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EFFECTS OF FERTILIZATION AND N-FIXING BACTERIAL INOCULATION ON NUTRIENTS OF COAL MINE SPOIL AND GROWTH OF BLACK LOCUST (*Robinia Pseudoacacia* L.)

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This study was conducted in greenhouse conditions to assess the effect of nitrogen fixing leguminous plant species, black locust (*Robinia pseudoacacia* L.), on the available nitrogen (N) and other soil nutrients such as phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) of the experimental coal mine soil. The growth and nitrogen (N) content of the plant grown in coal mine soil were also evaluated. Four treatments, including T_0-non-fertilized non-inoculation (control), T_1-fertilization, T_2-bacterial inoculation, and T_3-fertilization along with bacterial inoculation with three replications were used in the study. The results of the study showed that applied treatments significantly increased available N (NH_4^+-N and NO_3^--N) in the soil for black locust, as compared to control. Apart from control, NH_4^+-N was found significantly lower (1.75 ppm) at fertilized treatment, and NO_3^--N was recorded significantly lower (3.87 ppm) at inoculated treatment when compared with other treatments. Available P and K in the coal mine soil also increased significantly when NPK fertilizer was applied solely (30.7 mg kg^{-1} available P and 0.76 cmol (+) kg^{-1} available K), and together with inoculation (26 mg kg^{-1} available P and 0.67 cmol (+) kg^{-1} available K) to the plants but reduced at other treatments. The highest nodule volume (mL), number of nodules and nodule dry weight (g) per plant was documented 1.91, 27.65 and 0.60, respectively in black locust at rhizobia inoculated treatment. Fertilization and bacterial inoculation had positive effect on the growth of studied vegetative parameters of black locust as compared to control, except root dry weight. N content in plant also increased significantly when plants were treated by fertilizer and rhizobia inoculation. Therefore, it can be concluded that bacterial inoculation and fertilization in black locust species can significantly improve the fertility of coal mine soil as well as significantly improve the growth and nitrogen content of this legume plant while grown in coal mine soil. Hence, black locust could be a good option for the reclamation of degraded coal mine soil.

Keywords: Available nitrogen, black locust, coal mine soil, fertilization, nitrogen fixation, vegetative growth.

INTRODUCTION

Coal is one of the vital energy resources which has been widely used since long time to generate electricity in thermal power plants, and to produce steel and cement (Ram and Masto, 2014). However, coal mining is also responsible for land degradation of a vast area. The reasons for land degradation during coal mining activities are eradication of topsoil and vegetation cover, discarding of overburden materials, extensive soil damage, disturbance of microbial communities, change in mine fire, landscape, etc (Sheoran *et al.*, 2010). The disturbances in coal mine soil properties due to mining activities reduces the productivity of land; it may also result in severe air, water and soil pollution (Mukhopadhyay *et al.*, 2016). Reduced productivity of the coal mine spoil causes nutrient scarcity in the soil which can restrict the growth of plants (Gudadhe and Ramteke, 2012). Coal mining also has adverse effects on the soil properties, soil microbe populations, and nutrient cycles that are important for maintaining a sustainable and healthy ecosystem. Without nutrient cycling, nutrients will be immobilized or restrained, and as a result, plant regeneration will be limited in the coal mine spoil (Dutta and Agrawal, 2002).

To restore the productivity of the coal mine spoil, reclamation strategy can be an appropriate technique which may also bring improved landscape (Mukhopadhyay *et al.*, 2013). Therefore, the purposes of vegetation establishment in degraded mine land are accelerating soil forming processes, soil erosion control, improvement of organic matter, development of microbial communities, and initiating nutrient cycling (Josa *et al.*, 2012). N cycle re-establishment is very important to regulate the plant regeneration, but often it become difficult to establish in degraded coal mine land as N is found to be a limited nutrient in coal mine spoils (Mensah, 2015). Use of quick growing N-fixing legume plant species in revegetation for restoring degraded coal mine soil could be an effective method by enhancing nutrient cycling, reducing soil erosion, and increasing C assimilation in the soil. Symbiotic association between legume plant species and N-fixing rhizobia bacteria can significantly increase the soil fertility through the formation of root nodule and fast decomposable nutrient-rich leaf litter which influence N accumulation and organic matter development in the soil, and consequently, help in the process of N mineralization and cycling of nutrients (Moura *et al.*, 2016). Another alternative approach to maintain the sustainability and healthy growth of vegetation in coal mine land can be regular application of nitrogenous and other nutrient fertilizers to the soil (Song *et al.*, 2004).

Atmospheric N is discarded by N-fixing plants from the air and converted to organic form of N by plants to enrich the soil but plants cannot use the organic N. Only ammonium (NH_4^+) and nitrate (NO_3^-) forms of N can be used by plants for their growth. Organic N is broken down by microbes to form NH_4^+-N through a process known as mineralization. Under certain conditions, NH_4^+-N is converted to NO_3^--N by a specialized group of soil microbes while they are using NH_4^+-N for energy. This process is called nitrification. Some of that available nitrate N is absorbed by plants after nitrification and some of nitrate N discharge into the atmosphere (Sheoran *et al.*, 2010).

Black locust (*Robinia pseudoacacia* L.), which is also known as false acacia, is a leguminous multipurpose plant species. This species is native to North America and usually well adapted to temperate regions (Ferrari and Wall, 2007). This tree has the capacity to grow fast and fix atmospheric N in association with rhizobia bacteria which make this plant able to grow even in disturbed and nutrient deficient soils. Therefore, it can be used for erosion control, soil stabilization and the reclamation of degraded coal mine spoils (Keskin and Makineci, 2009). Apart from N-fixation, *Robinia* can also use mineralized N and fertilizer N as a source of N for its nutrition and growth.

