Impact of pyrochar and hydrochar on soybean (*Glycine max* L.) root nodulation and biological nitrogen fixation

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Abstract

The aim of this study was to identify effects of carbonized organic material ("biochar") on soybean growth, root nodulation and biological nitrogen fixation, and to elucidate possible underlying mechanisms.

Soybean (*Glycine max* L.) was grown in four arable soils amended with carbonized organic material produced from wood or maize as feedstocks, by pyrolysis ("pyrochar") or hydrothermal carbonization ("hydrochar"). Nodulation by *Bradyrhizobium*, biological nitrogen fixation (BNF) assessed by ^{15}N techniques, plant growth, nutrient uptake and changes in chemical soil properties after soil amendment were determined. Data were analyzed by means of a three way ANOVA on the factors soil, carbonization technique and feedstock. It turned out that soybean root nodulation and BNF was influenced by the carbonization technique used to prepare the soil amendment. Hydrochar, in average and across all soils, increased nodule dry matter and BNF by factors of 3.4 and 2.3, respectively, considerably more than pyrochar, which led to 1.8 and 1.2 fold increases, respectively. Nodule dry matter and BNF correlated positively with available soil sulfur and negatively with available soil nitrogen. Hydrochars provided more available sulfur than pyrochars, and hydrochars caused a decrease in nitrogen availability in the soil solution, thereby exerting a positive influence on nodulation and BNF. Pyrochar amendment increased soil pH but had no effect on nodulation and BNF. Plant growth was affected by the soil and by the feedstock used for the "biochar", and increased slightly more in treatments with pyrochar and hydrochar made from maize, which was richer in nitrogen and potassium.

The results show that carbonized organic materials, and specifically hydrochar, have the capacity to increase BNF in soils. We suggest that this enhancement in BNF in response to soil amendments with carbonized organic materials is due to an increase in available sulfur and a reduction of available soil nitrogen.

Key words: hydrochar / pyrochar / biochar / soybean / rhizobia / soil fertility / biological nitrogen fixation (BNF)

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1 Introduction

Soil application of carbonized organic material, commonly known as biochar (*Verheijen* et al., 2010), has recently attracted attention in research and among the public. The carbonization of organic material leads to a highly porous product with a skeleton of relatively stable carbon compounds. Carbonized organic material, which is enriched in plant nutrients in comparison to its feedstock (*DeLuca* et al., 2009), is proposed not only to ameliorate agricultural soils in various ways, but at the same time also to mitigate climate change by burying recalcitrant carbon compounds in soil (*Lehmann*, 2007; *Verheijen* et al., 2010).

However, even though many experiments adding carbonized organic material to soil have already been performed, the large physical and chemical variations, resulting from different carbonization conditions or feedstocks, make the understanding and prediction of effects on soil quality and crop growth difficult (*Verheijen* et al., 2010). Carbonized organic material is obtained by either pyrolysis of dry biomass leading to pyrochar, or hydrothermal carbonization (HTC) of wet biomass leading to hydrochar (*Libra* et al., 2011). Even if produced from the same feedstock, pyrochar and hydrochar are completely different in terms of fate and effects in soil. Pyrochar is highly recalcitrant in soil and soil properties are changed physically and chemically; in contrast hydrochar is degraded faster and changes in soil properties are possibly of shorter duration (*Lehmann* et al., 2011; *Schimmelpfennig* and *Glaser*, 2012; *Bamminger* et al., 2014). Besides the properties of the carbonized material, also soil physical, chemical

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and biological properties, the crop plant as well as site factor, such as climate, play key roles in modulating its effects in soil (*Biederman* and *Harpole*, 2013).

Many studies have observed enhanced yield of leguminous crops after hydrochar or pyrochar application (*Rondon* et al., 2007; *Ogawa* and *Okimori*, 2010; *Bargmann* et al., 2014; *Oram* et al., 2014; *van de Voorde* et al., 2014). Legumes are of particular interest because they are able to form a symbiosis—the root nodule symbiosis—with *Rhizobium* spp., which are able to fix atmospheric N. This symbiosis is of great agronomic and economic importance as legume crops are self-sustaining with respect to N and even help to reduce external N input for subsequent crops (*Salvagiotti* et al., 2008; *Kondorosi* et al., 2013).

