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EFFECTS OF BIOCHAR ON LEGUME-*RHIZOBIUM* SYMBIOSIS IN SOIL

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Abstract

An *in vitro* study was conducted to observe the effects of tannery waste and biochar on soil bacterial population particularly legume-*Rhizobium* symbiosis. The study comprised a total of seven different treatments including a control. Count of total bacteria and *Rhizobium* was observed on initial materials and on all treated soils. A leguminous plant, cowpea, was used to study the effects on nitrogen fixation which could be further linked to legume-*Rhizobium* symbiosis. Bacterial population was higher in tannery waste treated soils than the corresponding biochar treated ones. It was found that waste treated soils had higher *Rhizobium* count than the biochar treated ones. Nitrogen fixation was found to be higher in tannery waste than biochar treatments. Although there appeared to be no adverse impact on legume-*Rhizobium* symbiosis, growth of bacteria particularly *Rhizobium* was inhibited indicating that microbial functioning of the soil might be affected and thereby likely to jeopardize agricultural production and food security.

Introduction

Tannery industry is one of the most important sectors of any country and plays a vital role in national economy. Manufacturers of the industries constitute an indispensable source for export trade and foreign exchange earnings in many developing countries. Being an agro-based by-product with locally available indigenous raw materials, leather industries possess a potential for export development and sustained growth over the coming years. This industry is the second largest export sector of Bangladesh and currently there are approximately 206 tannery units in Bangladesh (BTA 2010). Of them 114 are large and medium units and the remainders are mostly of small types. About 190 tannery units are located at Hazaribagharea of the capital city, Dhaka covering 60 acres of land (Paul *et al.* 2013).

About 85,000 tonnes of raw material are processed in Bangladesh annually. Most of the tanneries in Hazaribagh do not have proper effluent treatment plants and generate about 20,000 m^3 effluent and 232 tonnes solid waste per day (Paul *et al.* 2013). About 40 to 50 litres of liquid required for processing each kilogram of hide is poured down in natural canal or low lying areas directly. These untreated solid and liquid wastes find their way into the sewer passing through the area leading to the pollution of water bodies and surrounding environment through destruction of its ecosystem and make the river water unusable (Buljan *et al.* 2000). Although tannery waste is rich in organic matter they contain significant amounts of chromium, sodium, sulphates, chlorides, inorganic nitrogen, pathogens and toxic compounds which pose serious threats to the microbial functioning of the soil (Sinha *et al.* 2002).

In the recent years, biochar has been accepted as an immediate, economically viable solution for reducing the global impacts of waste. Biochar, a highly porous and carbon rich product, produced from the waste materials through pyrolysis process, is used to manage waste, improve soil condition, enhance agricultural production that leads to sustainability in environment and reduction in greenhouse gas emissions. Biochar can act as a sorbent for organic and inorganic contaminants and can efficiently remove these waste materials from contaminated sources. Thus, biochar can help to improve food security by contributing to sustainable production systems and

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maintaining an eco-friendly environment (Qambrani *et al.* 2017). However, a big knowledge gap exists about the impacts of biochar on legume-*Rhizobium* symbiosis in soil. It thus becomes pertinent to assess the response of soil microbes particularly nitrogen fixing *Rhizobium* bacteria to biochar additions. As a part of this approach, the present study was undertaken to assess the effects of biochar produced from tannery waste on legume-*Rhizobium* symbiosis and *Rhizobium* count that could be linked to determine whether biochar brings the same advantages for soil microbes like the waste.

Materials and Methods

Waste samples were collected from three sites of Hazaribagh tannery area. The first spot was near the main outfall area of Kohinoor Tannery Industries, second spot was about 500 meters and third spot was nearly about one kilometer away from the first spot. The geo-references of the sampling spots are 23.738961ºN and 90.367615ºE, 23.846167ºN and 90.243324ºE, 23.845982ºN and 90.242906ºE, respectively. The surface soil (0-15 cm depth) of Chandina Series was used as a control. The depth of the soil was decided to represent the rhizosphere as legume-*Rhizobium* symbiosis was to be observed. According to USDA soil classification, the soil belongs to loamy, mixed, non-acid and the soil taxonomy is Aeric Haplaquept (Rahman 2005).

