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Biochar Influence the Production and Release of Exopolysaccharides on Plant Growth Promoting Bacteria

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Biochar is known as a multifunctional carbonaceous material mainly used in agrosystems. Little information is available on possible antagonistic, toxicological and negative effects of biochar on soil microorganisms. This work explored the effects of different doses of biochar produced from sugarcane bagasse and corn cob husk in the growth and development of plant growth promoting bacteria (*Leocobacter sp.* and *Bacillus aryabhattai*). Two types of low-dose solid culture media of biochar (0.4% w w^{-1}) were tested: biochar directly inserted into the medium and another one elaborated with the biochars extractable water compounds. The growth results indicated a deleterious effect on the survival of the colonies when in direct contact with the extractable compounds in water of the biochars. On the other hand, the strains grown in solid medium supplemented with biochar (high and low-dose) had some outbreaks with colonies of bacteria, where there was also the formation of exopolysaccharide films. The production of exopolysaccharides by *Bacillus sp*. was characterized by infrared chemical mapping at the nanoscale (AFM NANO-IR). The information obtained indicated that the dosage and the type of biochar modified the morphology and topography of the biofilms, as well as the presence of chemical groups such as Amide I and II, lipids and proteins. This fact suggests that adaptation and survival of the bacterial species depend on their interaction path with the molecular structure of the biochar surface. Thus, the binding force, or even biochar colonization, can be influenced by the type of dominant molecule readily available on the surface of the biochar, where hydrophobic components, covalent bonds, van der Waals forces, anionic or cation exchange or even substitution of ion can act as chemical signals that induce specific responses in bacteria.

1. Introduction

Billions of microorganisms are responsible for forming the pedological system. Through them, many biogeochemical cycles are kept in motion. For a soil to be healthy and fertile, the balance between the populations of organisms, such as fungi and bacteria, needs to be preserved or stimulated. In recent years the scientific literature has addressed the beneficial issues of biochar when introduced into soil as a conditioning, fertilizer and even a means to remove contaminants (Lau et al., 2017; Karunanayake et al., 2018). However, little is known about the existence of toxic behaviour, not only to plants, but to the soil microorganisms. Some of unintentional consequences of biochar application on soils include impacts on germination and soil biological processes, as well as the release of toxic chemicals that may be present in biochar (Belmonte et al., 2017). It is also known that biochar is currently a difficult material to be standardized, because characteristics such as biomass of origin, temperature of production, chemical characteristics, among others, can totally modify the behaviour of biochar in the environment due the origin of raw materials and pyrolysis temperature of biochar (Yang and Chen, 2017). In this context, the use of plant growth-promoting bacteria (PGPB) has been investigated more strongly in recent decades, mainly aiming to increase agricultural productivity. This growing interest in PGPB has made the endophytic microorganisms into new sources of bioactive molecules, as they produce chemical molecules resulting from the interaction between plant and PGPB as a molecular sign. Targeting the understanding of the of bacteria and biochar interaction, the objective of this work was to explore the effects of different doses of biochar produced from sugarcane bagasse and corncob husk on the bacteria survival and its ability of formation of colony forming units to the stress induced by the presence of

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biochar, as well as the influence on production of bioactive molecules (biofilms). Our main hypothesis was that biochar can disrupt the intracellular signalling on the environment, implicating on biochar production and dosage as parameters that could be tuned to regulate the rhizosphere colonization.

