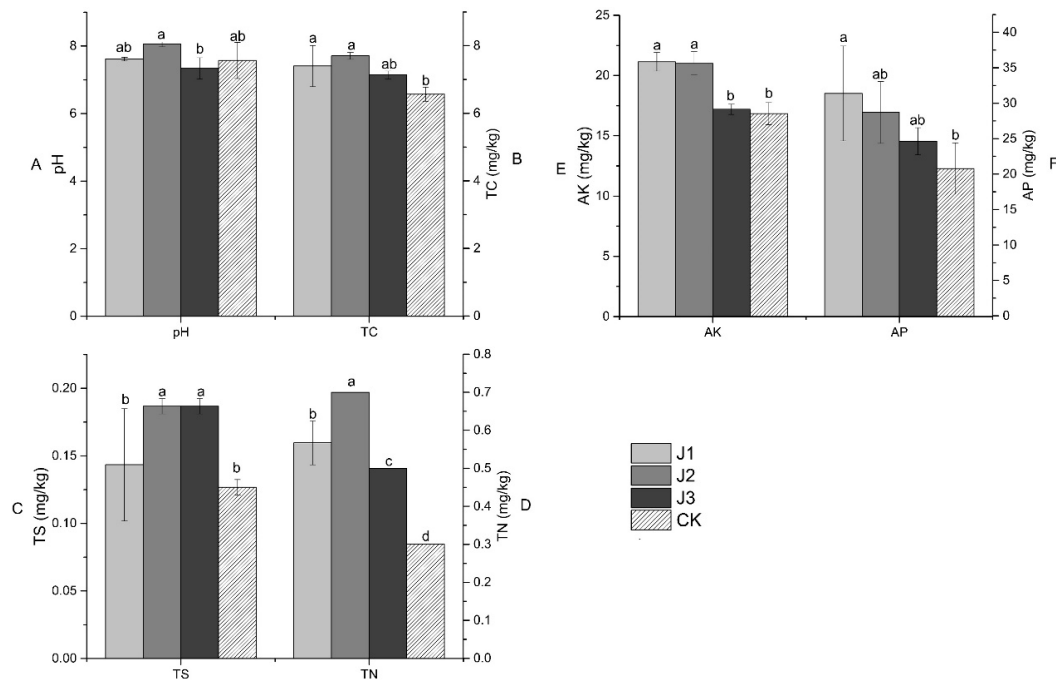
The addition of mineral-solubilizing microorganisms influenced the growth characteristics of *R. pseudoacacia* seedlings (Figure 2). Compared with the CK treatment, the J1 and J3 treatments increased the RL by 73.4%, on average. Furthermore, the RS was increased by 94.8% and 137.2%, respectively. For the RD and RV, microbial treatments had no significant difference to them. However, the J1, J2, and J3 treatments increased the RD by 75.8%, 17.3%, and 23.9%, respectively. The RV was increased by 143.5%, 234.1%, and 229.5%, respectively. These changes showed that the addition of microorganisms had a tendency to increase the RD and RV. In addition, the J2 treatment significantly increased the RL and RS by 141.7% and 181.7%, respectively ( $p < 0.05$ ).

#### 3.2. Soil Nutrients

The addition of mineral-solubilizing microorganisms influenced soil nutrients in the rhizospheres of *R. pseudoacacia* seedlings (Figure 3). The pH of the J2 treatment was significantly higher than that of the J3 treatment ( $p < 0.05$ ), and there were no significant differences between the J1, J3, and CK treatments. The J1 and J2 treatments significantly increased the soil total carbon (TC) and AK in the rhizospheres of *R. pseudoacacia* seedlings ( $p < 0.05$ ). In contrast to the CK treatment, the J1 treatment significantly increased the AP by 51% ( $p < 0.05$ ). The J2 and J3 treatments significantly increased the TS by 47.37% and 47.37%, respectively ( $p < 0.05$ ). Furthermore, the J1, J2, and J3 treatments significantly increased the TN by 88.9%, 133.3%, and 66.7%, respectively ( $p < 0.05$ ).