Sampling containers with proper labeling were used to collect waste and soil samples. Collection of soil samples was done by following composite sampling techniques as suggested by the USDA (1951). Collected samples were dried in air (at ~ 40°C) followed by removing visible roots and debris. Larger samples were broken down and screened through 2 mm sieve which will be used for various physical analysis. Another portion of the sample was screened through 0.5 mm sieve which will be used for chemical analysis. The bulk soil samples collected for pot experiment were air-dried, cleared-off the debris and crushed to make the bigger clods smaller which were then screened through 5 mm sieve (Carter and Gregorich 2007). Solid waste samples were air dried, ground and sieved through 0.2 mm sieve to assess physicochemical properties of these samples. For microbiological analysis, collected samples were kept in cool and dark place so that the sample does not deteriorate and the analytical results were representative.

A big earthen pot was taken and metal wires were arranged in a criss-cross arrangement in such a way that pots were uniformly heated from all sides. Tannery wastes were placed layer by layer in small earthen pots. These pots were covered with earthen lids. Finally, fire was lighted and accelerated by adding kerosene oil. After about an hour, when the waste was turned to biochar, fire was stopped. After cooling of the biochar, lids of the pots were opened and screened through 0.25 mm sieve (Khan *et al.* 2014).

Physical, chemical and physico-chemical properties of the soil, waste and biochar samples were analyzed by the standard procedures described by Hesse (1972). For every ten samples a Certified Reference Material (CRM) was included to ensure quality control/quality assurance (QC/QA).

In order to assess the impact of biochar on legume-*Rhizobium* symbiosis, a pot experiment was carried out with cowpea (*Vigna sinensis*). Tannery waste and biochar produced from the corresponding waste were mixed with Chandina soil at 5 t/ha. A total of 21 plastic pots including control were arranged in a completely randomized design. The seven treatments were designated as T0, T1, T2, T3, T4, T5 and T6 (Table 1).

Pour plate technique was followed as described by Khan and Huq (2014) for determining Total Viable Count (TVC) of bacteria which were expressed by the number of Colony Forming Units per gram of soil (CFU/gm).

The soil was sterilized at 120°C for 48 hrs after the treatments were applied (Goldman and Green 2008). After that, soil was cooled down and soaked with sterilized deionized water. Surface sterilized (with 0.5% potassium hypochlorite) certified seeds of cowpea were sown in the pots. After germination of seven days, purified *Rhizobial* strain was added to the rhizosphere of the seedlings. Each pot was watered with sterilized deionized water. Plants were harvested manually after 60 days by uprooting the plant carefully from the pot. Soil samples were also collected after harvesting. Collected plant samples were first air-dried and then oven-dried at 70 ± 5°C for 48 hrs. Both fresh and dry weight of plants was measured. The dried plant samples were then ground, mixed thoroughly and stored for further laboratory analysis. Plant samples were digested with H_2SO_4 and N content was determined by alkali distillation of the Kjeldahl digests (Jackson 1962).

Table 1. Design of the pot experiment and used symbols.

Treatment	Rate of application	Symbol
Control	5 t/ha	T0
Soil + waste of 1st spot	5 "	T1
Soil + waste of 2nd spot	5 "	T2
Soil + waste of 3rd spot	5 "	T3
Soil + biochar from waste of 1st spot	5 "	T4
Soil + biochar from waste of 2nd spot	5 "	T5
Soil + biochar from waste of 3rd spot	5 "	T6

Healthy and unbroken root nodules from each of treatment soils were randomly selected for isolation of *Rhizobia* following the method described by Somasegaran and Hoben (1985). Nodules were surface disinfected by immersion in ethanol (95% v/v) followed by immersion in 3.8% sodium hypochlorite and finally rinsed with sterile double-distilled water. The nodules were crushed in 100 µl of sterile distilled water using a sterilized blunt forceps. One loopful of each nodule suspension was aseptically streaked onto YEMA (10 g mannitol, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.5g K_2HPO_4, 1 g yeast extract, and 15 g agar) agar medium containing bromothymol blue having pH of 6.8 ± 0.2°C. The plates were then incubated at 28°C and observed periodically to characterize colony growth.

After incubation of 10 days, distinct colonies of *Rhizobia* started to appear as described in Somasegaran and Hoben (1985). Presence of *Rhizobium* was confirmed by observing morphological characteristics of the colonies and finally viable count was done manually. All microbiological experiments were conducted in aseptic condition, which prevented contamination and assured accuracy of result. The experimental data were statically analyzed by using the Microsoft Excel (version 2016) and MINITAB (version 18).