2. Materials and Methods

2.1 Biochar Production

The sugarcane bagasse and corncob husk residues used for the biochar production were separated and stored into a wooden bay for prior natural drying for 7 days. After this period the biomass was collected and washed in tap water to remove dust and then dried in an oven with mechanical air circulation for 48h at 80°C. Subsequently, the dried samples were ground in a Willey mill (MA048) with a fixed rotation of 1730 rpm, followed by sieving (Solotest Sieve, 35 MESH). The biochars (BC) were produced in a muffle furnace at two different temperature regimes: 300 and 600°C. The thermal processing condition selected was a slow pyrolysis cycle with an increase of 5°C min^{-1}. The retention time of the samples produced by slow pyrolysis on targeted temperature was 120 min. The samples were then naturally cooled at room temperature for 12h (overnight). Subsequently, the samples were stored in labelled polypropylene bottles at room temperature until their use. The biochars produced at 300 and 600ºC from sugarcane bagasse were labelled as BC-SC_3 and BC-SC_6, respectively; for the biochars produced from corncob husk they were labelled as BC-CH_3 and BC-CH_6.

2.2 Bacterial Incubation Assays

Two strains of plant growth-promoting bacteria were selected for the tests: *Bacillus aryabhattai* (CMAA-1363 - isolated from the rhizosphere of *Cereus jamacaru* - Brazilian mandacaru cactus) and *Leocobacter sp* (CMAA-1422 - isolated from Brazilian caatinga soils). A bacterial colony forming unit (CFU) of these strains was inoculated into a sterile culture medium for the growth of Trypticase Soy Broth (TSB) liquid (without agar) using an inoculating loop. For growth promotion, the vials were packed in an incubator at 35 °C with constant shaking until the medium showed turbidity. After the growth, with an inoculating loop, 1µL of the medium was inoculated in solid medium in plate to confirm the presence of the bacteria in the medium and verify if there was any contamination. For this purpose, the plates were conditioned in a microbiological incubator to grow bacteria at 35 °C and after a period of 4 days, the CFUs were analysed and accounted in order to promote the morphological characterization of the bacterial colonies. The liquid culture medium with bacterial strains were shaken, and then 0.1 mL of this medium was transferred to 9.9 mL of dilution liquid (1:100). After this dilution, the medium was slightly shaken and loaded with agar. After homogenization, the plating was performed. For this plating, 1 mL (inoculating loop) of the dilution was transferred to sterile Petri dishes (3 replicates). The plates were inverted and maintained in an incubator at 35 °C. After 4 days, the CFUs were counted with the help of an automatic colony counter. On the survival bacterial assays two types of low-dose solid culture media of biochar (0.4% w w^{-1}) were tested: biochar directly inserted into the medium and another one elaborated with the biochars extractable water compounds.

2.3 AFM with Nano Infrared Spectroscopy (NANO-IR)

B. aryabhattai strains were inoculated with an inoculating loop into culture media with and without biochar (control). Biochar was added to the media at a concentration of 0.4% m v^{-1} (low concentration - L) and 2.0% m v^{-1} (high concentration - H). The flasks were kept in a growth incubator at 35 °C, shaking at 150 rpm. After 24 hours, the controls were diluted with sterile 0.85% NaCl solution at 1:10 V V^{-1} ratio. Then the samples obtained were centrifuged (Eppendorf 5430/5430 R centrifuge) at 3500 rpm for 30 minutes at 4 °C. The extraction of exopolysaccharides (EPS) did not undergo purification. After this step, the samples were deposited on fresh mica substrates for topographic analysis by Atomic Force Microscopy (AFM). For the nano infrared spectroscopy (NANO-IR) analyses the samples were deposited on a gold plate and kept in a desiccator for 4 hours. The images were taken in a humidity controlled environment (5%) at room temperature. After the imaging, the samples were taken to the NANO-IR chamber for chemical analysis of the samples. For this purpose, NIR2 contact tips (PR-EX-nIR2-10) with a resonance frequency of 13 +/-4 kHz and an elastic constant of 0.07 N m^{-1} were used.

2.4 Surface Roughness

Surface roughness characterization was performed through AFM images using the free source 168 software, Gwyddion (http://gwyddion.net/). The amplitude parameters evaluated were roughness average (Ra), root mean square roughness (Rq), and average maximum height of the profile (Rz).

