Article

Humic Acid Mitigates the Negative Effects of High Rates of Biochar Application on Microbial Activity

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Abstract: Objective: Biochar and a commercial humic acid-rich product, Humac (modified leonardite), represent soil amendments with the broad and beneficial effects on various soil properties. Their combination has been scarcely tested so far, although the positive impact of their interaction might be desirable. Materials and Methods: The dehydrogenase activity (DHA), microbial biomass carbon (C_{mic}), soil respiration (basal and substrate-induced), enzyme activities, total carbon (C_{tot}), and both shoot and root biomass yield were measured and compared in the short-term pot experiment with the lettuce seedlings. The following treatments were tested: the unamended soil (control), the Humac-amended soil ($0.8 \text{ g} \cdot \text{kg}^{-1}$), the biochar-amended soil (low biochar $32 \text{ g} \cdot \text{kg}^{-1}$, high biochar $80 \text{ g} \cdot \text{kg}^{-1}$), and the soil-amended with biochar + Humac. Results: The effect of both amendments on the soil pH was insignificant. The highest average values of C_{tot} and C_{mic} were detected in high biochar treatment and the highest average values of basal and substrate-induced respiration (glucose, glucosamine, alanine) were detected in the low biochar treatment. The phosphatase activity and fresh and dry lettuce aboveground biomass were the highest in the low biochar + Humac treatment. Conclusions: Even though the combination of both biochar + Humac decreased the microbial activities in the amended soil (C_{mic} , DHA, enzymes, substrate-induced respiration) at the low biochar dose, they mitigated the detrimental effect of the high biochar dose on respiration (all the types) and the enzyme (phosphatase, arylsulphatase) activities. In contrast to the previously published research in this issue, the effects could not be attributed to the change of the soil pH.

Keywords: organic amendment; priming effect; soil dehydrogenase; microbial biomass carbon; respiration; enzymes; plant biomass; soil health

1. Introduction

The term biochar is used for a heterogeneous material produced by pyrolysis of the selected raw material under controlled conditions. Biochar is produced from a wide variety of feed stock [1,2]. Nowadays, it has been widely applied in various branches of anthropogenic activities: in the agriculture to increase productivity, management of soil health, and industry and technologies [3–5]. Biochar employed as a soil amendment has mostly been recognized as beneficial for the soil when being applied solely or in combination with other organic materials [6–9]. Soil rhizobacteria plays an significant role in phytoremediation of contaminated soil, increases nutrient uptake and promote plant growth and yield. One such co-amendment may represent humic acids (HA), which are the components of humus, a key factor in the persistence of soil organic matter (SOM) in soils. Biochar-derived HA seems to be important in the process of SOM enrichment in biochar-amended soils. However, their features differ from soil-derived HA, namely, they exert more surface COOH and higher cation exchange capacity, which may change biochar surface properties [10]. The biochar-derived HA render increased aromaticity of low-temperature biochar whereas their interaction with high-temperature biochar has pore-blocking effects due to the extension of HA into pore throats, where they artificially lower the biochar surface area [11,12].

Nevertheless, the interaction between biochar and HA improves soil health and enables many applications. Yet, mainly the effect of HA on the biochar sorption of sulfonamides [11], polar and nonpolar organic pollutants [13], and Cd(II) ions from aqueous solutions [14] has been evidenced. The benefits of combined HA and biochar in agriculture have not been extensively investigated although positive effects of co-amended biochar and HA have been reported in a few studies: In a pot experiment, the enhancement of characteristics of composted green waste and the growth and nutrition of the ornamental plant *Calathea insignis* has been proven [15]. In the field study, the combined application of the organic amendments (rock phosphate-enriched compost, biochar, and HA) along with *Alcaligenes* sp. showed improved maize growth and yield as well as soil health [16]. On the contrary, biochar and HA amendment did not improve performance under water-limited conditions in comparison with the amendment of pure biochar [17].

In the present study, a pot experiment was conducted to investigate the effect of biochar, commercial (mined) HA, and their combination on soil properties and crop yield. We hypothesized that the HA amendment in the biochar-treated soil would mitigate the potential negative priming effect of high dose of biochar on the certain soil properties (dehydrogenase and other enzyme activities, carbon decomposition, nutrient retention). Such a hypothetical synergistic effect of HA and biochar on crop growth and soil properties might be helpful in sustaining the soil health.

