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RESEARCH ARTICLE

EFFECT OF FERTILIZATION AND RHIZOBIUM INOCULATION ON THE GROWTH AND NITROGEN CONTENT OF SOYBEAN (*GLYCINE MAX*) AND SHRUB LESPEDEZA (*LESPEDEZA BICOLOR*) GROWN IN COAL MINE SOIL

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ABSTRACT

The current research was conducted in the greenhouse to figure out the fertilizer and rhizobium inoculation effect on the vegetative growth of two nitrogen fixing leguminous plant species, namely shrub lespedeza (*Lespedeza bicolor*) and soybean (*Glycine max*) grown in coal mine soil, as well as how their nitrogen (N), phosphorus (P), and potassium (K) contents changed. Three replicates of each of the four treatments were applied to both plants: T0- non-fertilized non-inoculation (control); T1- fertilization; T2- rhizobium inoculation; and T3- fertilization combined with rhizobium inoculation. Except for root dry weight, fertilization and fertilization combined with inoculation considerably enhanced the nourishment of all vegetative parameters of both plants in comparison to the control. Compared to the control, both the dry and fresh weight of the shoots, plant dry weight, the canopy spread, leaves, and node number on each plant, the leaf area index, and the leaf area increased. The fertilizer and rhizobia inoculation application dramatically raised the N content of soybean and shrub lespedeza. P and K content of the investigated plants increased significantly only when NPK fertilizer was administered. In conclusion, it could be stated that rhizobium inoculation and fertilization play an indispensable role in stimulating the outgrowth of shrub lespedeza and soybean in coal mine soil and increasing the nutrient content of examined legume species.

KEYWORDS

Soybean; shrub lespedeza; rhizobium inoculation; vegetative growth; N content

1. INTRODUCTION

Planting N-fixing legumes with rapid growth is an efficient method for restoring deteriorated coal mine soil (Sheoran, et al., 2010; Mukhopadhyay, et al., 2014). In addition to improving nitrogen cycling, increasing carbon absorption, and reducing soil erosion, these plants serve as a nursery for newly planted species (Moura, et al., 2016). N-fixing plants significantly increase soil fertility by creating nutrient-rich, rapidly decomposing litter and altering nodules and fine roots (Zhang, et al., 2001). In association with symbiotic N-fixing bacteria, legume species enhance increased nitrogen (N) accumulation and minimize the soil's C/N ratio, which aids N mineralization and nutrient cycling, an indispensable condition for degraded mine soil reclamation (Moura, et al., 2016). Regular fertilization with N fertilizer might also be a good alternative for maintaining healthy growth and sustainability of vegetation in coal mine spoil (Song, et al., 2004).

Soybean (*Glycine max*) is a high-value grain and oilseed legume crop. Soybean grain includes forty percent protein and twenty percent oil (Marinkovic, et al., 2018). As a legume, soybean utilizes atmospheric N_2 for growth through biological nitrogen fixation (BNF) in root nodules that form through symbiosis with rhizobia bacteria and give considerable amounts of available N to the soil (Ohyama, et al., 2011). Shrub lespedeza (*Lespedeza bicolor*) is a blooming legume plant also known as shrubby bush clover or bicolor Lespedeza. This plant stabilizes steep slopes and streambanks, reduces erosion, and improves wildlife habitat. This legume plant can be recommended for abandoned coal mines to restore N in degraded soil because of its nitrogen-fixing capability and ability to thrive in poor soil (Tesky, et al., 1992).

N is a crucial nutrient needed by plants for growth and development. According to reports, N can boost assimilation by promoting plant vegetative development and expanding the area available for photosynthesis (Bangar, et al., 2000; Mohammadi, et al., 2016). When plants are fertilized with microbiological fertilizer or the appropriate rhizobia, nodules are formed, growth and development are accelerated, atmospheric N is fixed, the biomass and N content of the plant rise, and yield are improved. This means it has the potential to be used in conjunction with or as a replacement for artificial fertilizer in plant cultivation (Marinkovic, et al., 2018). Even under ideal conditions, plants can't meet their entire nitrogen needs through symbiotic N fixation alone; therefore, N fertilization is also required for optimal growth and a high yield (Al-Chammaa, et al., 2014).