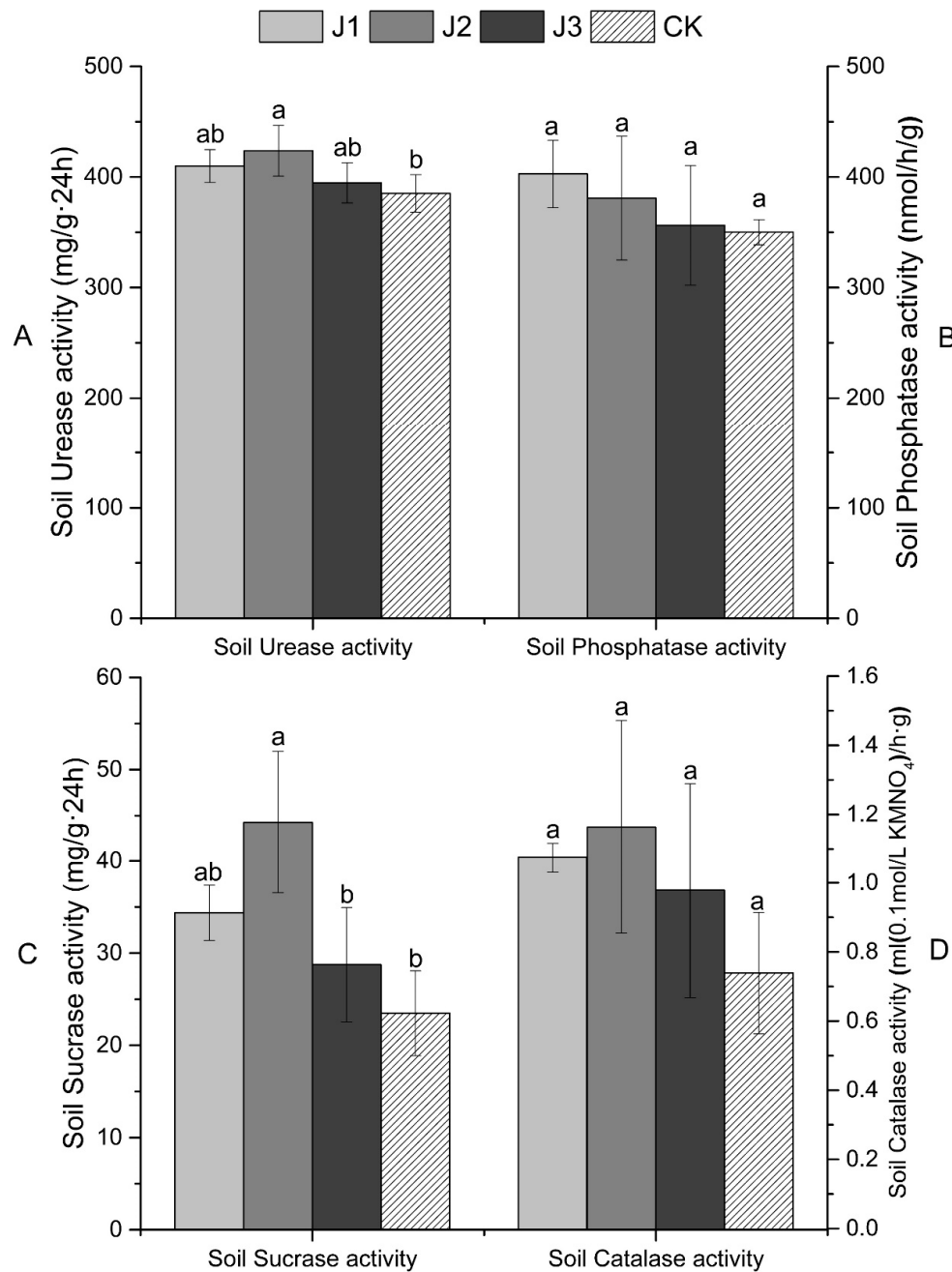
**Figure 2.** The root growth parameters of *R. pseudoacacia* measured with WinRHIZO. Empty circles within each box represent the average pH value, horizontal lines within each box represent the median pH value, and vertical lines represent the interquartile ranges. (A–D) Different letters show treatments that are significantly different ( $p < 0.05$ ) following a Duncan’s test. Experimental treatments: J1, NL-11; J2, NL-11 + NL-15; J3, NL-1 + NL-11 + NL-15.