2. Materials and Methods

2.1. Soil and Pot Experiment Preparation

The following soil amendments were used for the laboratory-scale experiments: biochar (middle-temperature biochar—approx. 400 °C—pyrolyzed from agricultural grain waste), HA-rich product Humac AGRO (commercial soil fertility stimulator, mined from leonardite-oxihumolite), and their combination. Composition of the Humac AGRO (according to the manufacturer Envi Produkt Ltd., Prague, Czech Republic) was as follows: pH 6.5; dry matter $\geq 85\%$; $\geq 50\%$ of free HA in dry matter; approx. 38.4% C, 18.6% O, 3.1% N, 1.5% Na, 1.5% Ca, 1.6% Fe, 1.1% K in dry matter; microelements Zn 64 mg·kg^{−1}, B 77 mg·kg^{−1}, Cu 19 mg·kg^{−1}, Se 1.67 mg·kg^{−1}. The experimental design was derived from the previous research by Wang et al. [15]. Nevertheless, it excluded the amendment of any extra

organic matter (e.g., composted green waste) and the usage of nonbuffered humic acids, which may significantly affect the soil pH.

The pot experiment with lettuce (*Lactuca sativa* L. var. *capitata* L.) cv. Smaragd was carried out under controlled conditions in the growth chamber with full-spectrum, stable white LED lighting, light intensity 20,000 lx ($200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) [18]. Environmental conditions were maintained at temperature 18/22 °C (night/day), a 12-h photoperiod, relative humidity 70% [19].

The topsoil (0–15 cm) applied for the growth substrate in this pot experiment was collected from the rural area near the town of Troubsko, Czech Republic (49°10′28″ N 16°29′32″ E). The soil type was clayey to loamy, modal brown earth, and chemical properties were determined at the beginning of the experiment. Prior to using, the soil was sieved through a grid of size 2.0 mm and mixed with fine quartz sand (0.1–1.0 mm) (1:1, w/w). The substrate nutrient composition is in the Table 1.

Table 1. The total content (mean values) of substrate macro- and microelements, calculated to the dry weight.

Soil Physical and Chemical Properties					
Parameter	Value	Unit	Parameter	Value	Unit
pH	7.29		C:N	8.75	
C _{tot}	7.0	g·kg ^{−1}	S	72.5	mg·kg ^{−1}
N _{tot}	0.80	g·kg ^{−1}	P	48.5	mg·kg ^{−1}
N _{anorg}	32.8	mg·kg ^{−1}	K	115	mg·kg ^{−1}
N-NO ₃	29.6	mg·kg ^{−1}	Ca	1629	mg·kg ^{−1}
N-NH ₄	3.2	mg·kg ^{−1}	Mg	118	mg·kg ^{−1}

The six different soil treatments—the five various amendments (according to the type and amount of the added matter mixed with the substrate) and the control (unamended soil)—were tested: (1) control, (2) Humac (0.8 g·kg^{−1}), (3) low biochar (32 g·kg^{−1}), (4) low biochar + Humac (32 g·kg^{−1} and 0.8 g·kg^{−1}, respectively), (5) high biochar (80 g·kg^{−1}), (6) high biochar + Humac (80 g·kg^{−1} and 0.8 g·kg^{−1}, respectively). Each treatment was performed in three repetitions (pots). The experimental pots (volume 1 L) [20] were filled with 1 kg of either sole growth substrate (soil–sand mixture), the control, or mixed substrate and soil amendments (five other variants) according to the doses of treatment.

All the pots were watered with 100 mL of distilled water. The lettuce seeds were sprouted on wet filter paper for two days. Three sprouted lettuce seeds were sown at about 2 mm depth in each pot and incubated in the growth chamber under defined conditions (see the above). After 10 days, the seedlings were thinned to one per pot; one representative was left in each pot. The pots were placed into the growth chamber randomly. All the plants were manually watered with 50 mL of distilled water every other day. Once per week, the pots were rotated variably [21]. The plants were harvested six weeks after sowing.

2.2. Plant Biomass

The lettuce shoots were cut at the ground level; the roots were removed from the soil and washed with water [21]. Fresh aboveground biomass (AGB) and roots were estimated gravimetrically by weighing the shoots and cleaned roots separately on the analytical scales. The weighed lettuce shoots were dried at 60 °C to the constant weight, and dry AGB was estimated gravimetrically by weighing the dried shoots on the analytical scales. Dried root biomass was not determined due to extremely small weight value under the limit of measurement on the scales.