Results and Discussion

The soil, tannery waste and biochar were analyzed to determine their nutritional status and heavy metal contents. It was found that Chandina soil was silty loam in texture and slightly acidic in character (pH 6.73). Its content of organic matter, total nitrogen, CEC and EC was 1.12%, 0.11%, 37.90 meq/100 g and 1.01 dS/m, respectively. Contents of heavy metals *viz.,* Fe, Mn, Zn and Cu were 24178, 381, 235 and 130 ppm, respectively. Contents of Cr and Cd were found to be negligible. Basic chemical properties of the tannery waste samples collected from three different spots and their corresponding biochars are presented in Table 2.

When the tannery waste turned into biochar, its properties changed. In almost all of the cases, values of pH, EC and organic matter increased. Contents of organic matter were almost doubled. Similar trend was also observed in case of heavy metal contents. Heavy metals *viz.*, Fe, Cu, Cr and Cd were found to increase from waste to biochar. On the contrary, no significant change was observed for Mn and Zn. Khan *et al.* (2015) observed that contents of heavy metal increased when biomass turned to biochar at different incubation periods while working with three types of biomass, namely rice husk, straw and saw dust.

Table 2. Characterization of tannery wastes and biochars.

Name of parameter	Parameter values of the waste and biochar samples					
	1st spot wst	2nd spot wst	3rd spot wst	1st spot bc	2nd spot bc	3rd spot bc
pH	5.30	6.90	5.80	5.40	7.20	6.35
EC (dS/m)	6.89	5.93	6.92	7.25	5.99	7.10
Organic matter (%)	16.78	13.89	12.89	35.23	27.17	24.90
Total N (%)	1.89	2.01	2.02	2.05	2.05	2.08
CEC (meq/100g)	84.58	93.75	98.75	92.65	95.40	98.85
Exchangeable PO_4 (ppm)	310.00	70.00	60.00	300.00	70.00	60.00
Exchangeable K (ppm)	3200.00	980.00	175.00	3200.00	900.00	160.00
Exchangeable SO_4 (ppm)	480.00	680.00	860.00	460.00	680.00	895.00
Exchangeable Ca (ppm)	815.00	760.00	895.00	810.00	750.00	895.00
Iron (ppm)	335.00	320.00	305.00	340.00	355.00	310.00
Manganese (ppm)	436.00	520.67	BDL	435.00	510.00	35.00
Zinc (ppm)	280.65	265.00	BDL	450.00	415.00	60.95
Copper (ppm)	75.00	65.00	63.00	120.00	95.00	110.00
Chromium (ppm)	35000.00	29000	20000	70000.00	60000.00	30000.00
Cadmium (ppm)	5.90	6.35	1.45	10.50	15.95	3.09

wst = Waste, bc = Biochar, BDL= Below detection limit.

Bacterial colonies started to appear after 24 hrs of incubation indicating the presence of bacteria in these materials. Initially, the soil had Total Viable Count (TVC) of 120×10^4 CFU/g. Waste of 1st, 2nd and 3rd spot possessed 40×10^4, 80×10^4 and 100×10^4CFU/g of TVC, respectively. T3 had more viable count which could be due to its long distance from the tannery. However, no count was observed in any of the biochar samples. The reason could be that, high temperature for producing char might have killed the microbes that are present in the waste samples. The present findings corroborate with the work of Khan *et al.* (2014) who showed that most of the microorganisms could not survive when biomass turned to biochar due to its antagonistic effects resulting from nutrients deficiency, decreased sorption of enzymes and formation of complex compounds that are not easily degradable by the microorganisms.

Fig. 1 shows that the highest bacterial population was in T3 as indicated by the highest number of TVC (70×10^4 CFU/g). Conversely, the lowest bacterial population was found in T4 and T5 (25×10^4). It was observed that soil treated waste treatments (T1, T2 and T3) contained less bacteria than that of the control soil (T0). It might be due to the high concentrations of salt and heavy metal present in tannery wastes. Moreover, after applying the biochar treatments, bacterial population was decreased from both the waste treated soil and control soil. De Luca and Gundale (2006) observed that biochar participated in rapid mineralization of labile carbon leading to

reduced soil nitrogen that led to decreased availability of total N and C for the microbes. Although no bacterial growth was observed in initial biochar materials produced from tannery wastes, growth appeared after mixing with the soil. Dioselina *et al.* (2006) found that effluents from leather processing tannery industries contains valuable nutrients as well as contaminants, particularly salts and Cr, that might affect soil processes and crop production. They revealed that untreated tannery waste increased soil organic matter and microbial biomass C while C mineralization and N mineralization remained low. AVOVA test indicates that both treatment and pH had highly significant effect ($p = 0.000$) on TVC. In all three cases the waste and biochar treatments showed significant differential effect on TVC.