N is one of the most important nutrients required for plant growth and development. It has a significant role in increasing the vegetative growth of plant and can also increase photosynthesis and assimilation in plants (Mohammadi *et al.*, 2016). Application of biofertilizer or inoculation with compatible N-fixing bacteria result in efficient nodule formation, better atmospheric N-fixation, and increasing content of N, biomass and yield in plants. So, bacterial inoculation can be an alternate for reducing chemical fertilizer use during plant growth (Marinkovic *et al.*, 2018). However, it is also necessary to apply decent amount of N fertilizer to enhance N fixation and to get maximum growth of plant because plant cannot achieve all its demand for N only through biological nitrogen fixation even under favorable situations (Gai *et al.*, 2017).

The symbiotic association of legumes with rhizobia provides a superior ability to the N-fixing legumes to grow quickly in infertile soils of degraded mine area and to tolerate the severe conditions of deteriorated soils. This could be an effective strategy to enhance the reclamation of degraded soil and initiate natural succession in the coal mine soil. There is scanty of information regarding the role of N-fixing legume species in changing the fertility status of degraded coal mine soil through the application of fertilizer together with rhizobia bacteria inoculation. Therefore, our present study aims to evaluate the performance of N-fixing tree species black locust (*Robinia pseudoacacia* L.) in improving the nutrient status of coal mine soil and to find out the effects of N fertilizer application and bacteria inoculation on the growth and N content of the same plant species while growing in coal mine soil.

MATERIALS AND METHODS

Soil collection: Coal mine soil for growing black locust seedling was collected from three different locations of an abandoned coal mine area located in the Taebaek city of South Korea. Clean plastic box was used for collecting the soil of coal mine. Firstly, collected coal mine spoil was air-dried, ground, and sieved (mesh size 2 mm). After that, each pot was filled with equal amount of experimental soil. The volume of each pot was 4.8 L and the shape of the pot was conical frustum.

Seed germination and transplantation of plant: Seeds for growing black locust seedling were directly collected from the black locust tree located at Chungbuk National University, South Korea, during the end of the fall season. To stratify the seeds, collected seeds were kept inside the fridge during whole winter season. After stratifying, seeds were washed four times with sterile water and planted in the plastic tray for germination. Plastic trays were kept in the growth chamber for seed germination with the relative humidity of 60%-90% and temperature of 28°C. Lamps of 400W were used to maintain the photoperiod of 16 h light and 8 h darkness (Ferrari and Wall, 2007). Two weeks after germination, required amount of black locust seedlings were transplanted in the experimental soil containing pots and all the pots were kept in the green house. After transplantation, seedlings were irrigated three to four times in a week.

Greenhouse experiment setup: The study was conducted in a greenhouse of the Forest Science Department, Chungbuk National University, South Korea, during the period of May 2018 to July 2018. The environments of greenhouse were maintained at 26-28°C temperature, approximately 90% relative humidity, and a photoperiod of 9–12-h light/24 h. A completely randomized design (CRD) comprising of four treatments with three replications was used for the experiment. Treatments were as follows: T_0—control (non-fertilized non-inoculated), T_1—fertilization (NPK fertilizer was applied to the coal mine soil), T_2—bacterial inoculation (N-fixing rhizobia bacteria was inoculated on the coal mine soil), T_3—fertilization together with bacterial inoculation (both NPK fertilizer and N-fixing rhizobia bacteria were added on the coal mine soil). Six seedlings were used for each replication; hence, eighteen seedlings were used for each treatment.

Application of fertilizer: NPK 20:20:20 fertilizer was applied to the seedlings by mixing with water. This fertilizer consists of 20% nitrogen (N), 20% phosphorus (P_2O_5), and 20% potassium (K_2O) macro elements. In the experiment, the rate of fertilizer application was 250 NPK kg/ha. Fertilizer was dissolved in the water at the rate of 0.5 g/L. 250 mL of dissolved fertilizer as applied to each seedling of treatment T_1 and T_3 through a broadcast irrigation system. Fertilizer was applied at 15, 30, and 45 days after germination of the plant, respectively. Fertilization was done according to the instruction of the fertilizer company.

Collection of bacterial strain: For inoculation, strain of *Rhizobium* sp. was collected from the microbial germplasm of National Institute of Agricultural Sciences, South Korea. The source of the strain (KACC No: 11053) was leguminous plant species, and the location of isolation was Suwon, South Korea. From the collected strain, a single yellowish colony was further sub cultured in YEM agar (Vincent, 1980), and the test of good growth of the new colony was done using liquid medium of YEM. This colony was maintained as the new isolates of *Rhizobium* sp.

Bacterial inoculation procedure: For inoculum preparation, *Rhizobium* strain was cultivated in 50 mL of YEM for 48 h at 28°C and 200 rpm. Bacteria were separated through centrifugation and resuspended in a modified 1/10 strength Hoagland solution (without N) of 50 mL (Hoagland and Arnon, 1950). Suspensions of bacteria were diluted to prepare an optical density of 0.15 (λ = 500 nm), which feature a concentration of 3×10^8 bacteria/mL, measured by the Bradford method (Bradford, 1976). Black locust seedlings were inoculated by prepared inoculum suspensions after 15 days of germination by pouring 2 mL rhizobia suspension directly onto the base of the root (Ferrari and Wall, 2007). Inoculation of bacterial strain was done for treatment T_2 and T_3.

Soil sampling and analysis: The experimental coal mine soil was analyzed to determine the physical and chemical properties (Table 1) of the soil before starting of the experiment.