2.3. Soil Sampling and Preparation

One soil sample was collected from each pot after lettuce harvesting for further analysis. The soil samples were homogenized by sieving through a 2-mm mesh under sterile conditions.

The samples for the enzyme activity assays (arylsulphatase = ARS, phosphatase = Phos, N-acetyl- β -D-glucosaminidase = NAG, β -glucosidase = GLU) were freeze-dried [22]. The samples for the measurement of dehydrogenase assay, pH, microbial biomass carbon (C_{mic}) quantification, and respiration (basal and substrate-induced) were stored at 4 °C for 14 days prior to the analysis.

2.4. Soil pH

The pH value of extracts from soil in $CaCl_2$ ($0.01 \text{ mol}\cdot\text{L}^{-1}$) in ratio 1:5 (volume fraction) was measured according to ISO 10390:2005 [23].

2.5. Total Soil Carbon

The level of total carbon (C_{tot}) as an essential parameter of the soil quality and fertility was analyzed, using LECO TruSpec analyzer (MI USA), on the samples of a fine particle size, prepared by sieving through a 0.15-mm mesh, and dried to the constant weight at 105 °C.

2.6. Microbial Biomass Carbon

C_{mic} represents an active fraction of SOM, mostly formed by bacteria and fungi. Soil C_{mic} was determined by the fumigation extraction method [24], based on the lysis of microbial cells upon contact with chloroform (24 h). The samples were duplicated in each set and only single set underwent fumigation, subsequent extraction of K_2SO_4 , and comparison of the fumigated and nonfumigated samples.

2.7. Microbial Respiration: Basal Respiration and Substrate-Induced Respiration

Soil respiration is another indicator of soil metabolic activity. The substrate-induced respiration (SIR) allows quantifying the catabolic activity of a functional group of organisms determined by the respective substrate specificity. The MicroResp method was carried out according to Campbell et al. [25] and the official supplier protocol (Technical Manual v2.1, The James Hutton Institute). Basal respiration (BR) was measured without any additive (energy source). The substrate-induced respiration was measured with the addition of specific energy source: D-glucose (in the dose of $30 \text{ mg}\cdot\text{g}^{-1}$ of soil water) and D-trehalose, N-acetyl- β -D-glucosamine, L-alanine, L-lysine (in the dose of $7.5 \text{ mg}\cdot\text{g}^{-1}$ of soil water). Each soil sample was measured in four replicates. The respiration was expressed in the unit $\mu\text{g CO}_2\cdot\text{g}^{-1}\cdot\text{h}^{-1}$.

2.8. Soil Enzyme Activities

The triphenyl tetrazolium chloride-dehydrogenase activity (TTC-DHA) was applied to determine the general microbial carbon mineralization activity in the soil. The method [26] was modified as follows: 3 g of the soil sample was mixed with MgO and sealed with the standard solution (TTC + distilled water). The samples were incubated in the incubator at 37 °C for 24 h. Afterwards, triphenyl formazan (TPF) was extracted from the samples using methyl alcohol, resulting in the color change of the solution. The spectrophotometer (DR 3900, Hach Lang, Dusseldorf, Germany) was used to measure the color intensity at a wavelength of 485 nm. DHA was calculated according to the calibration curve and expressed in $\mu\text{g TPF}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$.

The soil enzyme activities were determined according to ISO 20130 [27]. p-nitrophenyl (PNP) derivatives of the specific soil substrates were used for Vis spectrophotometric measurement at $\lambda = 405 \text{ nm}$. Each soil sample was measured in nine replicates.

2.9. Statistical Analysis

Data processing and statistical analysis were carried out with the help of the statistical program R version 3.6.1. [28] together with the additional packages “ggplot2” [29] for creating all the graphs.

Multivariate analysis of variance (MANOVA) and principal component analysis (PCA) with the dependence of different treatments were used for modeling the relation between the soil properties and selected treatments with the help of the additional packages “factoextra” [30] and “FactoMineR” [31]. One-way analysis of variance (ANOVA) and the Duncan’s multiple range test from package “agricolae” [32] at the significance level of 0.05 were applied to detect the difference among the treatments. The factor level means calculating (with 95% confidence interval (CI)) was carried out by using “treatment contrast”. Partial eta-squared (η^2) from package “BaylorEdPsych” [33] was employed for measuring the effect size, and the Pearson correlation coefficient (with 95% CI) was applied for measuring the linear dependence among the soil properties. The chart of the correlation matrix was created with the help of additional packages “PerformanceAnalytics” [34].