However, only a few experiments addressed the influence of hydrochar or pyrochar on the nodule symbiosis of legumes with rhizobia, although positive effects of charcoal (pyrochar) on nodulation have already been described in the middle of the last century (*Vantsis* and *Bond*, 1950; *Nutman*, 1952; *Turner*, 1955). Especially (*Turner*, 1955) searched in detail for underlying mechanisms and concluded that stimulation of nodule formation in clover by charcoal (pyrochar) is best explained by adsorption of inhibitory plant exudates. Additionally he argued that pyrochar might also contain compounds that influence nodulation. Further explanations were proposed by more recent studies: (1) decreased N availability due to sorption to organic compounds or microbial immobilization combined with increased availability of other nutrients such as P, K, Ca, B, and Mo (*Rondon* et al., 2007; *Mia* et al., 2014; *Nguyen* et al., 2017), or (2) interference with nodulation signaling pathways (*Quilliam* et al., 2013). Root nodulation is negatively affected by low pH (*Taylor* et al., 1991; *Zahran*, 1999). As pyrochar has a liming effect, this could also explain the finding of a positive effect on nodulation (*Rondon* et al., 2007). The data presented by (*Ogawa* and *Okimori*, 2010), who applied bark pyrochar loaded with 1% of different fertilizers at two different application rates to soil, illustrate the complexity of possible interactions. Pyrochar alone had a negative effect on plant growth and nodulation was also significantly lower than in control treatments without pyrochar, but loaded with different fertilizers it led to significantly higher nodulation (*Ogawa* and *Okimori*, 2010). Increased nodulation of legumes has also been described after hydrochar addition, but without further analysis of possible mechanisms (*Bever* et al., 2010; *George* et al., 2012). *Bever* et al. (2010) suggested stimulation of plant–rhizobia signaling pathways by hydrochar as a possible reason, whereas *George* et al. (2012) attributed it to a combination of effects on physical structure, nutrient inputs, and sorption of toxic compounds. One of the most important staple food crops worldwide, the legume soybean (*Glycine max* L.), is colonized by the bacterium *Bradyrhizobium japonicum*. We used this legume to get more insight into the effects of pyrochar and hydrochar. In particular, the aim of our study was (1) to identify the effects of nutrient poor and nutrient rich pyrochar and hydrochar on soybean growth and soybean root nodulation by the symbiont *Bradyrhizobium japonicum* across four different soils and (2) to find patterns in soil chemical properties changed by pyrochar or hydrochar that assist in identifying possible mechanisms. In more detail, we expected that plant growth and root nodulation will primarily depend on initial soil properties and be positively influenced by the amended materials. We assumed that pyrochar and hydrochar will alter soil chemical properties differently but in a distinct pattern across all soils. We hypothesized that the more recalcitrant pyrochars influence plant growth and root nodulation rather by physicochemical changes, *e.g*., an increase in soil pH and adsorption of nutrients from soil solution, than by providing additional macro- and micronutrients. We expected, furthermore, that due to faster mineralization, nutrients contained in hydrochars will be more plant available and, thus, have a stronger effect compared to pyrochars. Finally we anticipated that carbonized organic material from feedstock maize will have more pronounced effects on plant growth and nodulation than from the feedstock wood.

2 Material and methods

2.1 Soils and carbonized materials

The top 10 cm of four arable soils (Tab. 1) from northwestern Switzerland were collected in late autumn 2011, sieved (< 5 mm) and stored at 4°C until usage. Two soils with contrasting soil organic matter contents were taken from the DOK farming systems trial, Therwil, Switzerland. These are the soils BIODYN and CONMIN, sampled from plots under biodynamic and conventional management, respectively (*Mäder* et al., 2002). The two other soils, *i.e*., the acidic Caron soil and the alkaline ToMa soil, were selected to introduce a broad range of soil pH into the study.