**Figure 3.** The responses of soil nutrients in the rhizosphere of *R. pseudoacacia* to applications of mineral-solubilizing microorganisms. Error bars are standard errors of the mean ( $n = 3$ ). (A–F) Different letters indicate significant differences ( $p < 0.05$ ) among the different treatments following a Duncan’s test. Experimental treatments: J1, NL-11; J2, NL-11 + NL-15; J3, NL-1 + NL-11 + NL-15. TC, soil total carbon; TN, soil total nitrogen; TS, soil total sulfur; AP, available phosphorus; AK, available potassium.

### 3.3. Soil Enzyme Activities

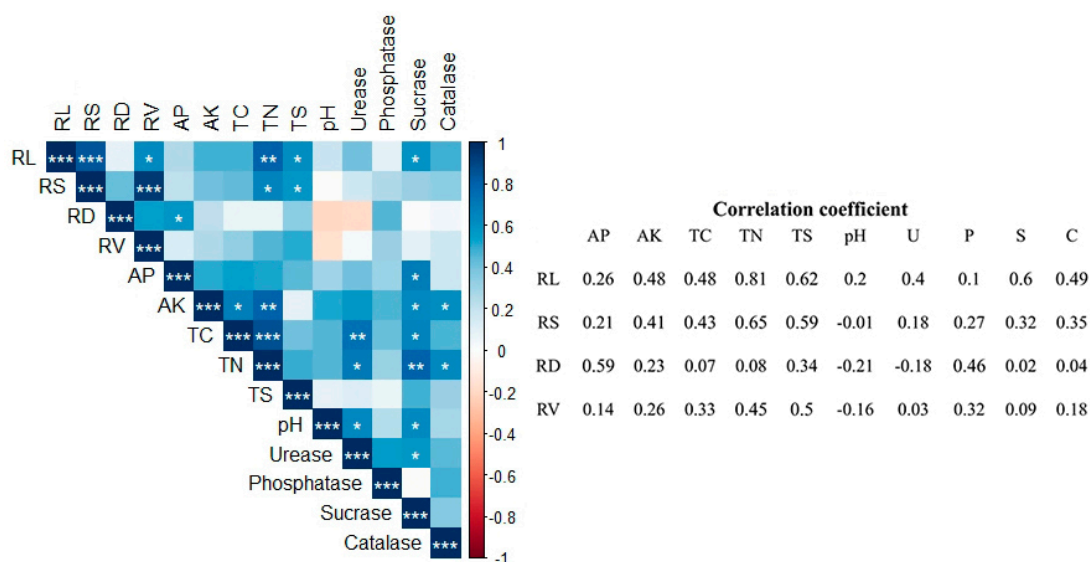
The addition of mineral-solubilizing microorganisms increased the soil enzyme activities in the rhizospheres of *R. pseudoacacia* seedlings (Figure 4). In contrast to the CK treatment, the J2 treatment significantly increased the soil urease and sucrase activities by 10% and 88.5% ( $p < 0.05$ ), respectively, and there was no significant difference between the J1, J3, and CK treatments. For the soil phosphatase and catalase activities, microbial treatments had no significant difference to them. However, the addition of microorganisms had the propensity to increase soil phosphatase and catalase activities.