3. Results and Discussion

3.1. Plant Biomass

Plant biomass measurement is an essential parameter in assessing how different soil amendments can help farmers in improving productivity. Result of one-way ANOVA showed that addition of Humac did not affect the fresh root biomass and dry AGB compared to the control. Moreover, the fresh AGB was significantly decreased. However, either sole biochar or biochar + Humac treatments significantly increased the dry AGB (both low and high biochar doses) and root biomass (not the high biochar + Humac) as compared to the control and Humac treatments (Figure 1a,b).

Figure 1. Lettuce biomass in soil amended with Humac, biochar, and biochar + Humac: (a) Fresh Aboveground biomass and fresh root biomass; (b) dry aboveground biomass. Bar plot with standard error of mean. Different letters indicate statistically significant differences at $p \leq 0.05$.

The current study findings are in agreement with the result of [35,36], where the application of biochar alone showed a significant increase in plant biomass. However, there are evidences of no or even negative effect of biochar amendment on the crop yield [37]. Also, the contradiction between laboratory and field test results of the biochar effect on plant biomass was observed [38]. Although authors of the metanalysis [39] concluded that biochar application had no significant effect on root biomass, we observed the opposite. However, an increase in the number of fine root hair, which may stimulate the soil microbial activity due to the increased root exudate, was observed in another study [40].

As a general pattern, plant growth increased in response to treatments of humic acid. In contrast to it, we observed a decrease in fresh AGB on treatment Humac (only) treatment as compared to control, although the decline was not significant. Arancon et al. [41] reported that plant growth initially increases on the application of humic acid, but with the increasing concentration of humic acid plant growth may also significantly decrease ($p \leq 0.05$) when the concentrations of humic acids exceed $0.5\text{--}1.0\text{ g}\cdot\text{kg}^{-1}$.

3.2. Soil pH

The pH values of all the experimental treatments did not differ significantly (Appendix A), even though the applied biochar had a high ($\text{pH} > 9.0$) basic soil reaction. On the contrary, the Humac was characterized by being close to the neutral pH (6.5). It might have partially neutralized the alkaline effect of biochar as it was similarly reported on peat amendment [42]. Nevertheless, it seemed that the soil also presumably possessed a significant pH-stabilizing effect. This assumption may be supported by the previously referred works, which did not show a significant effect of biochar amendments on the pH of neutral to basic sandy soils [43]. Biochar may initially lower soil pH due to dilution of the cations and the enhanced biochar-derived cation exchange capacity (CEC) would increase soil buffering ability [44]. Therefore, any significant impact of the soil reaction on the determined soil properties was not considered, and the pH values were not subjected to the PCA (Figure 2) and measurement of the Pearson correlation coefficient (Appendix B).

Figure 2. Rohlf PCA Biplot of Individuals (sample values) and Variables (soil properties).

3.3. Total Soil Carbon

The high correlation of C_{tot} with C_{mic} and the ABG and root biomass (Figure 2, Appendix B) was found. These findings correspond to the study in which biochar served as a type of organic amendment that efficiently enriched the soil with carbon [45]. The differences in C_{tot} were statistically significant among treatments with different biochar dosages (Figure 3b) (detailed results in Appendix C). There was a presumption that co-application of biochar and Humac could increase the C_{tot} content even more efficiently than pure biochar, as this was previously evidenced [15]. However, the treatments with biochar (low and high) + Humac showed no significantly different content of C_{tot} compared to the treatments amended only with biochar (low and high) (Figure 3b). Nevertheless, it was evidenced that biochar and humic acids could exert an opposite (and eventually antagonistic) effect on soil C losses/gain balance in a short-termed field trial [46], similarly to what we observed in a pot experiment. This finding is novel and contradictory to the up-to-now biochar recognition as an amendment that contributes to humic acid-mediated recalcitrance of soil carbon [47]. With respect to the negative correlation of DHA to both C_{tot} and C_{mic} (Figure 2) and in contrast with the study by Zhang et al. [15], it could be assumed that the soil carbon sequestration was more enhanced by biochar carbon access than by the pH-controlled effect of humic acid amendment on soil C mineralization activity and rates.

