Eötvös Loránd University Faculty of Science
Doctoral School of Environmental Science
Program of Environmental Biology

András Kari

THE PERSITENT ORGANIC AMENDMENTS OF SOILS

Monitoring of the biochar and plant growth promoting rhizobacteria soil amendments effect on deteriorated soils.

-PhD thesis-

Supervisor:
Dr. Károly Márialigeti
Professor

Head of Doctoral School:
Dr. Tamás Turányi
Professor

Head of Doctoral Program:
Dr. Erika Tóth
Associate professor

ELTETTKINSTITUTE OF BIOLOGYDEPARTMENT OF MICROBIOLOGY
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INTRODUCTION

In the last decades, due to the intensive agricultural practices (enhanced chemical fertilizer and pesticide application and genetic modification), not just the yield quantity per area and time has been increased, but the negative consequences related to the ecosystem, environment and climate change as well (Nkebiwe et al., 2016). The degradation of agricultural lands together with loss of nutrients and intensified soil erosion consist a major problem for the economies. The aim to preserve environmental quality, and to provide safe and healthy food in adequate quantities to overcome the needs of the growing population, lead to sustainable agriculture (Benbrook, 1999).

One of the possible tools of sustainable agriculture is the amendment of agricultural soils with biochar. Biochar is a high porosity and finely structured material with big amount of carbon content, which bases on renewable sources. It is produced by the thermal "degradation" of organic residues (animal and plant wastes) in the absence of oxygen (pyrolysis) (Lehmann et al., 2011). Biochar, as a soil amendment, has been described to have multiple benefits in improving soil fertility, enhancing crop yield and even sequestering carbon to mitigate climate change (Liu et al., 2012). Besides carbon sequestration there are growing interests to use biochar as soil amendment in order to bring deteriorated soils into agricultural production. Through its numerous properties as high surface area with functional groups, high porosity, capabilities to store nutrients due to its adsorption capacity and improving water-holding capacity, biochar application also influences the soil biota. The previous microbial community researches presented, that biochar induced soil microbial community and activity changes play the same significant role on the soil nutrient and organic matter content, as well as in the enhance of the plant growth (Ding et al., 2016).

Plant growth promoting rhizobacteria (PGPR) as biofertilizers and biocontrol agents also offer a sustainable way for agriculture. They competitively colonize plant roots and due to their various metabolic pathways enhance plant growth besides the stress handling capabilities of plants (Kloepper et al., 1980). A plant growth promotion due direct mechanism consists plant nutrient supply (e.g. nitrogen fixation), enhancing nutrient availability (e.g. phosphorus solubilization) and producing phytohormones as auxin and gibberelin. Indirect mechanisms consist substrate competition and induced systematic resistance, which enables PGPR to be used as biocontrol against plant pathogens (Aeron et al., 2011).

One of the major challenges in the use of commercialized bacterial inoculants is the un-predictable or poor survival of the inoculated bacteria in the soil (Hale és mtsai, 2014). Biochar due to the high porosity and adsorption capacity can provide efficient shelter (protect and nutrient).

There is a lack information related to the application of biochar as inoculant carrier, and also related to the effect of the combined application of biochar and PGPR on the rhizosphere microbial community structure. According to that it is important to have more knowledge from the effect of the combined application of biochar and PGPR, not just related to the soil physical-chemical properties, but also related to the effect on the rhizosphere microbial community structure and on the inoculated PGPR.

OBJECTIVES

Biochar based on renewable raw material, not just helps to bring deteriorated soils into agricultural production, but also offers the possibility, due to their physical-chemical properties, to be used as an effective inoculant carrier. Related to that our aims during the open-field trail were as it follows.

- Our aim was to establish a monitoring system, which enables us to *in situ* monitor the relative abundance changes of the inoculated PGPR in open field trials.
- Our aim was to determine the possible application of biochar as inoculant carrier, due to the monitoring of the inoculated PGP bacteria abundances, through the maize growing stages.
- Our aim was to detect the combined effect of biochar and PGPR application on the microbial community structure in acidic and calcareous sandy soil, through the maize growing stages.
- Our aim was to detect the long term (2 year) effect of biochar amendment in the acidic and calcareous sandy soils, due to the exploration of the bacterial community structure differences between the two different soil.