The occurrence of symbiosis between N-fixing bacteria and legume plant nodules is an effective way to initiate natural succession and boost soil reclamation effectiveness. Because of their symbiotic association, plants of N-fixing species flourish in the harsh conditions of degraded lands and grow swiftly despite the shortage of nutrients in the soil. A previous study investigated the capacity of two nitrogen-fixing crop and tree species, shrub lespedeza (*Lespedeza bicolor*) and soybean (*Glycine max*), to reclaim the fertility or productiveness status of degraded soil of coal mine in terms of pH, NO_3^--N and NH_4^+-N (Shin, et al., 2018). Although reproductive and vegetative outgrowth is not investigated, N fertilizer with rhizobium inoculation may boost the N content and growth of these two-plant species when cultivated in the soil of a coal mine. The prime goal of this initiated research was to evaluate or assess the influences of rhizobia inoculation and N fertilizer on the nutrient content (N, P, K) and vegetative outgrowth of the shrub lespedeza and soybean grown in coal mine soil.

2. MATERIALS AND METHODS

2.1 Soil collection

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The collection of soil was done from three distinct locations in the coal mine (an abandoned one) in the city of Taebaek, South Korea, to cultivate experimental plants. After collection, the soil was transferred to the greenhouse, where the research experiment was carried out, and the experimental mine soil was extracted, pulverized, sieved, and air-dried (mesh size 2 mm). The soil was then evenly distributed among the experimental pots. Each pot has a conical frustum shape and a capacity of 4.8 liters.

2.2 Seed Germination and Plant Transplantation

"The Crop Science Division of Chungbuk National University, South Korea," provided the soybean seeds. Seeds were put in the tray (plastic) for germination after rinsing with sterile water. Planted seeds were kept in a growth chamber by maintaining a temperature of 28°C and relative humidity of 60 to 90 percent (Ferrari, et al., 2007). After a week of sprouting, the seedlings were moved to pots with experimental soil and kept in the greenhouse. "The National Forest Seed Variety Center of South Korea" supplied seeds for the plant Lespedeza. Lespedeza shrub seedlings germinated similarly to soybean seeds in the greenhouse. When germination surpassed two weeks, seedlings of the shrub lespedeza were also placed in the test containers. Consistent watering was done while the seeds were germinating.

2.3 Experimental Design

From June 2021 to August 2021, the trial was guided in a greenhouse at the "Department of Forest Science, Chungbuk National University, South Korea." For this study, a completely randomized design (CRD) comprising treatments (four) and replications (three) followed in coal mine soil for each plant. Treatments were: T0 as the control (non-fertilized non-rhizobia injected), T1 was fertilization (coal mine soil was treated with NPK fertilizer), T2 was rhizobia inoculation (N-fixing rhizobia inoculated on soil), and T3 was fertilization and rhizobia inoculation (both NPK fertilizer and rhizobia added on soil). For each replication, six seedlings were used; hence, eighteen seedlings were used for each treatment.

2.4 Fertilizer Application

The NPK 20:20:20 fertilizer, which comprises 20 percent of each of the macro elements nitrogen (N), phosphorus (P_2O_5), and potassium (K2O), was administered to both plants after being mixed with water. The plants in treatments T1 and T3 were fertilized after 15, 30, and 45 days of germination. Fertilization was performed under the fertilizer company's recommendations.

2.5 R*hizobium* Strain collection

For the inoculation purpose, *Rhizobium* sp. strains (a total of two) were isolated from the microbial germplasm of the "National Institute of Agricultural Sciences, South Korea." The initial strain was produced from *Glycine max,* and the second strain came from *Lespedeza bicolor*. A single yellowish colony from both strains was sub cultured in Yeast Extract Mannitol (YEM) agar media (Vincent, et al., 1981). YEM liquid media was utilized to test the colony's nourishment or growth, which was kept alive as the fresh *Rhizobium* sp. isolates.