3. Results and Discussion

In the Petri dishes produced with the biochar's water extractable compounds (WEC) the bacteria *Leocobacter sp*. and *Bacillus aryabhattai* did not grow, indicating a deleterious effect on the survival of these colonies. On the other hand, the strains sown in solid medium loaded with biochar had formation of bacterial colony forming units, as shown in Table 1, where there was also the formation of exopolysaccharides (EPS) biofilm. This experiment suggests that the solid medium loaded with biochar allowed for better initial adaptation of the bacteria tested compared with the WEC medium were the possibly toxic compounds were readily available for assimilation. Thus, the biochar present in the medium should release some compounds that act as chemical signals by which the bacteria translate with the release and formation of biofilm. This fact suggests that the adaptation and survival of the bacterial species depend on their interaction pathway with the molecular structure of the biochar surface, which varies according to the production temperature and the dosage used. In other words, the binding force or even the biochar colonization by microorganisms is influenced by the type of dominant molecule available immediately on the surface of the biochar, where hydrophobic compounds, covalent bonds, van der Waals forces, anionic or cation exchange or even substitution of ion can act as chemical signals that induce specific responses in bacteria.

Table 1: CFU count per Petri dish

Sample	WEC Medium		BC Medium	
	Leocobacter sp	*B. aryabhattai*	*Leocobacter sp*	*B. aryabhattai*
Control	72	93	84	101
BC-CH_{3L}	0	2	42	56
BC-CH_{3H}	0	0	28	43
BC-SC_{3L}	0	1	52	88
BC-SC_{3H}	0	0	45	76
BC-CH_{6L}	0	0	43	34
BC-CH_{6H}	0	0	24	24
BC-SC_{6L}	0	0	40	32
BC-SC_{6H}	0	0	32	26

The production of biofilms by *Bacillus sp*. has been reported in the literature, mainly in response to stressful or changes in environmental conditions (Kumar et al., 2004, Chowdhury et al., 2011). The production of exopolysaccharides is a response mechanism, which may contribute, for example, against pH changes, periods of drought and root desiccation (Seminara et al., 2012). The biofilm formed through the interaction with biochar was shown to be highly hygroscopic, forming a gel structure. The Figure 2 shows the formation of different types of biofilms by the means of roughness, according to the type and dose of inoculated biochar, as shown in Table 2. By means of this response to inoculation with biochar, this material can be considered as a stressing material, as it induces to unfavourable external environmental conditions, triggering the formation of biofilms and altering in the microenvironment in which the strains were inserted, mainly significant changes in pH (Ahmad et al., 2013 and Velmourougane et al., 2017).

Table 2: EPS biofilm roughness parameters

Sample	Ra (nm)	Rq (nm)	Rz (nm)	Correlation with BC features
BC-CH_{3L}	0.39	0.57	3.19	
BC-CH_{3H}	0.27	0.43	2.94	
BC-SC_{3L}	0.08	0.12	0.72	Phenolic Groups (+ **)
BC-SC_{3H}	0.06	0.09	0.69	Dissolved Organic Carbon (- **)
BC-CH_{6L}	0.09	0.14	0.81	Wettability (+ ***)
BC-CH_{6H}	0.15	0.21	1.37	Si and Ash Content (+ **)
BC-SC_{6L}	0.12	0.16	0.88	
BC-SC_{6H}	0.07	0.10	0.77	