MATERIALS AND METHODS

In the present study the grain husk and paper fibre sludge based biochar were used together with PGPR inoculant in three different doses in acidic (pH 4.4) and alkaline (pH 7.9) sandy soils. Sampling time correlated with the maize main growth stages as sowing, V4–V6 stage, R1 – silking and R6 – physiological maturity were sampled as well. In order to detect the long term of biochar application on soil bacterial community structure, sampling was done 30 months after inoculation as well.

Prior to the open-field trial the nearly complete 16S rRNA gene of the strains was sequenced by Sanger method at LGC Ltd. (Berlin, Germany). In order to distinguish the strains of

the stress-tolerant bacterial inoculants, the 16S rRNA gene sequences of the PGPR were investigated *in silico*, in the 27F-519R (Lane, 1991), 341F-907R (Teske et al., 1996) and 968F-1401R (Heuer et al., 1997) enclosed regions. Thirty-four, mostly type II, restriction endonucleases were tested from the supply of Fermentas (nowadays Thermo Fisher, USA). The *in silico* analyses were conducted by the MEGA6 program and with the ISPaR (In Silico PCR and Re- striction) software of the web based Microbial Community Analysis (MiCA) program package (http://mica.ibest.uidaho.edu/) (Shyu et al., 2007).

Based on the results of the *in silico* analysis *Bsh*12361and *Bts*CI restriction endonucleases were selected for the molecular fingerprint T- RFLP (terminal restriction fragment length polymorphism) analysis of the 16S rRNA gene section determined by 27F-519R primer pairs. Sequence-aided T-RFLP analysis enabled the monitoring of the soil maize rhizosphere bacterial community changes based on the identification of dominant community by using partial 16S rDNA clone libraries. To assess the microbial community dynamics, besides sequence-aided genotypic (T-RFLP) analyses phenotypic phospholipid fatty acid (PLFA) analyses were also conducted. Statistical analyses have been done by using the PAST software version 3.13 (Hammer et al., 2001), while relationships between environmental variables and bacterial community composition were revealed by principal component analysis (PCA) ordination combined with vector-fitting. Environmental variables were fitted as vectors with 'envfit' function (package vegan, Oksanen et al., 2018) onto the PCA ordination (T-RFs, PLFA and OTU), and the significance of fittings was tested with random permutations in program R (R Core Team, 2016; http://www.r-project. org/).

DNA extracted from soils after 30 months of treatments were used to extract the bacterial community structure of soils, by sequencing the hypervariable V3 and V4 amplicons of 16S rRNA gene. From the community DNA isolates amplicon libraries were generated by PCR amplification of the V3-V4 region of the 16S rDNA using B341F and B805R primers. Sequencing was done by in the Genomics Core Laboratory of Michigan State University (USA) Research Technology Support Facility, on Illumina MiSeq platform. The examination of the biochar surface colonization was done by scanning electron microscope (SEM).

RESULTS AND DISCUSSION

The T-RFLP technique developed by us made it possible to distinguish the members of commercialized bacterial inoculants, to monitor the relative abundance changes of the applied PGPR and to detect the effect of inoculants on the bacterial community structure of the rhizosphere. By a similar concept, the T-RFLP technique was also successfully used by Conn and Franco (2004) to monitor the colonization levels of inoculated actinobacterial endophytes in wheat roots, and also detecting and discriminating *phlD*+ biocontrol pseudomonads (von Felten et al., 2011).

Our results reflected that combined application of biochar and PGPR inoculants had significant effects in both acidic and calcareous sandy soils. In both soils the N_2 fixing *Azospirillum* bacteria relative abundances have been significantly increased. In the acidic sandy soil the abundance of *Azospirillum brasilense* have been positively affected by the 30 t ha^{-1} biochar (A4, +1-3%) and by the 15 t ha^{-1} biochar as inoculant carrier treatment (C3 +0,5-3,2%). On the calcareous sandy soil *Azospirillum irakense* relative abundance have been increased with +1,5% abundances compared to the control, due to the high doses (30 t ha^{-1}) of biochar treatment. Amongst the other inoculated PGPR's *Arthrobacter crystallopoietes* and *Kocuria rosea* relative abundances have been positively, but not significantly, influenced by the applied treatments. The abundances of *Bacillus aryabhattai* és *Paenibacillus peoriae* were around 1%, which is the detection limit. This phenomenon reflects the limitation of the applied method, and also the large number of abiotic and biotic factors influencing the survival and abundance of microbes (including PGPR bacteria) in rhizosphere soil.