2.6 The procedure of *Rhizobium* Inoculation

Rhizobium inoculum was prepared by following the protocol of Hoaglandand Arnon (Hoagland, et al., 1950). *Rhizobium* suspensions were diluted using the Bradford method (Bradford, et al., 1976). After 8 and 15 days of germination, rhizobia inoculum suspension (2 mL) was poured directly into the root base of soybean and shrub lespedeza (Ferrari, et al., 2007). The first and second strains were used to inoculate soybean and shrub lespedeza, respectively.

2.7 Gathering of data on the parameter of vegetative growth

Different parameters of both plants were measured sixty days after inoculation. The stem heights (cm), branches, nodes, canopy (cm) from the final leaf of the plant (on one side) to the last leaf of the same plant (on the opposite side) and leaves were measured. A leaf area meter (Model LAI 3100C, LICOR, Lincoln, NE) was used to measure the leaf area (cm^{-2}) of each plant, and the leaf area index was then determined using the formula: leaf area per plant divided by plant ground area, which is known as LAI. Fresh and dry roots and shoots were weighed to determine their fresh and dry weight. The yield components like the pod number per plant, seeds number per plant and pod, grain yield per plant, and 100-grain weight were measured.

2.8 N, P, and K Analysis

For each treatment, all dried parts of both plants were finely crushed and filtered through a 1mm sieve. Each sample's ground material was used to determine the N, P, and K concentrations in the plant body. N concentration was determined using the Kjeldahl distillation techniques (Horwitz, et al., 1975). The P and K concentrations were measured using the digestion method described by APHA and AOAC, respectively (APHA, 1992; AOAC, 2005).

2.9 Analysis of Data

Standard one-way analysis of variance (ANOVA) was followed to assess the various treatments' effects. Using the GraphPad software, Tukey's test was utilized to distinguish between treatment means at a 5% significance level. (GraphPad Prism 8.00, GraphPad Software, California, La Jolla, United States).

3. Results

Figure. 1 displays the average stem height, root and shoot weight (fresh and dry), and plant dry weight for soybean and lespedeza under various conditions. The highest mean values for stem height (Figure. 1a), shoot dry weight (Figure. 1c), root fresh and dry weight (Figure. 1d and 1e), and plant dry weight (Figure. 1f) were seen in treatment T3, comprising fertilization and rhizobium inoculation. The T3 and T1 treatments produced the heaviest soybean and lespedeza fresh shoots, respectively (Figure. 1b). The control treatment did not add nitrogen to the soil, resulting in the lowest mean values of all parameters for both plants.

The treatments T1 and T3 significantly enhanced stem height, shoot fresh and dry weight, fresh root weight, and plant dry weight for both shrub lespedeza and soybean Figure. 1(a-f). At T2, the shoot weight (fresh and dry) and plant dry weight of both plants changed significantly compared to the control treatment (Figure. 1b, 1c, and 1f). Different treatments did not affect the dry weight of each plant's roots ($p > 0.05$) (Figure. 1e). In the case of soybean, treatments T1 and T3 raised shoot fresh and dry weight significantly ($p < 0.05$) compared to treatment T2 (Figure. 1b and 1c). Although treatment T3 resulted in a substantial increase in plant dry weight for both soybean and lespedeza relative to treatment T2, no difference was obtained between treatments T2 and T1 (Figure. 1f).