Before analysis, soil samples were dried at 60°C for 48 h and thrashed in a 2 mm sieve. The concentration of available nitrogen (NH_4^+ and NO_3^-), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), pH, and electrical conductivity (EC) in the experimental coal mine soil were determined. Final soil sampling was done after 60 days of inoculation, for determining the effect of fertilization and bacterial inoculation on the change of available nitrogen and other nutrients in the coal mine soil. Soil samples were collected from the base of the plant root. Each soil sample was divided into two parts. One part of the divided samples was for the determination of available nitrogen (NH_4^+ and NO_3^-) which was done by using stream distillation method. The other part of the soil samples was for the measurement of soil pH, available P, K, Ca, Mg, and EC. All the soil samples were sent to the Soil Science Department of Agricultural Research and Extension Services, Chungcheongbuk-do, South Korea, for analyzing the soil parameters.

***Collection of nodule data*:** After soil sampling, seedlings of black locust were uprooted from soil very carefully to keep the root intact to avoid the loss of nodules. The roots were then cleaned to remove the adhering soil using a stream of water from the tap and a metal sieve of 1 mm size was kept underneath to trap the detached nodules. Nodule volume per plant, number of nodules per plant, and dry weight of nodules per plant were recorded for each treatment. The number of nodules per plant was calculated by counting the nodule number of each plant and then averaged as per plant. To find out nodule volume, nodules of each plant were immersed in a plastic cylinder of 50 mL which was filled with 30 mL of water. The volume of water replaced by the nodules acquired from each plant was recorded, and the average was calculated as volume of nodules per plant. Finally, the nodules separated from each plant were dried at 70°C to constant weight and then weight of dried nodules was measured as g/plant.

Collection of data on vegetative growth parameter: The measuring growth parameters of the plant were stem height, root length, fresh and dry weight of root and shoot, ratio of shoot and root dry weight, plant dry weight, number of leaves, nodes and branches per plant, canopy spread, leaf

Table 1. Physical and chemical characteristics of the experimental coal mine soil

		Nutrient Level in the Soil							
Texture	**pH**	**P_2O_5 (mg kg^{-1})**	**K (cmol (+) kg^{-1})**	**Ca (cmol (+) kg^{-1})**	**Mg (cmol (+) kg^{-1})**	**NH_4 (mg L^{-1})**	**NO_3 (mg L^{-1})**	**Total N (%)**	**EC (dS m^{-1})**
Clay loam	5.7	6	0.4	7.5	2.3	1.09	4.36	0.12	0.01

area, and leaf area index (LAI). The stem heights (cm) and root lengths (cm) were recorded using a measuring tape. The measurement of leave, node and branch number per plant was done manually. The canopy spread (cm) was recorded from the last leaf at one side of the plant to the last leaf of other side of the same plant using a tape measure. Leaf area per plant was measured using a leaf area meter (Model LAI 3100C, LICOR, Lincoln, NE), then leaf area index (LAI) was calculated using the following equation: LAI = Leaf area per plant (cm^{-2})/Plant ground area (cm^{-2}). Plants were separated into shoots and roots. Shoots and roots were weighted in an electronic balance to determine the fresh weight of these parts. After that, these parts were dried in an oven at 65°C for 48 h and weighted again to determine the dry weight of shoot and root. Dry matter weight of plant was the sum of all oven dried plant parts.

Plant sample analysis: All the dried plant parts such as shoot, root, and nodule of black locust seedling for each treatment were finely ground and passed through a 1 mm sieve. Then, the ground material of each sample was sent to the Soil Science Department of Agricultural Research and Extension Services, Chungcheongbuk-do, South Korea for analyzing the concentration of N, P and K in the plant. Analysis of N concentration was done using sulfuric acid digestion, followed by distillation according to the Kjeldahl method, as described by AOAC (2005). The concentration of P and K in plants was determined using digestion method, as described by APHA (1992) and AOAC (2005), respectively.

Statistical analysis: Data were analyzed using a standard procedure for one-way analysis of variance (ANOVA) to determine the effects of different treatments. Differences between treatment means were separated by the Tukey's HSD test at significance level $P < 0.05$ using GraphPad software (GraphPad Prism version 7.00, GraphPad Software, La Jolla, CA, USA).

RESULTS

Concentration of Available N (NH_4^+-N and NO_3^--N): The increased concentration of NH_4^+-N and NO_3^--N in the coal mine soil, at different treatments for black locust, are shown in Figure 1 and Figure 2, respectively. The highest mean value of increased NH_4^+-N (2.44 ppm) was found in treatment T_2, where N-fixing rhizobia bacteria were inoculated on the soil, and the highest value of increased NO_3^--N (4.94 ppm) was observed in treatment T_3, where NPK fertilizer together with N-fixing rhizobia bacteria were added to the experimental soil. The lowest mean values of both increased NH_4^+-N (0.06 ppm) and NO_3^--N (0.83 ppm) were recorded in the control treatment (no source of nitrogen was given on the soil). Figure 1 and Figure 2 also present the effect of different treatments on the concentration of NH_4^+-N and NO_3^--N in the experimental soil by growing black locust species. The results showed that NH_4^+-N and NO_3^--N was significantly increased ($P \leq 0.001$) in the soil at different treatments as compared to control (T_0). There was also significant difference in increased concentration of NH_4^+-N and NO_3^--N between the treatments T_1, T_2, and T_3 when comparing with each other. In figure 1, black locust showed a low significant increase ($P \leq 0.05$) of NH_4^+-N at treatment T_3 (2.19 ppm) when compared to treatment T_1 (1.75 ppm). The results in the figure 1 also showed a significant difference ($P \leq 0.01$) in increasing NH_4^+-N between fertilizer application treatment (1.75 ppm) and bacterial inoculation treatment (2.44 ppm). The results in figure 2 revealed that NO_3^--N increased significantly ($P \leq 0.01$) at combined application of fertilizer and bacterial inoculation treatment (4.94 ppm), and fertilization treatment (4.59 ppm), comparing to bacterial inoculation treatment (3.87 ppm).