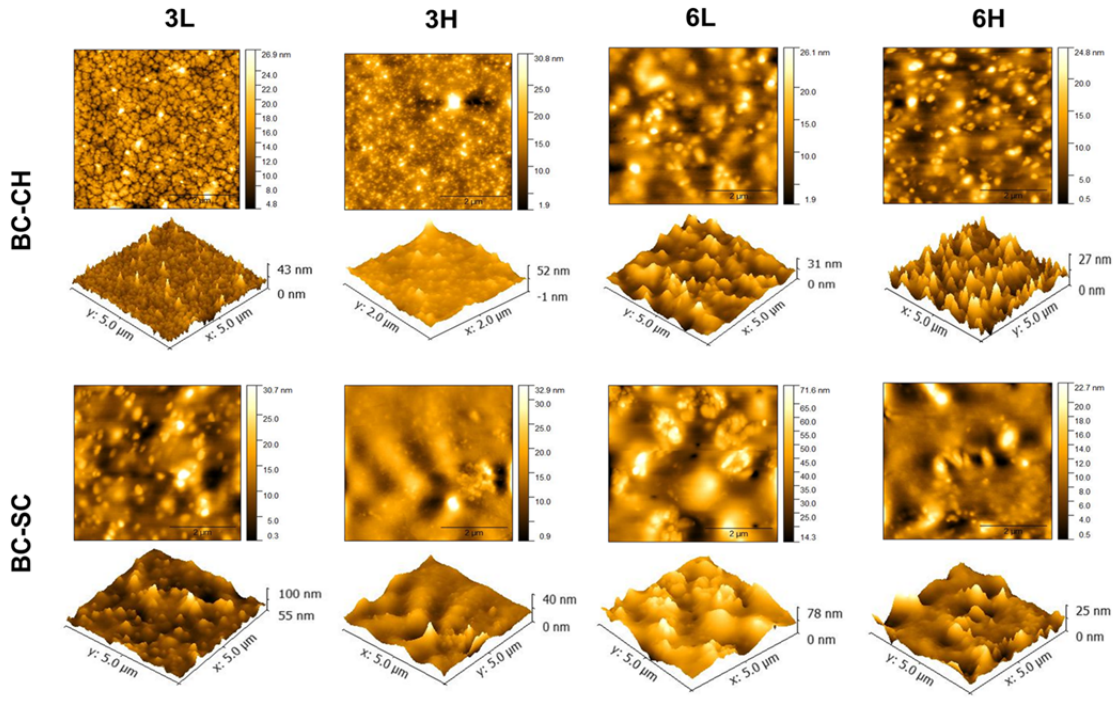

Figure 1: EPS biofilm formation as a result of the interaction of B. aryabhattai with biochar produced from corn cob husk (BC-CH) and sugarcane bagasse (BC-SC) produced at 300 and 600 ºC at low (L) and high-dose (H) (3L, 3H, 6L and 6H, respectively).