The four types of carbonized materials used in this study resulted from the combination of two feedstocks (maize and wood) with the two types of production methods: pyrolysis and hydrothermal carbonization (Fig. 1). Pyrowood and Hydrowood were produced from sieved residues of wood chips from a mixed forest of deciduous and coniferous. Pyromaize and Hydromaize were produced from ^{15}N labelled maize litter (leaves and stems) provided by the Institute of Soil Ecology, Helmholtz Zentrum München, Germany. The Pyrowood was produced using an industrial pyrolysis facility (Pyreg, Dörth,

Table 1: Acidity, soil organic carbon, and clay content of the four soils used in pot studies with soybean and wheat. Geographical coordinates of the sites are given (latitude/longitude). SOC = soil organic carbon.

Soil	pH_{CaCl2}	SOC (%)	Clay	Coordinates (WGS)
Caron	4.65	1.95	20%	47.335 / 7.093
CONMIN[ab]	5.62	1.07	16%	47.503 / 7.539
BIODYN[ac]	5.92	1.22	15%	47.503 / 7.539
ToMa	7.92	1.89	28%	47.488 / 7.545

[a]From the same site;
[b]under conventional farming;
[c]under organic farming.

Germany) by Franz Keiser, Neuheim, Switzerland. The Pyromaize was produced using a similar apparatus at the UK-Biochar Research Center in Edinburgh, UK (*Brownsort* and *Mašek*, 2011). The two hydrochars were produced by the group of Prof. Bottlinger in the HTC laboratory at Umwelt-Campus Birkenfeld (Hochschule Trier), Birkenfeld, Germany. The four carbonized organic materials were sieved to < 5 mm and stored at 4°C until use (Tab. 2).

2.2 Pot experiment

A pot experiment with four different agricultural soils was performed. Each of them was amended with four different carbonized organic materials. Batches of all 16 soil and biochar combinations were produced by mixing soil with carbonized organic material at a ratio of 0.7% (w/w). This ratio corresponded to an application rate of approx. 20 t ha^{-1}. Four replicates of each mixture were filled into 3-L pots and incubated for 9 weeks prior to planting in a climate chamber at 25°C/22°C (14 h day/10 h night) and at 60% of maximum soil water holding capacity. In addition, four replicate pots were established for each soil without any amendment or fertilization as control treatments. After the incubation, the soils were sieved (10 mm), mixed, and aliquots for nutrient availability measurements were prepared and stored at –20°C. The soils were refilled to the same pots. 100 g topsoil was inoculated with *Bradyrhizobium japonicum* (HiStick©, Becker Underwood, USA) at 5×10^5 cells g^{-1} soil. Seven pre-germinated soybean seedlings (*Glycine max* L.) of the variety Aveline (Delley Samen und Pflanzen AG, Delley, Switzerland) were transplanted to each pot and thinned out to five plants after two days. Soybean was grown under same climatic conditions as during soil incubation with a photon flux density (PFD) of 600 µmol m^{-2} s^{-1}. After a growing period of eight weeks, whole plants were harvested, soil was sieved (10 mm), and aliquots prepared. The remaining soil was refilled in pots, and spring wheat, variety Fiorina (Delley Samen und Pflanzen AG, Delley, Switzerland), was grown under the exact same conditions as soybean and served as a control plant for biological N fixation (BNF) estimation. Soils were not sterilized to keep the native microflora.