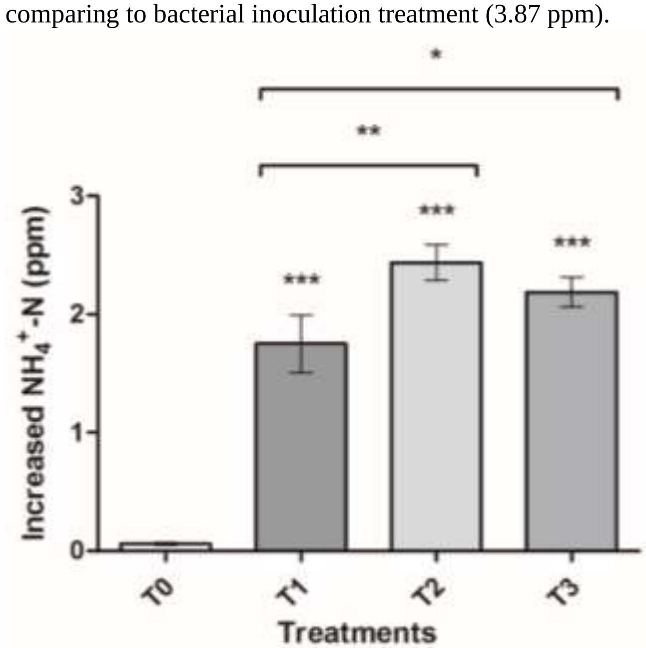

Figure 1. Effect of different treatments on the concentration of NH_4^+-N in coal mine soil by growing black locust. Here, T_0= control, T_1= Fertilization, T_2= Bacterial inoculation and T_3= Fertilization along with bacterial inoculation. Asterisk sign indicate significant differences with control and between treatments (* indicate $P \leq 0.05$, ** indicate $P \leq 0.01$, * indicate $P \leq 0.001$).**

Soil pH and Available Nutrients: The effect of different treatments on soil pH and other available nutrients (P, K, Ca, and Mg) in the black locust growing coal mine soil are recorded in Table 2. There was a significant increase in the soil pH of collected coal mine spoil at all treatments after growing black locust plant species in the experimental coal mine soil. The soil pH was recorded highest when both fertilizer and bacterial inoculation (treatment T_3) were applied to the plant. The highest mean values of available P

Table 2. The effect of different treatments on soil pH and different available nutrients in coal mining soil by growing black locust seedling (Mean±SD).

	Measured soil pH and available nutrients				
‡ Treatments	Soil pH	P (mg kg^{-1})	K (cmol (+) kg^{-1})	Ca (cmol (+) kg^{-1})	Mg (cmol (+) kg^{-1})
T_0	7.0 ± 0.06	5.1 ± 0.17	0.30 ± 0.02	6.2 ± 0.20	1.93 ± 0.15
T_1	7.2 ± 0.06	30.7 ± 3.06	0.76 ± 0.05	5.4 ± 0.20	1.70 ± 0.10
T_2	7.2 ± 0.00	4.7 ± 0.15	0.26 ± 0.04	5.7 ± 0.10	1.60 ± 0.10
T_3	7.3 ± 0.06	26.0 ± 2.65	0.67 ± 0.04	5.5 ± 0.20	1.53 ± 0.06

‡ T_0 = Control (No source of nitrogen was given on the soil), T_1 = Fertilization (NPK fertilizer was added on the soil), T_2 = Bacterial inoculation (Nitrogen fixing rhizobia bacteria was inoculated on the soil), T_3 = Fertilization along with bacterial inoculation (Both NPK fertilizer and nitrogen fixing rhizobia bacteria were added on the soil).

and K in the soil were noted when only NPK fertilizer (treatment T_1) was applied, whereas highest values of available Ca and Mg were found in the control treatment followed by fertilization treatment (treatment T_1).

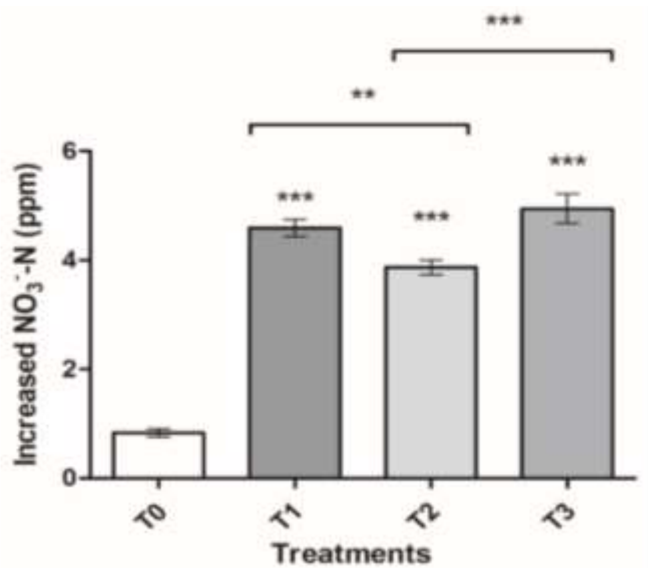

Figure 2. Effect of different treatments on the concentration of NO_3^--N in coal mine soil by growing black locust. Here, T_0= control, T_1= Fertilization, T_2= Bacterial inoculation and T_3= Fertilization along with bacterial inoculation. Asterisk sign indicate significant differences with control and between treatments (* indicate $P \leq 0.05$, ** indicate $P \leq 0.01$, * indicate $P \leq 0.001$).**

Vegetative Growth Parameters: The mean values of stem height, root length, fresh and dry weight of shoot and root, ratio of shoot and root dry weight and plant dry weight of black locust at different treatments are shown in Table 3. Highest mean values of stem height (51.2 cm), fresh weight (79.5 g plant^{-1}) and dry weight (27.9 g plant^{-1}) of shoot, dry weight of root (6.2 g plant^{-1}), ratio of shoot and root dry weight (4.50) and plant dry weight (34.56 g plant^{-1}) of plants were observed in treatment T_3 where both NPK fertilizer and N-fixing rhizobia bacteria were added on the coal mining soil. Highest value of root length (32.1 cm) and root fresh weight (19.5 g plant^{-1}) of plant were recorded in treatment T_2 where only rhizobia bacteria were added to the coal mine soil. Lowest mean values of all these parameters were found in the control treatment where no source of nitrogen was applied to the soil (Table 3). Fertilization, inoculation and fertilization along with inoculation had significant effect ($P \leq 0.05$) on the growth of studied vegetative parameters of black locust as compared to control, except root dry weight.