The molecular results both reflected the significant differences between the acidic sandy (Lamellic Arenosol) and calcareous sandy (Mollic Umbrisol) soils rhizosphere bacterial community structures. The sequences-aided T-RFLP method identified the main reasons of differences due to the high abundance of Acidobacteriaceae family in the acidic soil and to the high abundance of Solibacteres class EU445199 family in the calcareous soil. On the other hand, new generation sequencing 30 months after inoculation, enlightened that besides the high Acidobacteriaceae abundance in the acidic soil, the high abundance of Pyrinomonadaceae family (Acidobacteria phylum) in the calcareous sandy soil was the main driven factor in the differences between the two soils. These results reflecting not just the different resolution of the molecular technologies (Gong et al., 2013), but also to the significant community shifts driven by the changed soil nutrient form and content. Acidobacteria phylum with organism adapted to variable habitats,

can be considered as a good indication for the changing of the soil physicochemical properties (Bartram és mtsai, 2014).

In both soils the major influencing factor on the bacterial community changes were the maize development stages. On the 2015 genotypic sequence-aided T-RFLP and phenotypic PLFA methods reflected the maize growth-related bacterial community changes. On the acidic sandy soil the genotypic methods also reflected, that above 15 t ha^{-1} grain husk and paper fibre sludge based biochar treatment, not just the previously shown (Rékási et al., 2018) soil physicochemical properties have been influenced significantly, but also the bacterial community assemblages as well.

Our results reflected, that labile carbon sources introduced by the high doses of biochar treatment triggered the bacterial community changes, as the K-strategist (Acidobacteriaceae, Ktedonobacteraceae, Solibacteraceae) organisms have been outcompeted by the r-strategist copiotroph (Sphingomonadaceae és Chitinophagaceae) organisms. This phenomenon is in accordance with the previously described "copiotroph bacterial community change" after biochar application (Jenkins és mtsai, 2017). The phenotypic PLFA analysis proved that due to the high doses of biochar treatment the changing ratio of Gram-negative/ Gram-positive bacterial groups are related to the altered quality and quantity of the organic matter in the soil (Kourtev et al., 2003; Zhong et al., 2010).

Our research enlightened, that the high doses of biochar treatment (15 és 30 t ha^{-1}) influenced not just the organisms which are responsible for soil carbon cycling, but also increased the relative abundance of the N_2 fixing organism (Rhodospirillaceae, Bradyrhizobiaceae). These phenomena support the hypothesis of Anderson et al. (2011) that biochar plays an integral role in enhancing the proliferation of these bacteria by supplying them with utilizable carbon compounds, and biochar can act as a "nitrogen island" in the soil (reducing N losses due to leaching and gas fluxes).

Our results clarified, that besides biochar induced soil physicochemical and microbiological changes, biochar as an inoculant carrier had significant beneficial effect on maize biomass. Biochar immobilized PGPR inoculant (C3 treatment) not just significantly enhanced *Azospirillum brasilense* relative abundance, but significantly promoted the maize biomass between extreme acidic soil environment (4.4 pH). The prerequisite of the successful plant growth promotion is effective root colonization (Buddrus-Schiemann et al., 2010). Besides the measured relative abundances enhancement in this study, the described maize biomass increment in case of biochar with immobilized inoculant by Rékási et al. (2018) reflects the fact that PGPRs survived.

Our study reflects that biochar with a well-developed pore structure may provide better protecting living environment for inoculated PGPR, which can enhance maize tolerance to drought stresses. The results of the study reflect the possible applicability of biochar as a carrier material, and also cleared that the combined application of biochar and PGPR soil amendments are significantly efficient in case of acidic soil environment. Contrary findings related to the biochar raw material, pyrolysis parameters and applied quantity, reflects the need of further open-field, long term studies, in order to optimize the use of biochar as inoculum carrier and soil amendment.

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