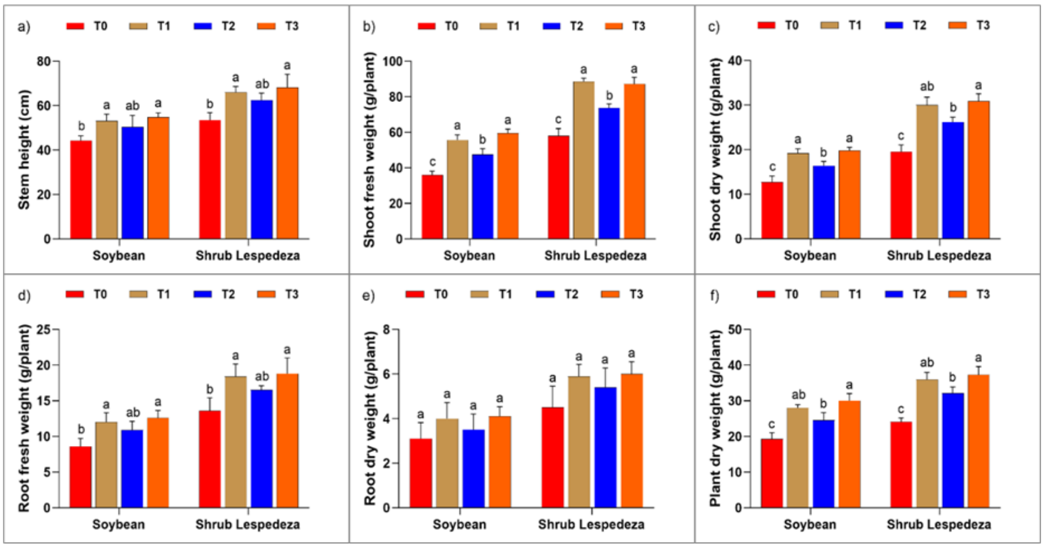

Figure 1: Effect of fertilization and rhizobium inoculation on (a) stem height, (b) shoot fresh weight, (c) shoot dry weight, (d) root fresh weight, (e) root dry weight, and (f) plant dry weight of soybean and shrub lespedeza while cultivated in coal mine soil. Here, T_0= Control (without nitrogen source on soil), T_1= Fertilization, T_2= *Rhizobium* inoculation, and T_3= Fertilization along with *Rhizobium* inoculation. Bars are mean ± SD (*n=3*). Different lowercase letters above bars show a significant difference ($p \leq 0.05$) between treatments.

Thet treatment T3 had the utmost average values for canopy spread and the number of nodes per plant for both plant species in the coal mine soil (Figure. 2a and 2d). The treatment T3 of the shrub lespedeza and T1 of soybeans attributed the greatest mean values for the leaf and branch number per plant, respectively (Figure. 2b and 2c). The mean values for leaf area and leaf area index (LAI) were most significant for soybean and shrub lespedeza in the T3 and T1, respectively (Figure. 2e and 2f).

Canopy expansion, leaf count, node count, and leaf area were all significantly higher in T1, T2, and T3 treated soybean and lespedeza in contrast to the control ($p<0.05$) (Figure. 2a, 2b, 2d, and 2e). The branches number per plant and leaf area index of soybean were not substantially different ($p > 0.05$) between treatments T1, T2, and T3 (Figure. 2c and 2f). Treatments T1 and T3 significantly enhanced the LAI in both soybean and shrub lespedeza, but T2 showed a significant change in LAI only for shrub lespedeza (Figure. 2f).