Table 4 is showing the mean values of canopy spread, number of leaves, branches and nodes per plant, leaf area, and leaf area index of black locust at different treatments while grown in coal mine soil. Mean values of canopy spread (56.3 cm), leaf number (44.2), branch number (7.7), and node number (49.2) per plant were recorded highest at fertilized inoculated treatment (T_3) followed by fertilized treatment (T_1). Maximum mean values of leaf area (37.4

Table 3. The effect of different treatments on stem height, root length, fresh and dry weight of shoot and root, ratio of shoot and root, and plant dry weight of black locust seedling grown in coal mining soil (Mean±SD)

‡Treatments	Parameters							
	Stem height (cm)	Root length (cm)	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Root dry weight (g plant^{-1})	Shoot/Root ratio	Plant dry weight (g plant^{-1})
T_0	37.5±1.61	26.3±2.21	51.0±3.12	15.9±1.93	14.8±0.96	4.8±0.98	3.31±0.17	20.82±1.79
T_1	48.0±3.58	31.1±1.76	78.2±2.86	26.2±2.78	18.8±2.04	5.9±1.06	4.44±0.27	32.19±2.16
T_2	44.4±2.26	32.1±2.88	66.8±2.44	23.0±2.55	19.5±1.35	6.1±0.70	3.77±0.17	29.71±2.71
T_3	51.2±2.27	31.5±1.73	79.5±4.27	27.9±2.80	19.1±2.18	6.2±0.62	4.50±0.25	34.56±2.35

‡ T_0 = Control (No source of nitrogen was given on the soil), T_1 = Fertilization (NPK fertilizer was added on the soil), T_2 = Bacterial inoculation (Nitrogen fixing rhizobia bacteria was inoculated on the soil), T_3 = Fertilization along with bacterial inoculation (Both NPK fertilizer and nitrogen fixing rhizobia bacteria were added on the soil).

Table 4. The effect of different treatments on canopy spread, number of leaves, number of branches, number of nodes, leaf area, and leaf area index (LAI) of black locust seedling grown in coal mining soil (Mean±SD).

‡ Treatments	Parameters					
	Canopy spread (cm)	Number of leaves ($plant^{-1}$)	Number of branches ($plant^{-1}$)	Number of nodes ($plant^{-1}$)	Leaf Area (cm^2)	Leaf Area Index (LAI)
T_0	41.8 ± 2.29	29.5 ± 1.37	3.5 ± 0.62	32.4 ± 1.82	21.9 ± 2.61	3.53 ± 0.59
T_1	55.1 ± 1.57	42.5 ± 1.80	7.2 ± 0.61	46.9 ± 2.54	37.4 ± 3.52	7.70 ± 0.80
T_2	49.7 ± 2.72	37.0 ± 2.29	6.2 ± 0.62	40.5 ± 3.32	30.0 ± 1.93	5.92 ± 0.40
T_3	56.3 ± 2.54	44.2 ± 1.82	7.7 ± 1.14	49.2 ± 2.49	36.7 ± 1.78	7.35 ± 0.61

‡ T_0 = Control (No source of nitrogen was given on the soil), T_1 = Fertilization (NPK fertilizer was added on the soil), T_2 = Bacterial inoculation (Nitrogen fixing rhizobia bacteria was inoculated on the soil), T_3 = Fertilization along with bacterial inoculation (Both NPK fertilizer and nitrogen fixing rhizobia bacteria were added on the soil).

Table 5. The effect of different treatments on nodule volume, number of nodules, and nodule dry weight of black locust seedling grown in coal mining soil (Mean±SD).

‡ Treatments	Parameters		
	Nodule volume (mL $plant^{-1}$)	Nodule number ($plant^{-1}$)	Nodule dry weight (g $plant^{-1}$)
T_0	0.43 ± 0.09 ^{c}	6.12 ± 0.25 ^{c}	0.11 ± 0.04 ^{b}
T_1	0.32 ± 0.08 ^{c}	4.96 ± 0.24 ^{c}	0.08 ± 0.03 ^{b}
T_2	1.91 ± 0.17 ^{a}	27.65 ± 1.08 ^{a}	0.60 ± 0.09 ^{a}
T_3	1.32 ± 0.16 ^{b}	21.30 ± 0.96 ^{b}	0.45 ± 0.13 ^{a}

‡ T_0 = Control (No source of nitrogen was given on the soil), T_1 = Fertilization (NPK fertilizer was added on the soil), T_2 = Bacterial inoculation (Nitrogen fixing rhizobia bacteria was inoculated on the soil), T_3 = Fertilization along with bacterial inoculation (Both NPK fertilizer and nitrogen fixing rhizobia bacteria were added on the soil).

cm^2), and leaf area index (7.70) of black locust were observed at fertilized treatment (T_1) followed by fertilized inoculated treatment (T_3). Lowest mean values of canopy spread (41.8 cm), number of leaves (29.5), branches (3.5) and nodes (32.4) per plant, leaf area (21.9 cm^2), and leaf area index (3.53) of black locust seedling were found in the control (T_0).