Figure 3. (a) Dehydrogenase activity and microbial biomass carbon in the soil amended with Humac, biochar, and biochar + Humac; (b) Total carbon in the soil amended with Humac, biochar, and biochar + Humac. Bar plots with standard error of mean. Different letters indicate statistically significant differences at $p \leq 0.05$.

3.4. Microbial Biomass Carbon

Microbial biomass carbon is a carbon contained in living part of soil organic matter and it reflects the soil health [48]. Our experiment results showed a significant increase in C_{mic} value as compared to control for all the treatments amended with biochar (detailed results in Appendix C), whereas an increase in C_{mic} value was not significant in the case of Humac treatment. It is also important to note that Humac presence with either high or low dosage of biochar tends to decrease the value of C_{mic} as compared to respective treatment without Humac. Although the decline is not statically significant, there is an important trend that cannot be ignored. Usually, HA amendment enhances soil carbon and microbial biomass [49], but it is not always true. It may decrease or not affect soil carbon and microbial biomass [50]. HA application inhibits a certain type of bacteria while it may also increase some useful bacteria and fungi. So, the outcome of C_{mic} is the combined result of inhibition and promotion of certain microorganisms [50], which vary with the soil microbial environment. Earlier studies confirm that organic matter added to soil increases beneficial bacteria, whereas it decreases harmful bacteria [51,52]. Our results correlated with the changes in the total carbon values (Appendix B). The low C_{mic} value in low biochar + Humac treatment could be due to stabilization of available carbon via the HA-derived hydrophobic protection and pore-blocking effect mediated by humic acids. It might have caused lowered carbon to C_{mic} assimilation [53]. This presumes that the retention of labile carbon leaching from biochar was suppressed by the excess of biochar in high biochar + Humac treatment (Figure 3a). However, this effect did not correspond with the C_{tot} and the soil fertility (discussed in Section 3.3).

3.5. Basal and Substrate-Induced Respiration

The results of the measurement of respiration are in Figure 3. BR and SIR with various substrates strongly correlated with each other (Figure 2, Appendix B).

Pure Humac did not exert a significant priming effect on BR as compared to the unamended control; HA impacted the soil respiration differently when it interacted with biochar (Figure 3a). Humac significantly inhibited BR for the low biochar + Humac treatment as compared to low biochar treatment, presumably via affecting the soil aggregation [54]. On the other hand, its interaction with high biochar seemed to mitigate the negative effect of high biochar dose on BR and resulted in the higher respiration value for treatment high biochar + Humac, however, without a significant difference in comparison with the pure high biochar treatment (Figure 4a).

Glucose is a source of energy that is readily oxidized and reflects the catabolic capacity of soil microbial community. The treatment of the soil amended with low biochar exerted significantly increased glucose-induced respiration (Glc-SIR) in comparison with the control and the Humac treatment (Figure 4b). A similar result has also been reported by Hamer et al. [55]. Nonetheless, the other biochar-amended treatments showed no significance in altered Glc-SIR compared to the control (Figure 4b).

Trehalose degrading activities could be assigned to the Gram-positive bacterial and fungal community. Alanine and lysine are indicators of the nitrogen mineralization process in the soil due to the presence of easily degraded nitrogen. Trehalose-induced respiration (Tre-SIR) and lysine-induced respiration (Lys-SIR) were significantly decreased in the treatment with Humac, low biochar + Humac, and high biochar compared to the control. These results supported the observed features: Humac, contrarily to low biochar, decreased respective SIR respirations. Low biochar showed increased Tre-SIR and Lys-SIR compared to the Humac treatment, whereas high biochar reduced the SIR compared to the control: This negative priming effect of high biochar was, however, suppressed via the Humac amendment in the high biochar + Humac treatment. Alanine-induced respiration (Ala-SIR) (Figure 4a) respiration was significantly decreased only in the high biochar treatment, compared to the control. Nevertheless, this Ala-SIR decrease was mitigated in the high biochar + Humac treatment.

Figure 4. Respiration in soil amended with Humac, biochar, and biochar + Humac: (a) basal and substrate-induced (alanine, lysine) and (b) sugar substrate-induced (glucosamine, glucose, trehalose). Bar plot with standard error of mean. Different letters indicate statistically significant differences at $p \leq 0.05$.