The ^{15}N content of the soybean plants was considered to be a result of the respective ^{15}N contents in the compartments

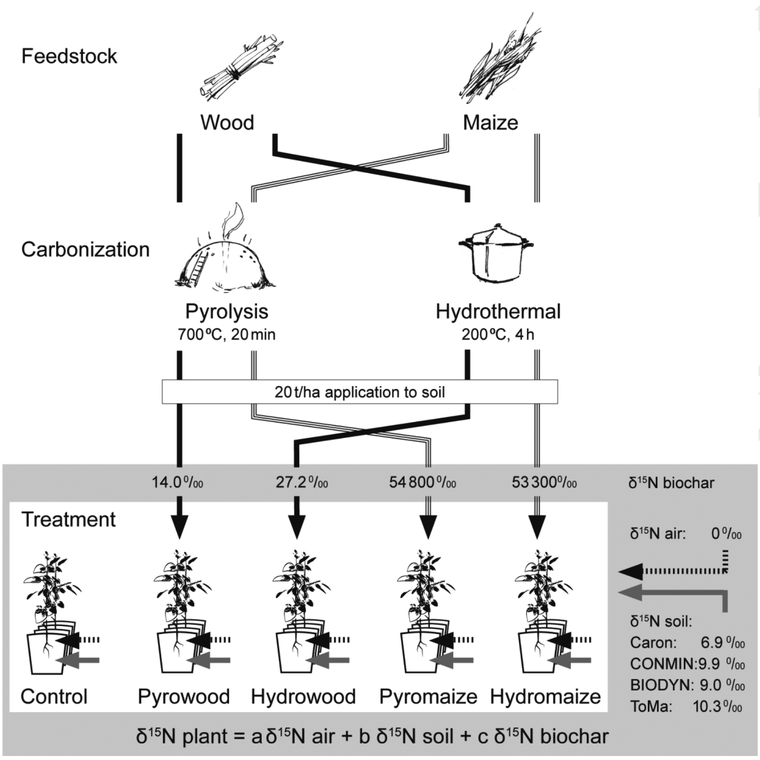

Figure 1: Carbonized materials were produced by processing the feedstocks, wood chips residue (Wood), and maize straw (Maize) by pyrolysis or hydrothermally, resulting in four biochars: Pyrowood, Hydrowood, Pyromaize, and Hydromaize. Five treatments, one control and one of each biochar at an application rate of 20 t ha^{-1} (0.7%) (w/w), at four replicates of each soil, (Caron, CONMIN, BIODYN, and ToMa) were prepared in pots. Soybean was grown for eight weeks in a climate chamber. The ^{15}N content of the plants at harvest is a linear combination of the ^{15}N content in air, soil and biochar: $\delta^{15}N$ Plant = a $\delta^{15}N$ Air +b $\delta^{15}N$ Soil + c $\delta^{15}N$ Biochar.

Table 2: Process conditions and chemical properties of the four carbonized materials amended to four soils described in Tab. 1.

	Temperature	Time	Pressure	Feedstock humidity	pH	C (mg g^{-1})	H (mg g^{-1})	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})	S (mg g^{-1})	$\delta^{15}N$ (‰)
Pyrowood	695°C	20 min	–	max 40%	12.15	679	13.2	6.6	1.25	7.39	0.25	14.0
Pyromaize	700°C	20 min	–	max 10%	9.47	631	16.8	10.4	2.69	65.54	1.51	54800
Hydrowood	200°C	4 hours	≈ 20 bar	water bath	4.75	544	54.6	6.5	0.34	1.02	2.50	27.2
Hydromaize	200°C	4 hours	≈ 20 bar	water bath	4.17	559	56.3	14.9	0.73	9.42	3.98	53300

air, soil and carbonated organic materials (Fig. 1). Wheat served as a non-leguminous control plant, grown after soybean. N mineralization from soil organic matter (SOM) in case of soybean, and both from SOM and soybean residues in the case of wheat dilute the ^{15}N values and, thus, the calculated BNF. Nitrogen from SOM to the dilution of biochar N must have been the same or slightly higher in the control plants. Neglecting the dilution by mineralization of SOM leads thus to very conservative BNF estimations.