Nodule volume, Nodule Number and Nodule Dry Weight per Plant: Table 5 presents the mean values of nodule volume, nodule number per plant and dry weight of nodules per plant at different treatments applied to black locust while grown in coal mine soil. The highest nodule volume (mL), number of nodules and nodule dry weight (g) per plant were recorded 1.91, 27.65 and 0.60, respectively. The highest mean value of these parameters was recorded at bacteria inoculated treatment followed by fertilization with inoculated treatment (Table 5). On the contrary, all these parameters produced lowest mean values at fertilization treatment (T_1). Nodule volume, number of nodules and nodule dry weight per plant were documented significantly higher ($P \leq 0.05$) at bacteria inoculated treatments (T_2 and T_3) compared to uninoculated treatments (T_0 and T_1).

N, P and K content in Plant: The effect of different treatments on N, P and K content (%) of black locust seedling grown in coal mine soil are shown in Figure 3, Figure 4 and Figure 5, respectively. The concentration of N has shown highly significant increase ($P \leq 0.001$) in the plant due to the effect of different treatments (T_1, T_2 and T_3) when compared with control treatment (Figure 3). Highest content of N in black locust was recorded at fertilization with inoculated treatment (1.68%) followed by fertilization treatment (1.61%) and both of these treatments were significantly higher than bacteria inoculated treatment (1.52%). The results of P content in black locust are given in Figure 4. P content in black locust seedling increased significantly ($P \leq 0.001$) while grown in coal mine soil due to the application of NPK fertilizer and NPK fertilizer with bacteria inoculation comparing to control (T_0) and bacteria inoculation (T_2) treatment.

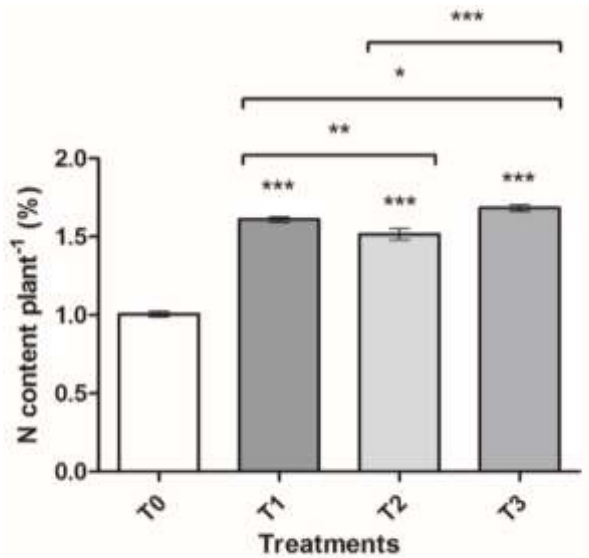

Figure 3. Effect of different treatments on nitrogen (N) content of black locust seedling grown in coal mine soil. Here, T_0= control, T_1= Fertilization, T_2= Bacterial inoculation and T_3= Fertilization along with bacterial inoculation. Asterisk sign indicate significant differences with control and between treatments (* indicate $P \leq 0.05$, ** indicate $P \leq 0.01$, * indicate $P \leq 0.001$).**

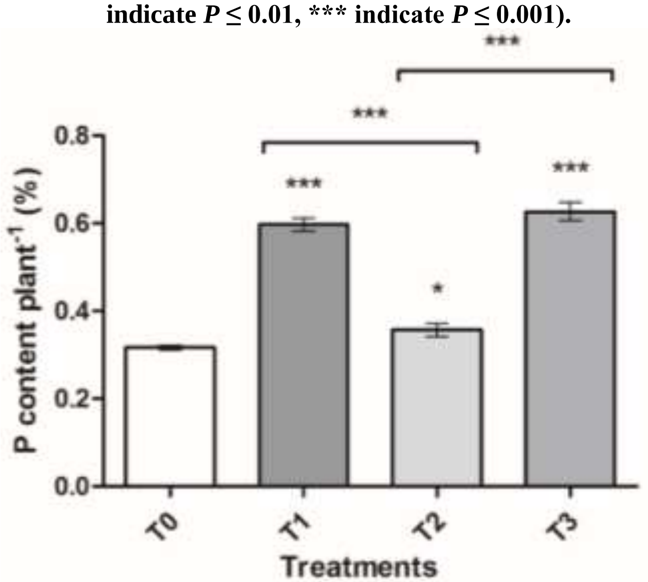

Figure 4. Effect of different treatments on phosphorus (P) content of black locust seedling grown in coal mine soil. Here, T_0= control, T_1= Fertilization, T_2= Bacterial inoculation and T_3= Fertilization along with bacterial inoculation. Asterisk sign indicate significant differences with control and between treatments (* indicate $P \leq 0.05$, ** indicate $P \leq 0.01$, * indicate $P \leq 0.001$).**

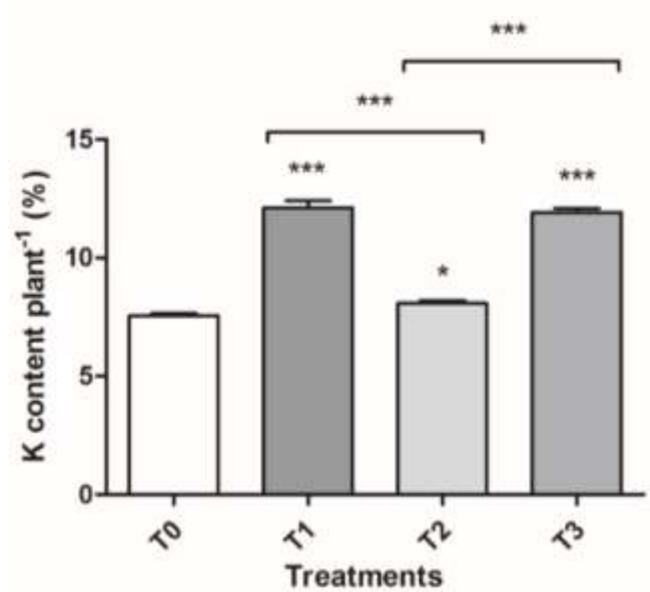

Figure 5. Effect of different treatments on potassium (K) content of black locust seedling grown in coal mine soil. Here, T_0= control, T_1= Fertilization, T_2= Bacterial inoculation and T_3= Fertilization along with bacterial inoculation. Asterisk sign indicate significant differences with control and between treatments (* indicate $P \leq 0.05$, ** indicate $P \leq 0.01$, * indicate $P \leq 0.001$).**