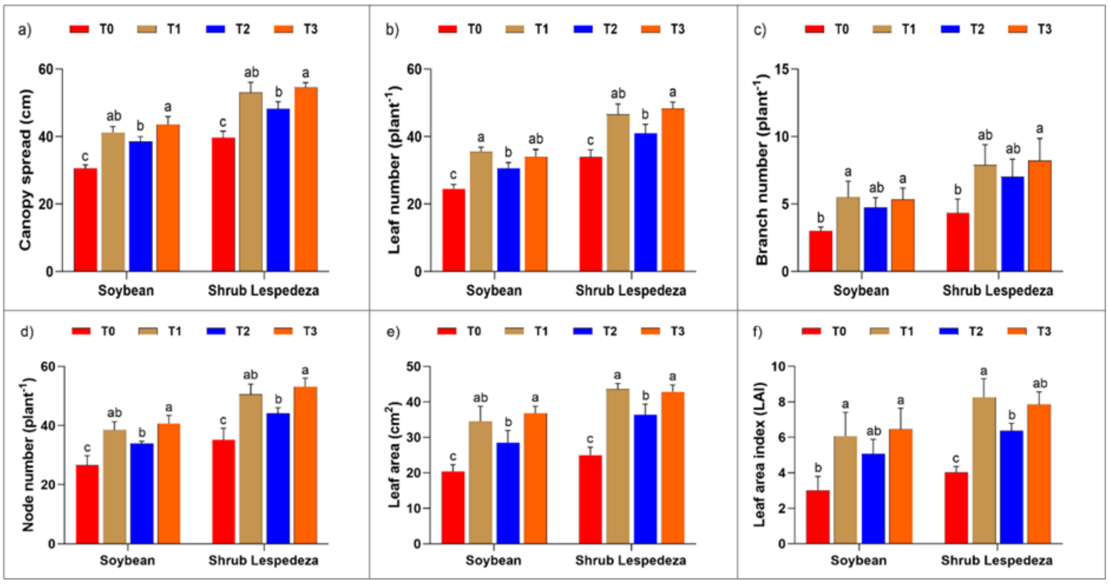

Figure 2: Effect of fertilization and rhizobium inoculation on (a) canopy spread, (b) leaf number, (c) branch number, (d) node number, (e) leaf area, and (f) leaf area index of soybean and shrub lespedeza while cultivated in coal mine soil. Here, T_0= Control (without nitrogen source on soil), T_1= Fertilization, T_2= *Rhizobium* inoculation, and T_3= Fertilization along with *Rhizobium* inoculation. Bars are mean ± SD (*n=3*). Different lowercase letters above bars show a significant difference ($p \leq 0.05$) between treatments.

Various treatments' influence on the yield components of soybean. Treatment T3 had the most significant average values of pod number, seed number per plant, pod dry weight, grain yield, and 100-grain weight, while treatment T1 had the greatest average value for seed number per pod. All of these yield components revealed the lowest mean values for the control treatment (Figure. 3).

Compared to the control treatment, the pod number, seeds per plant, grain yield, and weight of 100 grains rose significantly ($p<0.05$) in all treatments (Figure. 3a, 3c, 3e, and 3f). Despite the fact of no statistical significance distinction ($p>0.05$) between treatments of bacteria-inoculated (T2) and the control (T0), the seed number per pod and pod dry weight for treatments T1 and T3 significantly differed from the control treatment (Figure. 3b and 3d). Compared to the treatments T1 and T3, seed number per plant and grain yield per plant of soybeans were significantly ($p<0.05$) lower at treatment T2, and the number of pods per plant at treatment T2 has decreased significantly in comparison to treatment T3 (Figure. 3a, 3c and 3e).