**Figure 4.** The responses of soil enzyme activities in the rhizosphere of *R. pseudoacacia* to applications of mineral-solubilizing microorganisms. Error bars are standard errors of the mean ( $n = 3$ ). (A–D) Different letters indicate significant differences ( $p < 0.05$ ) among the different treatments following a Duncan’s test. Experimental treatments: J1, NL-11; J2, NL-11 + NL-15; J3, NL-1 + NL-11 + NL-15.

### 3.4. Correlation Analysis between Root Growth Parameters and Soil Nutrients/Enzyme Activities

The results of Pearson's correlation analysis between the root growth parameters and soil nutrients and enzyme activities in the microbial treatments are depicted in Figure 5. The results revealed that the RL was positively correlated with the AK, TC, TN ( $p < 0.01$ ), TS ( $p < 0.05$ ), urease, sucrase ( $p < 0.05$ ), and catalase. The RS was positively correlated with the AK, TC ( $p < 0.05$ ), TN ( $p < 0.05$ ) and TS. Furthermore, the RV was positively correlated with the TN and TS, whereas the RD was positively correlated with the AP ( $p < 0.05$ ) and phosphatase.



**Figure 5.** Correlation analysis between root growth parameters, soil nutrients, and soil enzyme activities. Notes: \*\*\* indicates significant correlation at  $p < 0.001$ ; \*\* indicates significant correlation at  $p < 0.01$ ; \* indicates significant correlation at  $p < 0.05$ .

## 4. Discussion

In our study, the plant root growth parameters of *R. pseudoacacia* seedlings were enhanced in the presence of mineral-solubilizing microorganisms. This might have been related to the generation of plant-growth-promoting substances. In previous studies, the effects of *Azospirillum* on root development were related to indole 3-acetic acid, gibberellic acid, and ethylene, which promoted root growth [50,51]. Furthermore, certain microorganisms might indirectly benefit plant growth by preventing the harmful effects of phytopathogenic organisms, or by inducing systemic resistance in the host plant [52–54].

### 4.1. Soil Nutrients

Soil pH is one of the most important physical and chemical properties of soil. In previous studies, the applications of microorganisms could decrease pH, thus promoting the release of Ca and Mg. Finally, the dissolution rate of mine was increased [41,42,55]; however, for the pH levels in our study, microbial treatments had no significant difference to them. A potential explanation for this was that the pH was influenced not only by the microorganisms, but also some other natural factors (climate or parent materials) [56,57].

Microorganisms have the ability to effectively convert inaccessible soil nutrients into those that are useful while promoting their effective absorption and utilization by plants [35]. For this study, the addition of mineral-solubilizing microorganisms increased the AK and AP contents, which was consistent with previous work. Mineral-solubilizing microorganisms participated in the soil-phosphorus cycle through the excretion of organic acids, which may be converted to soluble forms, to facilitate the released phosphorus into the soil [24,58–60]. The microorganisms also played a critical role in the solubilization of elements such as K from acid-leached soil [61–63]. The reason why J1 has the best effect on AK and AP might have

been that NL-11 possessed the best solubilization effect. Furthermore, there might be certain antagonistic effects between NL-1, NL11, and NL15 [64,65].

Nitrogen is the most important nutrient for plant growth and productivity [66]. The increase in TN showed that the addition of microorganisms enhanced the N fixation capacity of *R. pseudoacacia*. This may have been due to an interesting phenomenon in this study, involving the increased population of root nodules, which might improve root growth through N-fixation [66–69]. Sulfur is the fourth most important plant nutrient following nitrogen, phosphorus, potassium, and is also one of the sixteen nutrients that are essential for the growth of plants [70]. The increase in TS might have been due to microorganisms that promoted the conversion of S [71]. Furthermore, the combined *B. thuringiensis* and *G. butleri* treatment had optimal effects on the increase of TC, TS, and TN. This phenomenon might be initiated via synergistic effects between *B. thuringiensis* and *G. butleri* [64,72]. Correlation analysis indicated that soil nutrients are intimately related to root growth, particularly TN and TS. The relationship between root growth and TN and TS also demonstrated the effects of nodules for N fixation and the effects of S conversion for plant growth.