2.3 Sample analysis

Samples of the soil-amendment mixtures were taken from all pots directly after soybean harvest and analyzed for pH in a suspension with 0.01 M $CaCl_2$ (1:2.5, w/w). The suspension was shaken and left over night at room temperature. Available nutrients (P, S, K, Fe, and Mn) were extracted according to the reference method FM-AAE10-ICP with ammonium acetate + EDTA (1:10), and measured with ICP-OES (Vista-MPX, Varian, USA) from soil sampled immediately before planting soybean (*Reckenholz*, 1999). Mineral N (N_{min} = NO_2^-, NO_3^-, NH_4^+) was measured with a SAN-plus Segmented Flow Analyzer (Skalar Analytical B.V., Breda, Netherlands). Nodules were picked off the roots, counted, dried (40°C / 48 h with ventilation), and nodule dry matter (DM) was determined. Soybean total biomass, root DM and shoot DM were determined using the same procedure. Plant samples were ground using a Retsch ZM200 titanium mill. Ground material (4 g) was mixed with 0.9 g of wax and pressed into tablets. The total element concentrations of P, K, and S of the tablets were determined using a Spectro X-lab 2000 X-Ray Fluorescence (XRF) spectrometer.

For total N measurements, about 2 mg of ground plant samples were filled into tin capsules and weighed. N content was determined with an elemental analyzer (Euro EA, Eurovector, Italy) coupled to an isotope ratio mass spectrometer (delta V Advantage, Thermo Fisher, Germany).

For general description of the isotopic ratios in plants and biochars we used the $\delta^{15}N$ notation, where the ^{15}N content in the samples is given as relative deviation from the natural occurrence of ^{15}N in the air [$^{15}N_{air}$ = 0.36647 atom% (*Werner* and *Brand*, 2001)],

$$\delta^{15}N\ (per\ mille) = \frac{atom\%^{15}N_{sample} - atom\%^{15}N_{air}}{atom\%^{15}N_{air}} \times 1000 \qquad (1)$$

Isotopic ratios of N in plants were used to estimate N fixation (Ndfa: N derived from atmosphere). For the control treatments with non-labelled amendments from wooden feedstock we used the natural abundance method (*Unkovich* et al., 2008):

$$\%\ Ndfa = \frac{\delta^{15}N_{wheat} - \delta^{15}N_{soybean}}{\delta^{15}N_{wheat} - B} \times 100. \qquad (2)$$

The *B* value adjusts for isotopic fractionation within the legume and was considered as –1.83‰ as described in (*Unkovich* et al., 2008) as a mean for soybean. This is likely to cause slight underestimation of BNF, as higher B-values (–0.88‰ and – 1.17‰) were measured by *Oberson* et al. (2007) while working with the same soils originating from the DOK system comparison experiment, but with a different soybean variety. The same plant variety and the same *Bradyrhizobium* inoculum were used throughout the whole study, which reduces the importance of the B-value.

The *% Ndfa* of labelled treatments was derived using the dilution method based on *atom% excess* (APE) in ^{15}N. APE is calculated by subtracting the ^{15}N content of the air from the measured ^{15}N content in the plant (*Unkovich* et al., 2008):

$$\%\ Ndfa = \left(1 - \frac{atom\%^{15}N\ excess_{soybean}}{atom\%^{15}N\ excess_{wheat}}\right) \times 100. \qquad (3)$$

The calculations using the dilution method are based on 100 times larger changes in ^{15}N contents than the results of the natural abundance method in our experiment, whereas standard deviations were only increased by a factor of 10. Therefore, the dilution method is considered to be more robust for monitoring N fluxes than the natural abundance method (*Unkovich* et al., 2008).

2.4 Statistical analysis

The experiment was designed as a randomized complete block system. The factors soil (Caron, CONMIN, BIODYN or ToMa), carbonization method (pyrolysis or hydrothermal carbonization), and feedstock (wood or maize), as well as their interactions in relation to not amended soil (control) were analyzed using three-way analysis of variance (ANOVA) followed by a post-hoc Tukey HSD test. If not stated otherwise, reported differences in the text were always significant at $p < 0.05$ level. Residuals were checked for normal distribution and data were transformed where necessary. Relationships between parameters were analyzed using Pearson's correlation coefficients and linear regression models over all treatments in each soil alone and across all soils. All statistical calculations were performed using the software package R (Version 3.1.2) in RStudio (Version 0.98.953).