Glucosamine-induced respiration (NAG-SIR) (Figure 4b) also reflects the aerobic decomposition of fungal-derived SOM. However, it corresponds with the results of BR and Glc-SIR. NAG-SIR was significantly decreased due to the treatment with low biochar + Humac and with high biochar, compared to the control and low biochar treatment. Combination of high biochar + Humac suppressed the high biochar-mediated NAG-SIR decrease.

All the resulting BR and SIR (details in Appendix C) cannot be assigned to any changes in the pH, as was evidenced (Section 3.1). The observed differences in the respiration values were hardly affected by the microbial diversity among all the treatments, as well, because different SIR types showed

similar patterns. Nearly all the types of respiration (BR, SIR) showed the significantly decreased values of the low biochar + Humac and high biochar treatment compared to the control and pure low biochar amendment, whereas low biochar + Humac seemed to inhibit soil respiration. The negative priming effect of high biochar on carbon mineralization was presumably mitigated by Humac amendment. However, these interactions may assume an impact on soil fertility and crop yield (fresh and dry AGB, fresh root biomass). The only significant changes were observed in comparison with the Humac-amended treatment (Section 3.3).

3.6. Dehydrogenase and Other Soil Enzyme Activities

DHA reflects the living microbial cell in the soil; it is considered as the best indicator of microbiological redox systems [56]. Any slight disturbance in the soil environment can be simultaneously seen with disruption in DHA as well [57]. In the present study, only treatment (high Biochar + Humac) showed a significant decrease in DHA value as compared to Humac treatment (Figure 3a), whereas other treatments did not show a significant change in DHA as compared to control and only Humac treatment (Appendix C). Also, The DHA activity showed a moderate positive correlation with respiration (basal and substrate-induced) and β -glucosidase and negative correlation with C_{mic} and C_{tot} (Appendix B). Figure 3a shows the behavior of DHA activity for all applied treatments. Previous studies showed that application of Humic acid has a substantial impact on soil enzyme activity [58–62]. Dehydrogenase is an intracellular enzyme. If unused, released enzymes either get immobilized by a humic acid molecule [63] or quickly get mineralized by other enzymes like protease present in the soil [64]. In our experiment, an increase in DHA value upon Humac treatment was observed, although the increase was not significant as compared to control. No significant difference in the value of DHA was observed in case of soil sample treated with biochar (low and high) as compared to the control. Other studies reported a decline in DHA activity upon biochar treatment [65,66]. Although microbial biomass indicator, the extracted phospholipidic fatty acids concentration, was not affected in the referred research. The possible reason could be due to the interaction (sorption and desorption) among the substrate, enzyme, and biochar surface [67] or owing to the negative priming effect of biochar on native SOM, as observed previously [68]. This is evidenced by significant differences in DHA values measured between Humac treatment and low biochar + Humac. However, treatment (high biochar + Humac) resulted in even more significantly lower DHA, as compared to Humac treatment.

All the treatments showed a significant lowering of β -glucosidase (GLU) activity compared to the unamended control (Figure 5a) Therefore, the negative priming effect on the GLU activity was manifested by both the Humac and biochar amendments, purely or in the combination. The decrease in GLU activity could be due to unavailability of the substrate to the enzyme due to high porosity and adsorptive power of biochar. A similar result was also reported by [69,70].

On the other hand, Phos was significantly decreased only at the high biochar dose (Figure 5a). The putative mitigation of the high biochar negative priming effect on Phos activity by interaction with Humac was observed as compared to sole biochar amendment treatments (Figure 5a). Such effect of biochar + Humac on Phos is novel and contradictory to the previous observations [71].

We observed a decrease in ARS activity for all applied treatments, but the low biochar amendment did not significantly affect the ARS activity. The decline in ARS activity due to high biochar and its combination with Humac was probably due to the sorption and enzyme inactivation [69,70]. Moreover, the negative priming effect of low biochar on ARS increased in combination with Humac (Figure 5b).

No significant change in NAG activity was measured for Humac and low biochar + Humac addition as compared to the control, whereas other treatments showed a significant decrease in NAG activity. The case of NAG assumed that the mitigating effect of Humac on the negative priming effect on biochar did not occur at the high biochar + Humac treatment but was detectable at low biochar + Humac (Figure 5b).