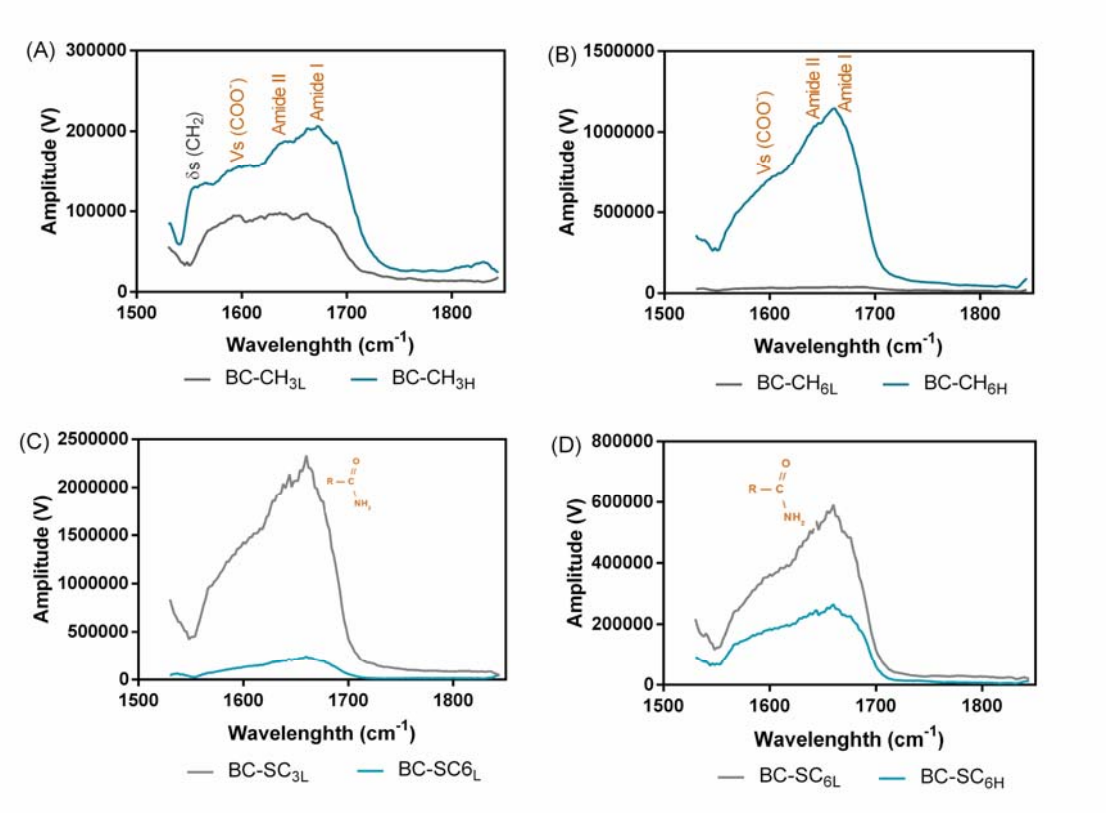

Figure 2: Infrared nano spectra obtained for B. aryabhattai biofilms when in contact with the biochars samples tested.

AFM images (Figure 1) showed that the nanoarchitecture of biofilms is featured with a roughness-induced superhydrophobicity (contact angle θ = 0) (Bazaka et al., 2011). This implies a great protection potential against drought in the rhizosphere, as well as in the microorganisms attachment. Surface roughness of the biofilms is dependent on the amount of phenolic groups, silica and ash content present on the surface and structure of the biochar, as well as the wettability, since values founded for Ra, Rq e Rz showed positive correlations with these chemical characteristics of the biochar samples (Table 2). The presence of silica and metal oxides showed an important correlation with the roughness and biofilm formation. This correlation is an important one because silica and oxides bounded with biofilms are an essential bridge on soil structure formation and strongly influences the biogeochemical interfaces in the rhizosphere area (Ma et al., 2017). Ma et al., found evidence that the silica and metal oxides may also induced cell lysis and, because of that, contributing with the decrease of CFUs for high dosages of biochars in the culture medium. The dissolved organic carbon was negative correlated with the roughness possibly due to the nature of carbon.
The characterization of the biofilms was performed by means of infrared chemical mapping at the nanoscale (AFM NANO-IR). The spectra (Figure 2) indicated the presence, mainly of Amida I and II groups. The biofilms formed from the interaction with BC-CH_{3L} and BC-CH_{3H} also showed indications of vibration clusters (δs) CH_2, which indicate the presence of lipids and also of stretching vibrations (νs) of COO^- groups, indicating the presence of proteins in the formed material (Baker et al., 2014).

4. Conclusions

The results obtained indicated that the dosage and the type of biochar modified the morphology and topography of the biofilms, as well as the adaptation and survival of the bacterial species depend on their interaction pathway with the molecular structure of the biochar surface. Thus, the binding force, or even biochar colonization, can be influenced by the type of dominant molecule readily available on the surface of the biochar, where phenolic components, silica and metal oxides (ash portion of biochar) can act as chemical signals that induce cell lysis and biofilm formation in bacteria. For each sample analysed, it was observed that the pyrolysis temperature, biomass of origin and dosage used are variables that need to be analysed case by case according to the type of soil to be applied, native microorganisms and species of plant to be treated. It is necessary to take into account that the inhibitory effects observed in the development of *Leocobacter sp*. when transferred to the field, can be diluted given the appropriate ratios. But they should not be disregarded, given the high rates of negative effects observed. Still, on *B. aryabhattai* scenario the biofilm formation is an important feature whereas the biofilm establishment in the rhizosphere promotes symbiotic association between microorganisms and plants. In addition, the biofilm may contain bioactive compounds of industrial importance and for agriculture, where it may have great potential as multi-species inoculants for biofertilizers. Finally, significant progress needs yet to be made in understanding the role on bacteria/ biofilm formation and biochar interactions. Identifying a direct correlation between the bacterial response metabolism and the chemistry of biochar is an essential advance to assemble the necessary data for design and use of agricultural materials containing biochar to promote the food and environment security.