3 Results

3.1 Effects of carbonized materials on soil properties

The pyrochars generally increased soil pH and available K and P, while the hydrochars increased available soil S and decreased mineral soil N (N_{min}) (Tab. 3). The effects were in general weaker for the amendments made from wood feedstock (Pyrowood and Hydrowood) than those from maize feedstock (Pyromaize and Hydromaize).

The soil pH_{CaCl2} was in average increased by 0.4 units by the two pyrochars, whereas the hydrochars had no or only very slight pH effects depending on the soil. The pyrochar amendments led to changes in P, K, and Zn availabilities in some soils: P availability was increased by both pyrochars, except in Caron soil, K availability was strongly increased in Py-

Table 3: Soil properties and nutrient availability as influenced by biochar amendments in four different soils measured before planting. Soils without charred amendments served as control treatment. ANOVA with the post-hoc analysis Tukey HSD was performed ($p < 0.05$), significant differences between treatments are indicated by different smaller case letters ($p < 0.05$). ± standard error between four replicates is displayed.

Soil	Treatment	pH ($CaCl_2$)	N_{tot}[a] (mg g^{-1})	N_{min}[b] (μg g^{-1})	P[c] (μg g^{-1})	K[c] (μg g^{-1})	S[c] (μg g^{-1})	Fe[c] (μg g^{-1})	Mn[c] (μg g^{-1})
Caron	Control	4.73 ± 0.03 c	2.80 ± 0.02 a	51.6 ± 2.5 a	3.6 ± 0.1 a	73 ± 4 d	9.9 ± 0.1 c	196 ± 11 a	243 ± 14 a
	Pyrowood	5.00 ± 0.11 ab	2.79 ± 0.04 a	58.5 ± 13.0 a	3.7 ± 0.3 a	120 ± 1 c	12.6 ± 1.1 c	206 ± 9 a	269 ± 6 a
	Pyromaize	5.19 ± 0.03 a	2.83 ± 0.06 a	41.2 ± 6.9 ab	4.4 ± 0.2 a	638 ± 20 a	14.4 ± 0.4 c	210 ± 10 a	269 ± 17 a
	Hydrowood	4.91 ± 0.16 bc	2.84 ± 0.03 a	12.5 ± 2.4 bc	4.0 ± 0.3 a	73 ± 2 d	25.7 ± 1.6 b	198 ± 8 a	262 ± 14 a
	Hydromaize	4.79 ± 0.02 c	2.87 ± 0.01 a	6.2 ± 1.0 c	4.1 ± 0.2 a	170 ± 6 b	38.8 ± 0.7 a	216 ± 2 a	275 ± 13 a
CONMIN	Control	5.76 ± 0.04 c	1.51 ± 0.02 b	45.3 ± 4.3 a	17.6 ± 0.2 b	86 ± 3 b	17.9 ± 2.7 b	170 ± 8 a	202 ± 10 a
	Pyrowood	6.45 ± 0.06 a	1.57 ± 0.02 ab	39.1 ± 0.7 a	21.2 ± 0.3 a	109 ± 4 b	14.6 ± 1.3 b	165 ± 7 a	199 ± 8 a
	Pyromaize	6.52 ± 0.03 a	1.63 ± 0.03 a	47.1 ± 5.7 a	24.2 ± 0.4 a	416 ± 50 a	21.7 ± 5.3 b	184 ± 5 a	218 ± 5 a