Highest content of P was observed at treatment T_3 (0.63%) followed by treatment T_1 (0.60%) and lowest content of P was recorded when there was no application of fertilizer (0.32%) and bacteria inoculation (0.36%) to the plant. Bacteria inoculated treatment (T_2) showed a low significant increase ($P \leq 0.05$) of P content in plant than control treatment, but no significant change ($P > 0.05$) was found between treatment T_1 and treatment T_3 (Figure 4). The content of K in the plant at different treatments was found similar to the results of P content which are illustrated in Figure 5.

DISCUSSION

Effect of fertilization and bacterial inoculation on NH_4^+-N in coal mine soil: N-fixing species because of their N-fixing ability through symbiotic association with N-fixing bacteria, can increase soil N and have been used widely for the reclamation of degraded land in the tropical and subtropical regions (Johnson and Curtis, 2001). Previous studies have documented that *Robinia* stands can increase N mineralization better than non-N fixing trees resulting in strong enrichment of soil N (Malcolm *et al.*, 2008). In our study, the increasing rate of NH_4^+-N in the experimental coal mine soil was highest when rhizobia bacteria were inoculated on the soil, followed by fertilizer application along with bacterial inoculation. This illustrates the significance of legume plants in enhancing NH_4^+-N in coal mining soil through biological nitrogen fixation (BNF). Nitrogen is found naturally in the atmosphere as N_2 gas and most of the N found in the soil also originated as organic N_2 gas. It is not possible for plant to use this N in organic form. Plant only can uptake the available ammonium (NH_4^+) or nitrate (NO_3^-) forms which are converted from organic nitrogen. N-fixing rhizobia bacteria form a symbiotic association or mutual beneficial relationship with legume plants. N is removed from the air by these rhizobia bacteria and transformed into the available NH_4^+-N in soil which is used by plants (Gil-Sotres *et al.*, 2005). A small concentration of NH_4^+-N also increased in the control treatment of the study without any application of nitrogen sources might be due to the presence of fewer root nodules and soil organic N but it was significantly lower in the control than other treatments. Shin *et al.* (2018) reported a significant increase of NH_4^+-N in the coal mine soil after 60 days of inoculation and fertilization applied to shrub lespedeza and soybean, and Cakmakci *et al.* (2007) also showed the same result in the soil of barley plant.

Effect of fertilization and bacterial inoculation on NO_3^--N in coal mine soil: In the study, the concentration of NO_3^--N in the coal mine soil increased significantly as a result of fertilization and bacterial inoculation into black locust species. NO_3^- is formed in the soil by organic N mineralization and oxidation of NH_4^+ produced from supplied fertilizer and N-fixation, and this NO_3^- form of N is available for plant use (Zahran, 1999). NH_4^+-N does not remain in the soil for long time; it is oxidized to NO_3^--N by a specialized group of soil microbes through a process known as nitrification. Application of mineral N-fertilizer is a general practice to minimize the N deficiency and increase the fertility of degraded soils. It has been reported that when symbiotic N fixation cannot provide enough N for gaining maximum soil fertility and plant growth, then N fertilizer can be applied to the N-poor soils to improve soil fertility (Zahran, 1999). There was no significant difference for NO_3^--N in soil between the fertilization and fertilization along with bacterial inoculation treatments of the study. The reason behind this might be the formation of less active and smaller nodules on the roots of black locust because the presence of soil NO_3^- added by fertilization inhibits nodulation and N-fixation by rhizobia (Hungria *et al.*, 2015). Therefore, fertilized inoculated treatment added only a small concentration of NO_3^--N to the soil through nodulation because of weak nodule formation and mostly added through fertilization. A previous study showed significant increase of NO_3^--N in the coal mine soil through bacterial inoculation and fertilization when applied to soybean and shrub lespedeza (Shin *et al.*, 2018).

Effect of fertilization and bacterial inoculation on pH, P, K, Ca, Mg in coal mine soil: The mean pH value of the investigated coal mine spoil was slightly acidic (5.7). The soil pH was found to increase significantly at different treatments, and the coal mine soil become neutralized after the study. Soil acidity can be neutralized with anything that supplies alkalinity. The base cations are the sources of alkalinity, principally calcium, or magnesium (Sobek *et al.*, 2000). Sheoran *et al.* (2010) reported that the presence of $CaCO_3$ or $MgCO_3$ tend to increase the pH of mine soil. Generally, the pH of rhizocylinder area increased by NO_3^--N and decreased by NH_4^+-N. The pH of rhizosphere region affected by N source by different reactions of nitrification and denitrification, and the displacement of H^+/OH^- uptake on the solid phase (Gahoonia and Nielsen, 1992). So, fertilizer can change the pH of soil apart from changing the soil nitrate and soil ammonium content (Brady and Weil, 2002).