Figure 5. Soil enzyme activities in soil amended with Humac, biochar, and biochar + Humac: (a) GLU (β -glucosidase), Phos (phosphatase), (b) ARS (arylsulphatase), NAG (N-acetyl- β -D-glucosaminidase). Bar plot with standard error of mean. Different letters indicate statistically significant differences at $p \leq 0.05$.

The decrease in GLU [72], Phos [73], and ARS activity [74] due to the addition of high doses of biochar has been reported before by other scientific groups. The decline in GLU activity could be due to nonselective sorption of enzymes on the surface of biochar. The findings of this study support these outcomes, too. Nevertheless, it was evidenced (Figure 5) for some enzymes (Phos, NAG) that the soil treatments amended with Humac may mitigate this negative priming effect of biochar. High biochar + Humac exerted the significant increase of the Phos activity compared to the treatment with sole high biochar. A similar trend in the NAG activity of low biochar + Humac treatment was apparent as compared to the pure low biochar treatment (Figure 5b). It was hypothesized that the interaction of humic acids with biochar in a certain ratio would mitigate the supposed sorption of proteins, products, and substrates to biochar [75] and, subsequently, would suppress the inhibition of enzyme activities. Soil physical chemical properties changes with the land use [76–78]. Use of Biostimulants reduce the need for fertilizers [79–81]. This hypothetical role of the biochar–Humac interaction in biochemical soil processes together with mitigation of the detrimental effect of high biochar doses on soil respiration

may represent (apart from the increased soil fertility, determined as dry AGB+) a promising beneficial feature of humic acids in the improvement of the soil quality properties.

4. Conclusions

Research was conducted to compare the effects of two organic amendments, biochar and HA (Humac), and their combination on the soil microbial activity and the crop yield in the short-term pot experiment. We concluded that the combined application of both amendments exerted a positive effect on C_{mic} , C_{tot} (+157% and +166%, respectively), and lettuce crop yield (+26%) under experimental conditions in comparison with the control and the pure Humac treatment. In the present study, we also observed the positive effect of low biochar (32 g·kg⁻¹) treatment on several other soil properties, C_{mic} , C_{tot} , and Glc-SIR as compared to the control and C_{tot} , BR, and Glc-SIR as compared to the Humac treatment, whereas Humac treatment decreased the values of soil respiration (Lys-SIR, Tre-SIR) and enzyme activities (GLU, ARS) compared to the control. The decrease of all respirations (except for Glc-SIR) and enzyme activities were detected in the high biochar (80 g·kg⁻¹) treatment, assuming the negative priming effect of high biochar on the respective properties. Although the Humac amendment did not significantly increase any of the monitored properties, the addition of HA in the treatment with high biochar (80 g·kg⁻¹) + Humac helped to mitigate the negative priming effect on the following properties: BR (+77%) and all SIR, Phos (+21.5%), and ARS (+3.1%, HA+ compared to no-HA variants) activities. Presumably, it was due to the interaction of biochar and Humac, which may result in the suppression of negative effects of both amendments. We concluded that applied combination of amendments was not in favoring optimum to get higher soil quality and fertility and the crop yield. Therefore, the next step should be to test the effect of Humac under a modified experimental design to get better and enhanced positive effects of the Humac amendment on the monitored soil properties and crop yield.

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Appendix A

Table A1. pH mean values $\pm$ standard deviation (SD) of soil extracts, $n = 3$. Results of the one-way Anova did not show significant differences at $p \leq 0.05$ between the treatments.

Treatment	pH (CaCl ₂)
Control	7.190 $\pm$ 0.057
Humac 1 t/ha	7.239 $\pm$ 0.071
Biochar 40 t/ha	7.268 $\pm$ 0.098
Biochar 40 t/ha + Humac 1 t/ha	7.171 $\pm$ 0.054
Biochar 100 t/ha	7.134 $\pm$ 0.010
Biochar 100 t/ha + Humac 1 t/ha	7.171 $\pm$ 0.133

Appendix B

Figure A1. Correlation Matrix of Soil Properties (numbers indicate the Pearson's correlation coefficient). Notes: * statistically significant difference at 5% significance level; ** statistically significant difference at 1% significance level; *** statistically significant difference at 0.1% significance level.

Appendix C

Table A2. Results of Duncan's Multiple Range Test: statistical differences in the respective properties of the soil variants amended with Humac (1 t·ha⁻¹), biochar (BC-40 t·ha⁻¹ and 100 t·ha⁻¹) and both amendments; * statistically significant difference at 5% significance level. ** statistically significant difference at 1% significance level. *** statistically significant difference at 0.1% significance level.