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Reference

Ahmad M., Lee S.S., Rajapaksha A.U., Vithanage M., Zhang M., Cho J.S., Lee S.E., Ok Y.S., 2013, Trichloroethylene adsorption by pine needle biochars produced at various pyrolysis temperatures, Bioresource Technology, 143, 615-622.

Bazaka K., Crawford R.J., Ivanova E.P., 2011, Do bacteria differentiate between degrees of nanoscale surface roughness?, Biotechnology Journal, 6, 1103-114.

Baker M.J., Trevisan J., Bassan P., Bhargava R., Butler H.J., Dorling K.M., Fielden P.R., Fogarty S.W., Fullwood N.J., Heys K.A., Hughes C., Lasch P., Martin-Hirsch P.L., Obinaju B., Sockalingum G.D., Sulé-Suso J., Strong R.J., Walsh M.J., Wood B.R., Gardner P., Martin F.L., 2014, Using Fourier transform IR spectroscopy to analyze biological materials, Nature Protocols, 9, 1771–1791.

Belmonte A.B., Tan R.R., Benjamin M.F.D., 2017, A Two-Stage Optimization Model for the Synthesis of BiocharBased Carbon Management Networks, Chemical Engineering Transactions, 61, 379-384.

Chowdhury S.P., Schmid M., Hartmann, A., Tripathi, A.K., 2011, Diversity of 16Sr RNA and nifH genes derived from rhizophere soil and roots of an endemic drought tolerant grass, *Lasiurus sindicus*, European Journal of Soil Biology, 45, 114-122.

Karunanayake A.G., Todd O.A., Crowley M., Ricchetti L., Pittman Jr C.U., Anderson R., Mohan D., Mlsna T., 2017, Lead and cadmium remediation using magnetized and nonmagnetized biochar from Douglas fir, Chemical Engineering Journal, 331, 480-491.

Kumar C.G., Joo H.S., Choi J.W., Koo Y.M., Chang C.S., 2004, Purification and characterization of an extracellular polysaccharide from haloalkalophilic *Bacillus sp*. I-450, Enzyme and Microbial Technology, 34, 673-681.

Lau A.Y.T., Tsang D.C.W., Graham N.J.D., Ok Y.S., Yang X., Li X.D., 2018, Surface-modified biochar in a bioretention system for Escherichia coli removal from stormwater, Chemosphere, 169, 89-98.

Ma W., Peng D., Walker S.L., Cao B., Gao, C.H., Huang Q., Cai P., 2017, *Bacillus subtilis* biofilm development in the presence of soil clay minerals and iron oxides, npj Biofilms and Microbiomes, 3(4), 1-9.

Seminara A., Angelini T.E., Wilking J.N., Vlamakis H., Ebrahim S., Kolter R., Weitz D.A., Brenner M.P., 2012, Osmotic spreading of Bacillus subtilis biofilms driven bt an extracellular matriz, Proceedings of the National Academy of Sciences of the United States of America, 109, 1116-1121.

Velmourougane K., Prasanna R., Saxena A.K., 2017, Agriculturally important microbial biofilms: Present status and future prospects, Journal of Basic Microbiology, 57, 548–573.

Yang Z., Chen J., Preparation, Characterization and Adsorption Performance of Reed Biochar, Chemical Engineering Transactions, 62, 1243-1248.