The concentration of available P and K in the coal mine soil after growing black locust was found significantly high when NPK fertilizer was applied to the plants. Generally, NPK fertilizer is expected to increase the concentration of P and K in the soil (Cakmak *et al.*, 2010). On the other hand, P and K cations decreased in the non-fertilized and control treatments. Application of N can increase soil acidity which may induce decreased solubility of P compounds, and finally, result in a declined P concentration in soil (Marsh *et al.*, 1987). Usually, NH_4^+ enhance the absorption of available P and NO_3^- enhance the absorption of available K by plants (Aulakh and Malhi, 2005). After growing black locust in the coal mine soil, concentration of Ca and Mg cations in the soil decreased at different studied treatments. Usually, Ca and Mg uptake by plants is also enhanced by soil nitrate due to the interaction of anion–cation. Cakmak *et al.* (2010) reported that imbalance between the input and output of exchangeable Ca and leaching of Ca that happened by nitrification processes and soil acidification, may result in the decreased concentration of Ca cations. Though Ca requirement of plant for its growth and metabolism is low, but it has an important role in maintaining the balance of other nutrients, including N (Marsh *et al.*, 1987).

Effect of fertilization and bacterial inoculation on the growth of black locust while grown in coal mine soil: In our study, fertilizer application and bacteria inoculation showed positive effect on most of the studied growth parameters of N-fixing black locust species (*Robinia pseudoacacia* L.) grown in coal mine soil as compared to control. From the results of the study, it is apparently clear that supply of N sources played a significant role in the vegetative growth of black locust even in degraded coal mine soil. There is evident from the previous study that N must be required for the growth and development of plant, and it can stimulate photosynthesis in plants by making more area available for photosynthesis process, and subsequently increase assimilation (Mohammadi *et al.*, 2016). Adeyeye *et al.* (2017) and Gan *et al.* (2002) reported that maximum growth of plants can be achieved through continuous application of N as compared to unfertilized and uninoculated plants. Fertilization and rhizobia inoculation could supply sufficient nutrient to the soil required for improving plant growth (Daramola *et al.*, 2006). Effect of bacterial inoculation was significantly higher in the study compared to control, but in contrary, bacterial inoculation showed significantly lower mean values of some growth parameters than fertilized treatments. Inadequate supply of N to the soil could be the reason behind this condition because previous studies reported that N fixation in legume species can provide only 50-60% of the demanding N in soybean and some other legume plants (Al-Chammaa *et al.*, 2014; Gai *et al.*, 2017).

Effect of fertilization and bacterial inoculation on nodule volume, nodule formation and nodule dry weight of black locust while grown in coal mine soil: According to the result of the study, bacteria inoculation and bacteria inoculation with fertilization increased nodule volume, nodule number and nodule dry weight of black locust seedling significantly higher than uninoculated fertilized and control treatments. Janagard and Ebadi-segherloo (2016) reported that nodule growth, and nodule dry weight of bacteria inoculated plants were increased significantly higher than uninoculated plants. Hungria *et al.* (2015) also found the similar results when soybean plant was inoculated with rhizobia bacteria. On the other hand, nodule volume and nodule number of black locust seedlings were observed significantly lower at fertilized inoculated treatment than non-fertilized inoculated treatment because N fixation by rhizobia in legume plants become affected by nitrogenous fertilizer application. Presence of more nitrate ion in the soil supplied by N fertilizer is the reason for producing poor and fewer nodules on the plant roots (Hungria *et al.*, 2015). The results about nodule parameters obtained from our study are similar with the study carried out by Shin *et al.* (2015) who also noted significant difference between bacteria inoculated and uninoculated treatments applied to soybean and shrub lespedeza grown in coal mine soil.

Effect of fertilization and bacterial inoculation on N, P and K content of black locust while grown in coal mine soil: Several authors observed a significant effect of rhizobium inoculation and N fertilizer on the plant N content while grown in low fertile soil (Mrkovacki *et al.*, 2008, Moreira *et al.*, 2010). The findings of our study also showed a significant increase in N content of black locust grown in coal mine soil due to fertilization and bacterial inoculation. N fixation in legume plant and N fertilization helped in providing enough available N in the soil for plant use and resulted in significant increase of plant N content than control treatment of the study. NPK fertilizer provide P in the soil which could enhance more uptake of N by plants than rhizobia inoculated legume plants by increasing the absorption capacity of the root system and photosynthesis of plants (Siam *et al.*, 2008). In a previous study, P was found to be effective in increasing the content of N in bacteria inoculated plant (Kouki *et al.*, 2016). The outcome of the study also showed that both P and K content of black locust were significantly high when treated with fertilizer and fertilizer with bacterial inoculation. P and K are vital nutrients for the growth and development of plants, and also enhance photosynthesis process in plant. Application of NPK fertilizer (20:20:20) was found to have a positive effect on N, P and K content of plants (Ouda and Mahadeen, 2008). The findings of the study are also in line with Ottman *et al.* (2010) who concluded that application of P had significant effect on the plant P content. There was low significant increase of P and K content in plants when inoculated with bacteria compared to control. Afzal and Asghari (2008) also showed a better content of P in wheat plant when inoculated with rhizobia but it was statistically indifferent from control.

Conclusions: Fertilized and inoculated treatments of the study applied to black locust exhibited significant increase of NH_4^+-N and NO_3^--N concentration in the experimental coal mine soil compared to control. Application of fertilizer and bacterial inoculation also had a significant influence on the vegetative growth parameters of black locust while grown in coal mine soil. Inoculation of rhizobia bacteria increased the nodule volume, number of nodules, and nodule dry weight per plant significantly higher than the non-inoculated plants. N content of black locust seedlings increased significantly in both inoculated and fertilized treatments. Therefore, it can be summarized that plantation of black locust for enhancing the fertility of coal mine soil could be a better option because of its N fixation ability, and fertilization and N fixation can significantly improve the growth and nutrient content of black locust while grown in coal mine soil. Thus, black locust could assist in restoring the nutrient cycling in the declined coal mine soil.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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