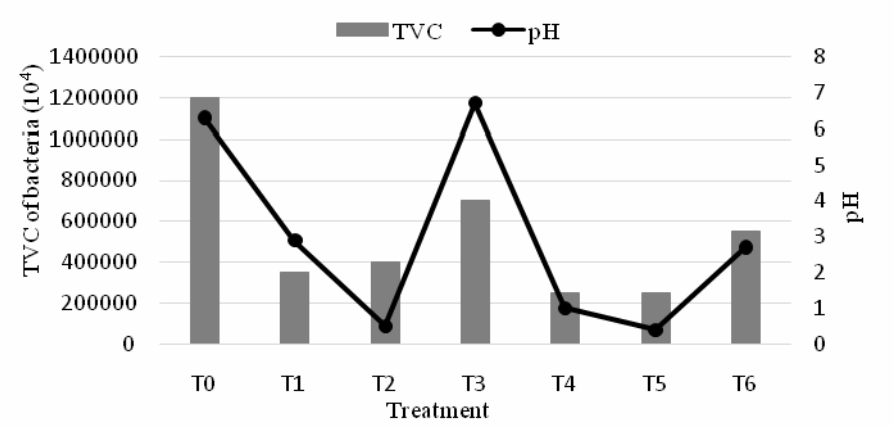

Fig. 1. Relationship between bacterial population and pH affected by different treatments.

The cowpea did not show any visual symptoms of toxicity after 20 days of germination grown in tannery waste treated soils. However, at the later stage, plant growth rate decreased and chlorosis was observed. On the other hand, biochar treated soil showed visual symptoms after 15 days of germination which might indicate toxicity or deficiency. Yellow spot was observed, which eventually spread throughout the whole plant. Growth of plant was reduced followed by chlorosis, stunting, reduced branching and abnormal darkening. Stems and lower leaf surfaces of the plants turned to purple grown on T4.

Table 3 shows that fresh and dry matter contents of cowpea were lower in biochar treated soil than those of the waste treated and control soil. The LSDs showed significant effect of waste or biochar treatments on the fresh and dry matter production of cowpea as well as amount of fixed N in plants at 5% level of significance (Table 3). It is apparent from Table 3 that amount of nitrogen fixation by cowpea was high in control soil (2433 mg) compared to the treatment soils. Among the soils treated with wastes, nitrogen fixation was maximum in T2 (1639 mg) than T1 (775 mg) and T3 (880 mg). It might be due to low contents of heavy metal and high contents of organic matter and nitrogen present in waste of 2nd spot. High contents of Zn, Cd, Cr, Cu and Co reduce nitrogen fixation and slow down the activity of *Rhizobium* (Madariaga and Angle 1991). Araújo *et al.* (2007) observed the effect of composted textile sludge on growth, nodulation and nitrogen fixation of soybean and cowpea in a greenhouse experiment. They showed that composted textile sludge did not exert any negative effect on nodule number, weight and nodule activity. Zereen *et al.* (2013) reported that tannery waste water is not suitable for irrigation purpose due to extremely high mineral and heavy metal contents. They mentioned that the effluent is highly alkaline with high values of Fe, Mn, Zn, Cr, EC, BOD, COD and SAR which leads to reduced growth and yield of plants.

It was observed that nitrogen fixation in the soils treated with tannery waste is lower than that of the corresponding biochar treated ones except T2. In the present study, among the biochar treatments, maximum N was fixed in T6 (2476 mg) and minimum in T5 (12 mg), because T6 was the farthest spot from the tannery industry whereas T5 was collected from the vicinity of the discharging site. Chowdhury *et al.* (2015) noted that pollution from the effluents of leather processing tannery industries decreases with distance.

Table 3. Fresh and dry matter content of cowpea and amount of fixed nitrogen at different treatments.