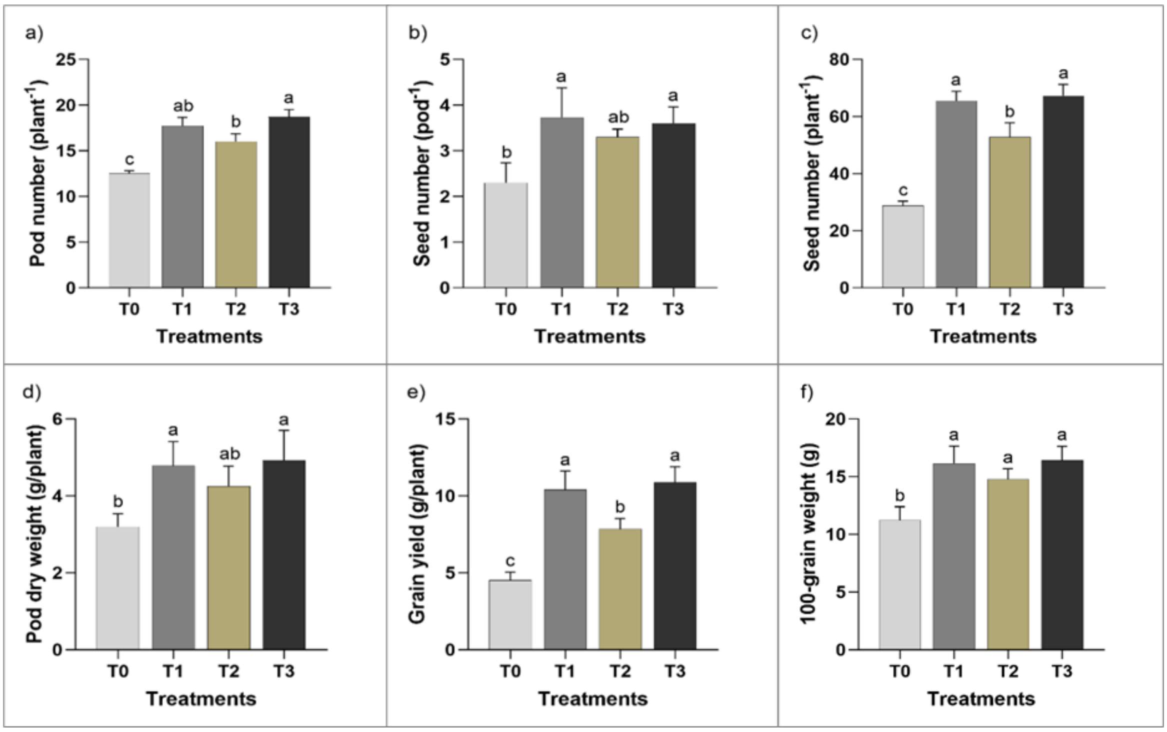

Figure 3: Effect of fertilization and rhizobium inoculation on (a) pod number, (b) seed number per pod, (c) seed number per plant, (d) pod dry weight, (e) grain yield, and (f) 100-grain weight of soybean while cultivated in coal mine soil. Here, T_0= Control (without nitrogen source on soil), T_1= Fertilization, T_2= *Rhizobium* inoculation, and T_3= Fertilization along with *Rhizobium* inoculation. Bars are mean ± SD (*n=3*). Different lowercase letters above bars show

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a significant difference ($p \leq 0.05$) between treatments.

The various treatments' effects on the N, P, and K content (percent) of soybean and shrub lespedeza cultivated on coal mining soil. When compared to the control, the increase in N content caused by the effect of treatments T1, T2, and T3 in both studied plants was found to be significant ($p \leq 0.05$) (Figure. 4a). The fertilization with inoculation treatment (T3) had the highest N content in both plants. Treatments T1 and T3 were statistically different ($p<0.01$) from treatment T2 (Figure. 4a). When treatments T1 and T3 were administered, the P content of both plants was significantly uplifted ($p<0.05$) compared to rhizobia inoculation (T2) and control treatment (Figure. 4b). Treatment T3 showed the highest increase of P content, followed by Treatment T1, and the lowest P content occurred when neither fertilizer nor rhizobia inoculation was used. Between the control and treatment T2, as well as between treatment T1 and T3, there were no appreciable differences in the P concentration of either plant ($p>0.05$) (Figure. 4b). The results for K content of both plants were found to be similar to the P content (Figure. 4c).