Variant-Combination	DHA	C _{mic}	Basal Respiration	Glc-SIR	Tre-SIR	Glc-SIR	Ala-SIR	Lys-SIR	Fresh AGB	Dry AGB	Fresh Root	ARS	Phos	NAG	GLU	C _{tot}
control-Humac 1 t·ha ⁻¹	0.57	0.07	0.09	1	0.02 *	0.42	0.55	0.01 *	0.2	0.99	1	0.009 **	0.99	0.49	0.046 *	0.86
control-BC 100 t·ha ⁻¹	0.83	<0.001 ***	0.003 **	0.5	<0.001 ***	0.002 **	0.03 *	<0.001 ***	0.46	0.11	0.04 *	<0.001 ***	<0.001 ***	0.02 *	<0.001 ***	<0.001 ***
control-BC 100 t·ha ⁻¹ + Humac 1 t·ha ⁻¹	0.5	<0.001 ***	0.29	1	0.06	0.4	0.93	0.06	0.54	0.03 *	0.71	<0.001 ***	0.82	0.004 **	<0.001 ***	<0.001 ***
control-BC 40 t·ha ⁻¹	0.98	<0.001 ***	0.66	0.04 *	0.97	0.97	0.98	0.57	0.33	0.13	0.35	0.22	0.87	0.04 *	0.007 **	<0.001 ***
control-BC 40 t·ha ⁻¹ + Humac 1 t·ha ⁻¹	0.99	0.03 *	0.12	0.44	<0.001 ***	0.01 *	0.06	<0.001 ***	0.17	0.11	0.43	<0.001 ***	0.98	0.99	<0.001 ***	<0.001 ***
Humac 1 t·ha ⁻¹ -control	0.57	0.07	0.09	1	0.02 *	0.42	0.55	0.01 *	0.2	0.99	1	0.009 **	1	0.49	0.046 *	0.86
Humac 1 t·ha ⁻¹ -BC 40 t·ha ⁻¹	0.91	0.07	0.001 **	0.01 *	0.15	0.09	0.17	0.51	0.006 **	0.048 *	0.29	0.83	0.64	0.82	0.99	<0.001 ***
Humac 1 t·ha ⁻¹ -BC 100 t·ha ⁻¹	0.11	<0.001 ***	0.86	0.75	0.38	0.24	0.69	0.79	0.009 **	0.04 *	0.03 *	<0.001 ***	<0.001 ***	0.72	0.48	<0.001 ***
Humac 1 t·ha ⁻¹ -BC 40 t·ha ⁻¹ + Humac 1 t·ha ⁻¹	0.27	1	1	0.68	0.37	0.66	0.84	0.69	0.003 **	0.04 *	0.36	0.02 *	1	0.74	0.66	<0.001 ***
Humac 1 t·ha ⁻¹ -BC 100 t·ha ⁻¹ + Humac 1 t·ha ⁻¹	0.04 *	0.003 **	0.99	0.99	1	1	0.98	1	0.01 *	0.01 *	0.63	0.002 **	0.58	0.38	0.11	<0.001 ***
BC 100 t·ha ⁻¹ + Humac 1 t·ha ⁻¹ -control	0.5	<0.001 ***	0.29	1	0.06	0.4	0.93	0.06	0.54	0.03 *	0.71	<0.001 ***	0.82	0.004 **	<0.001 ***	<0.001 ***
BC 100 t·ha ⁻¹ + Humac 1 t·ha ⁻¹ -BC 40 t·ha ⁻¹	0.2	0.86	0.007 **	0.07	0.29	0.09	0.54	0.82	1	0.95	0.98	<0.001 ***	1	0.98	0.38	<0.001 ***
BC 100 t·ha ⁻¹ + Humac 1 t·ha ⁻¹ -BC 40 t·ha ⁻¹ + Humac 1 t·ha ⁻¹	0.83	0.008 **	1	0.3	0.2	0.68	0.41	0.38	0.95	0.98	1	0.96	0.39	0.01 *	0.9	<0.001 ***
BC 100 t·ha ⁻¹ + Humac 1 t·ha ⁻¹ -BC 100 t·ha ⁻¹	0.99	0.71	0.51	0.36	0.21	0.25	0.26	0.48	1	0.98	0.36	0.93	0.04 *	1	0.97	1

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