Treatment	Fresh weight (g/plant)	Dry weight (m/plant)	Amount of N in 100 plants (mg)	Amount of N fixed by 100 plants (mg)
T0	8.08	0.97	2813	2433
T1	2.36	0.33	1155	775
T2	5.07	0.66	2019	1639
T3	3.46	0.45	1260	880
T4	3.08	0.37	1180	800
T5	0.21	0.07	392	12
T6	6.0	0.84	2856	2476
LSD (at 5%)	0.00	0.00	0.00	0.00

Following incubation at 28°C for 10 days, distinct colonies of *Rhizobium* were identified based on their size (2 to 4 mm), color (white), shape (round, ellipsoid, irregular) and elevation (raised, flattened). Under microscope they were observed as short rod. Initially, the viable count of *Rhizobium* in the soil was found to be 60×10^3 CFU/g. Viable counts of treated soils are presented in Fig. 2. However, no count of bacteria was found in T1. It could be due to the adverse effects of heavy metals present in waste samples. Heavy metals might reduce *Rhizobial* count by directly affecting its root nodule function, physiology, available N and plant growth. Studies found that

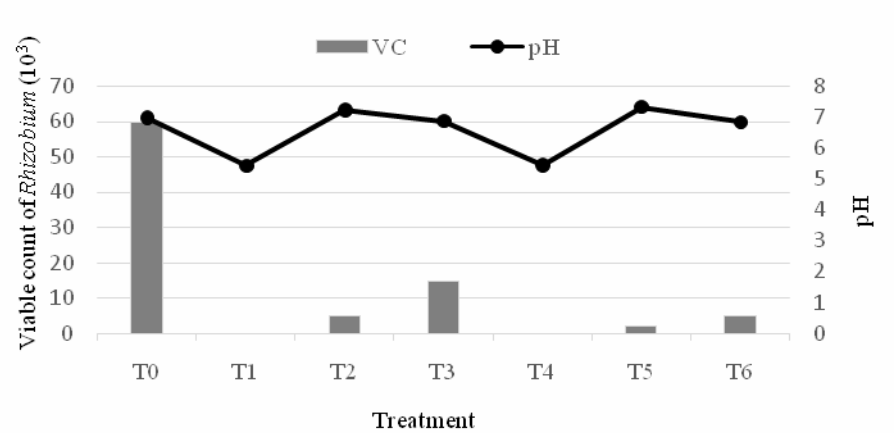

Fig. 2. Relationship between *Rhizobium* count and pH affected by different treatments.

heavy metals hampered growth and nodulation of *Rhizobium* up to a certain limit (Paudyal *et al.* 2007). As T1 was located near to the tannery industry, its effect was found to be worst on *Rhizobium* species. Bacterial growth was found to increase in T3 (15×10^3 CFU/g). In case of biochar treatments, *Rhizobium* was absent in T4 while it appeared in T5 (2×10^3 CFU/g) and T6

(5×10^3 CFU/g). It was observed that soils with waste treatments had higher bacterial growth than that of corresponding biochar treatments. Khan and Huq (2014) mentioned that *Rhizobium* are able to use NH_4^+ or NO_3^- as nitrogen source but when char is added utilization of these compounds might be hampered. Extreme pH hampers nodulation of *Rhizobium* which leads to reduced growth and population (Mensah *et al.* 2006). It was revealed in present study that soil alkalinity increased significantly when waste was converted to char (Table 1) which might have adversely affected proliferation of *Rhizobium.* Moreover, pH of T1 and T4 was not conducive for the growth of *Rhizobium* in the laboratory condition as it needs pH of 6.8 ± 0.2. AVOVA test indicates that both treatment and pH had highly significant effect ($p = 0.000$) on viable count of *Rhizobium*. Except for the T2 and T5 ($p = 0.096$), all waste and biochar treatments showed significant differential effect on viable count.

Although biochar is one of the burning questions of today's modern world to manage waste as well as to maintain soil fertility and ecological resilience, it is not beneficiary with respect to soil microbial population. Both tannery waste and biochar exert adverse effects on soil bacteria, particularly *Rhizobium*. The effects are much more pronounced for biochar. Biochar produced from tannery waste exerts tremendous negative impacts on the growth and survival of *Rhizobium* due to the relative stability of biochar and lack of readily utilizable nitrogen sources which might hamper legume-*Rhizobium* symbiosis. However, no noticeable impact was observed for legume-*Rhizobium* symbiosis although nitrogen fixation was higher in biochar treated soils. It can be concluded that biochar is not a suitable option with respect to soil microbial population. In this context, any potential risks should be thoroughly examined before adopting the biochar due to the irreversibility of applying biochar to agricultural soils.

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