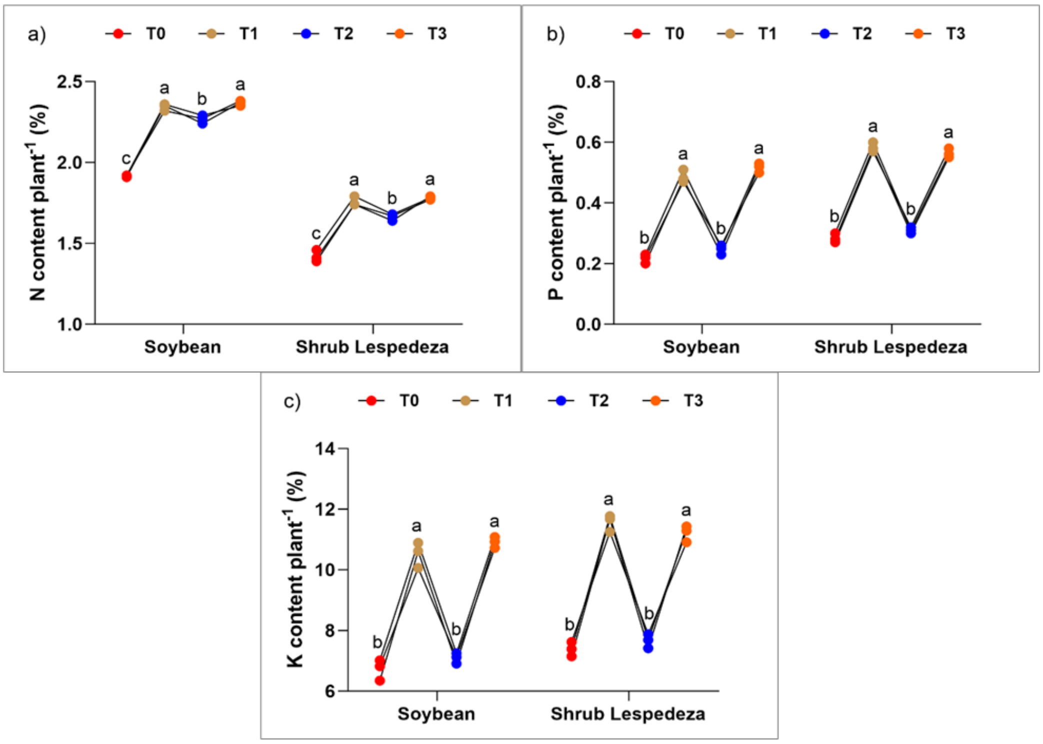

Figure 4: Effect of fertilization and rhizobium inoculation on (a) nitrogen (N), (b) phosphorus (P), and (c) potassium (K) content of soybean and shrub lespedeza while cultivated in coal mine soil. Here, T_0= Control (without nitrogen source on soil), T_1= Fertilization, T_2= *Rhizobium* inoculation, and T_3= Fertilization along with *Rhizobium* inoculation. Different lowercase letters above round symbols show a significant difference ($p \leq 0.05$) between treatments.

4. Discussion

4.1 Fertilization and rhizobium inoculation effects on the vegetative growth of soybean and shrub lespedeza grown in coal mine soil

Plant species of legumes that fix N have been the focus of restoration efforts for mine areas. In rehabilitating subtropical and tropical systems, such as agroforestry, degraded mining and deforested land, and N-fixing species are being exploited as a source of N (Wang, et al., 2010). Fertilizer application and rhizobia inoculation positively impacted many vegetative development indices of *Lespedeza bicolor* and *Glycine max* cultivated in the soil of coal mine that showed better performance than unfertilized and uninoculated samples. Since nitrogen is a prominent nutrient indispensable for plant development and growth, nitrogenous fertilizer application significantly improved the vegetative outgrowth features of both plants. N can also boost photosynthesis and plant absorption, which can enhance plant growth (Bangar, et al., 2000). It was reported that fertilizer-treated plants had significantly greater vegetative metrics than unfertilized plants (Adeyeye, et al., 2017). Applying bacterial inoculation and fertilizer could give the soil the necessary nutrients, which may substantially improve plant growth (Smith, et al., 1989; Daramola, et al., 2006). Although fertilizer treatment combined with bacterial inoculation to both plants produced most of the vegetative growth metrics in this study at their greatest levels, it showed no statistical disparities from those obtained when fertilizer application alone was utilized. Smaller and fewer nodules on the legume plants' roots may be the reason for this, as soil NO_3^- ions provided through fertilization considerably limit nodulation and the following N fixation process by rhizobia (Silsbury, et al., 1989). Therefore, when fertilizer and rhizobia are applied to the soil together, most of the N added to the soil comes from fertilizer, and very little N is added through N fixation. The results of the study demonstrated that nodulation in the rhizobia-inoculated plants caused the rhizobia inoculation effect to be significantly higher in both plants than in the control. Besides, in various vegetative parameters of both plants, the inoculation effect was noticeably less than fertilization and fertilization combined with inoculation therapy. According to some studies, N fixation can only supply 50–60% of the nitrogen needs of plants like soybeans and some other plants (Al-Chammaa, et al., 2014).