This suggested that part of the first and second hypothesis was supported by our results. It demonstrated that the application of mineral-solubilizing microorganisms promoted soil nutrients, and the root growth was intimately related to the soil nutrient, particularly for TN and TS. In this research, the availability of individual elements had changed, but the morphologic transformation of the individual element from soil to plant was unknown, it also required further research.

#### 4.2. Soil Enzyme Activity

The activities of soil enzymes are particularly useful for the assessment of soil quality as they play an important role in maintaining soil fertility and rapidly responding to environmental changes [73–75]. Among soil enzymes, catalase and sucrase are essential enzymes for the conversion of different nutrients required for plant growth [74,76], where urease is the only amidase in soil that can convert urea into useful N [77]. Phosphatases also mineralize organic phosphorus and improve phosphate nutrition and plant growth by increasing the concentration of soluble phosphate in the soil [78,79].

For this study, the addition of microorganisms promoted soil urease and sucrase activities, which was consistent with previous researches [36,80,81]. This confirmed the role of mineral-solubilizing microorganisms for soil enhancement. Moreover, the effects of double microbial strains on soil enzyme activities remained the most potent. The reason for this phenomenon might have been that the boost from NL-1 was greater than that from NL-11. However, the specific synergistic and antagonistic effects involved will require further experimentation to verify. In our previous studies, for *Lespedeza bicolor*, the root growth parameters are significantly related to sucrase and catalase. In this research, correlation analysis revealed that there was no significant correlation between the activities of soil enzymes and root growth. This was also not consistent with our second hypothesis. However, there were significant correlations between the activities of soil enzymes and soil nutrients. This suggested that soil enzyme activities might influence root growth via soil nutrients. Therefore, although *R. pseudoacacia* and *L. bicolor* were dominant tree species in abandoned mine restoration, their selection and collocation still need further study.

In previous studies, the addition of these microorganisms decreased the diameters of soil particles [40,41], where the results of SEM images also directly demonstrated this conclusion (Figure S1). Roots attained greater depths under loose soil conditions, as they could explore a larger soil volume to increase the quantity of available water for uptake [82]. Furthermore, changes in soil particle size classes also impacted the activities of soil enzymes [83], which were enhanced in more finely textured soils [84]. Therefore, changes in soil particle dimensions also impacted root growth by altering the activities of soil enzymes.



It should be noted that siderophore production and the synthesis of several other growth-promoting compounds were also influenced by mineral-solubilizing microorganisms [50–54]. Since each of these indices affected root growth, they warrant further investigation. Furthermore, specific synergistic and antagonistic effects also require further research. Most importantly, as the survival state of our added mineral-solubilizing microorganisms in the indigenous microbial community was unknown, we need to design an experiment to monitor them in the following research. Finally, our experiment was only monitored for one year; however, microbial communities may require far longer to stabilize. Therefore, we propose to investigate the changes in microbial communities over longer timelines.

## 5. Conclusions

In this research, mineral-solubilizing microorganisms were added to ESSS substrates to potentially augment the content of soil nutrients and soil enzyme activities in the rhizosphere soil of *R. pseudoacacia*. Furthermore, the nutrient content of the soil was intimately related to root growth, particularly for TN and TS. In general, the effects of NL-11+NL-15 (combined *B. thuringiensis* and *G. butleri*) on soil nutrients, enzyme activities, and plant growth were the strongest.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/1999-4907/12/1/60/s1>, Figure S1: SEM images.

**Author Contributions:** Conception and design of the research: C.L. and Z.J.; acquisition of data: X.P.; analysis and interpretation of data: C.L.; statistical analysis: C.L. and Z.J.; drafting the manuscript: B.Z. and L.Z.; revision of manuscript for important intellectual content: X.L. and J.Z. All authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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