	Hydrowood	5.93 ± 0.07 b	1.55 ± 0.03 ab	12.7 ± 2.7 b	16.5 ± 0.9 b	93 ± 3 b	33.5 ± 2.6 b	168 ± 3 a	211 ± 4 a
	Hydromaize	5.88 ± 0.06 bc	1.62 ± 0.02 a	5.7 ± 0.7 b	17.3 ± 1.0 b	133 ± 5 b	52.9 ± 6.3 a	171 ± 6 a	214 ± 5 a
BIODYN	Control	5.92 ± 0.08 c	1.80 ± 0.02 b	60.9 ± 3.9 ab	6.3 ± 0.8 b	83 ± 4 c	19.5 ± 1.9 b	184 ± 3 a	253 ± 2 a
	Pyrowood	6.59 ± 0.05 a	1.83 ± 0.02 ab	51.5 ± 5.0 abc	11.0 ± 0.3 a	109 ± 4 bc	26.9 ± 3.9 b	177 ± 4 a	248 ± 12 a
	Pyromaize	6.62 ± 0.01 a	1.87 ± 0.07 ab	77.5 ± 17.7 a	10.5 ± 0.7 a	377 ± 12 a	28.4 ± 1.4 b	174 ± 9 a	232 ± 11 a
	Hydrowood	6.02 ± 0.03 b	1.87 ± 0.02 ab	25.3 ± 6.5 bc	6.7 ± 0.3 b	83 ± 2 c	45.2 ± 3.3 a	182 ± 4 a	255 ± 3 a
	Hydromaize	5.94 ± 0.02 bc	1.98 ± 0.02 a	17.0 ± 4.8 c	7.6 ± 0.4 b	132 ± 3 b	46.8 ± 2.1 a	188 ± 7 a	252 ± 5 a
ToMa	Control	7.32 ± 0.01 b	2.75 ± 0.01 b	41.7 ± 5.2 a	12.3 ± 0.3 b	207 ± 9 b	19.7 ± 2.2 b	180 ± 8 a	484 ± 19 a
	Pyrowood	7.38 ± 0.02 a	2.73 ± 0.05 b	45.5 ± 7.6 a	13.0 ± 0.5 b	237 ± 3 b	19.5 ± 1.4 b	183 ± 3 a	491 ± 8 a
	Pyromaize	7.41 ± 0.01 a	2.84 ± 0.01 ab	43.4 ± 2.4 a	15.9 ± 0.7 a	496 ± 70 a	28.2 ± 3.7 b	190 ± 5 a	492 ± 12 a
	Hydrowood	7.30 ± 0.01 bc	2.73 ± 0.03 b	11.6 ± 4.0 b	12.5 ± 0.6 b	285 ± 74 b	36.2 ± 6.2 b	184 ± 4 a	489 ± 6 a
	Hydromaize	7.28 ± 0.01 c	2.89 ± 0.02 a	9.2 ± 1.9 b	11.5 ± 0.4 b	266 ± 3 b	58.4 ± 4.3 a	185 ± 6 a	478 ± 21 a
All soils	Control	5.93 ± 0.95 d	2.21 ± 0.29 b	49.9 ± 5.2 a	9.9 ± 2.8 b	112 ± 29 c	17.7 ± 2.6 c	182 ± 9 a	296 ± 58 a
	Pyrowood	6.35 ± 0.89 b	2.23 ± 0.28 b	48.6 ± 8.0 a	12.3 ± 3.3 a	144 ± 28 bc	19.1 ± 3.6 c	183 ± 9 a	302 ± 58 a
	Pyromaize	6.43 ± 0.82 a	2.29 ± 0.29 a	52.3 ± 11.7 a	13.0 ± 3.7 a	482 ± 65 a	24.4 ± 4.1 c	190 ± 10 a	303 ± 58 a
	Hydrowood	6.04 ± 0.88 c	2.21 ± 0.28 b	15.5 ± 4.8 b	9.9 ± 2.6 b	134 ± 56 bc	35.2 ± 5.0 b	183 ± 7 a	304 ± 56 a
	Hydromaize	5.97 ± 0.91 d	2.34 ± 0.29 a	9.5 ± 3.3 b	10.1 ± 2.6 b	175 ± 28 b	49.3 ± 5.2 a	190 ± 10 a	305 ± 54 a

[a]N_{total} = Total nitrogen measured with a mass spectrometer.
[b]N_{min} = Total mineral nitrogen (NO_3^- + NO_2^-)-N + NH_4^+-N.
[c]Available elements: extracted from soil with ammonium acetate + EDTA (1:10).

romaize amended soil, and Zn availability in Pyrowood amended soil.