4.2 Fertilization and rhizobium inoculation effects on the yield components of soybean grown in coal mine soil

The growth of yield components was significantly influenced by fertilization and bacterium inoculation of soybeans, according to earlier research (Marinkovic, et al., 2018; Daramola, et al., 2006). In this study, rhizobia inoculation, fertilization, and fertilization combined with inoculation all considerably outperformed controls in the soybean pod number, seeds per pod and plant, pod dry weight, grain yield, and weight of 100 grains. Additionally, it was discovered that fertilization and inoculation with N-fixing bacteria asserted a favorable impression on the yield components of soybean (Mrkovacki, et al., 2008). The presence of readily available nutrients provided by NPK fertilizer and N-fixation by the soybean plant may be the cause of the improvement in the yield characteristics of soybeans in coal mine soil. N is a crucial nutrient that significantly impacts soybean grain yield (Gai, et al., 2017). As a result of N fixation and fertilization in soybean plants, it could be hypothesized that the number and weight of seeds and pods in soybean greatly increased. This could have improved the photosynthesis translocation from the sources to the sink (Adeyeye, et al., 2017). According to reports, various environmental elements have an impact on the soybean yield metrics, with N fertilizer and N fixation being two of the most significant ones (Marinkovic, et al., 2018; Adeli, et al., 2005).

4.3 Fertilization and rhizobium inoculation effects on the N, P, and K content of soybean and shrub lespedeza grown in coal mine soil

Findings unveiled that the most significant increase in N content of shrub lespedeza (25.4%) and soybean (23.5%) in soil (coal mine) was noted when inoculation and fertilizer were provided to the plants. According to some writers, rhizobium inoculation and N fertilizer have a good impact on plants cultivated in soil with low fertility (Mrkovacki, et al., 2018; Adeli, et al., 2005). The availability of N in the soil due to fertilization and BNF resulting from rhizobia inoculation helped increase the N content of both plants significantly higher than the control. The findings of our study showed that the N content of inoculated soybean and shrub lespedeza was significantly lower than that of fertilized and fertilized with inoculated plants. By promoting photosynthesis and the effectiveness of the root system's ability to absorb nutrients, the presence of P in the NPK fertilizer may cause plants to absorb more N than inoculated plants (Siam, et al., 2008).

As expected, the fertilized and fertilized with inoculated treatments considerably increased the P and K content of soybean and shrub lespedeza. P and K are crucial nutrients for plant development and growth. The previous study remarked that NPK fertilizer significantly increased plants' N, P, and K concentrations (Ouda, et al., 2008). The positive effect of P application on the P content of plants was supported by the study findings (Reid, et al., 2004; Ottman, et al., 2010). According to earlier research, P and K have a synergistic effect on the content and uptake of plant nutrients (Mohammad, et al., 2004). When both plants were inoculated with *Rhizobium*, there was a slight rise in P and K content. A similar result was found in the study, which showed a satisfactory P content in the rhizobia-inoculated wheat plant over the control (Afzal, et al., 2008).

5. Conclusions

In this study, the effects of fertilization and rhizobium inoculation were examined on the vegetative growth of N-fixing species shrub lespedeza (*Lespedeza bicolor*) and soybean (*Glycine max*) while they were growing in coal mine soil, as well as the variation in N, P, and K concentrations in these plants. Except for root dry weight, all investigated vegetative parameters of both plants increased significantly due to the administered treatments compared to the control. Soybean yield components and grain yield produced the same result. As anticipated, the P and K content of both plants increased significantly only in fertilized treatments. In contrast, the N content of soybean and shrub lespedeza increased significantly in fertilized and inoculated treatments. Therefore, it can be remarked that fertilization and N fixation in deteriorated coal mine soil can greatly improve the development and nutrient content of soybean and shrub lespedeza. As a result, these plants may contribute to restoring nitrogen cycling in the deteriorated coal mine soil.

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Author Contributions

D.J.K. and C.S.S. co-conceptualized and co-supervised the study. M.O.S., C.S.S. and D.J.K. prepared the data sets. M.O.S. and U.B.R. generated the figures. M.O.S., B.D. and M.N.Z. co-wrote the manuscript. A.H. and C.S.S. revised the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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