Remarkably, S availability was increased in all soils by 179% in mean with Hydromaize. An increase was also observed with Hydrowood in Caron (159%) and BIODYN (132%) soils. Hydrowood and Hydromaize additionally decreased N availability in soil (N_{min}). In contrast, Pyrowood amendments did not lead to changes in N_{min}. Total soil N (N_{tot}) was increased by Pyromaize and Hydromaize across all soils. However, when the analysis was performed for each soil alone, a significant increase was only found for Pyromaize and Hydromaize addition to CONMIN soil and Hydromaize application to BIODYN soil. In the ToMa alkaline soil and the Caron acidic soil no significant changes in total N were observed.

3.2 Plant growth

Soybean plant dry matter (DM), shoot and root DM depended on soil used as potting ground (Tab. 4). In contrast, the different amendments had little effect on growth. While amendments produced from wood feedstock did not affect soybean DM, Pyromaize and Hydromaize treatments increased soybean biomass.

Table 4: Nodule dry matter (DM), nodule number, nitrogen derived from atmosphere (Ndfa), and soybean plant DM (shoot and root) per pot as influenced by amendments in four different soils. Soils without amendment addition served as control treatment. Significant differences of the least square means between treatments in respective soil are indicated by smaller case letters ($p < 0.05$). ± standard error between four replicates is displayed.

Soil	Treatment	Nodule DM (mg)		Nodule number (#)		Ndfa (%)		Plant DM (g)	
Caron	Control[a]	23 ± 3	d	51 ± 5	c	19 ± 3	b	11.4 ± 0.2	b
	Pyrowood[a]	49 ± 13	cd	107 ± 15	b	21 ± 5	b	11.4 ± 0.3	b
	Pyromaize[b]	67 ± 4	c	111 ± 5	b	nd[c]		13.3 ± 0.3	a
	Hydrowood[a]	109 ± 11	b	137 ± 19	b	nd		11.1 ± 0.3	b
	Hydromaize[b]	177 ± 9	a	192 ± 5	a	60 ± 1	a	12.9 ± 0.5	a
CONMIN	Control[a]	262 ± 33	b	73 ± 10	c	25 ± 3	c	18.4 ± 0.5	a
	Pyrowood[a]	355 ± 10	b	83 ± 7	bc	41 ± 3	b	19.3 ± 0.3	a
	Pyromaize[b]	411 ± 43	b	109 ± 22	bc	nd		19.6 ± 0.8	a
	Hydrowood[a]	670 ± 29	a	135 ± 11	ab	47 ± 2	b	17.9 ± 0.4	a
	Hydromaize[b]	669 ± 46	a	174 ± 13	a	76 ± 2	a	20.1 ± 0.7	a
BIODYN	Control[a]	133 ± 15	b	58 ± 9	b	17 ± 2	b	19.1 ± 0.3	b
	Pyrowood[a]	179 ± 47	b	79 ± 10	ab	36 ± 9	b	18.4 ± 0.3	b
	Pyromaize[b]	294 ± 12	ab	98 ± 8	ab	nd		21.5 ± 0.3	a
	Hydrowood[a]	394 ± 61	a	123 ± 19	a	25 ± 6	b	18.7 ± 0.7	b
	Hydromaize[b]	419 ± 61	a	114 ± 5	a	65 ± 2	a	21.4 ± 0.5	a
ToMa	Control[a]	183 ± 19	b	153 ± 20	b	42 ± 1	c	15.1 ± 0.3	a
	Pyrowood[a]	243 ± 24	ab	174 ± 32	ab	49 ± 3	c	14.7 ± 0.3	a
	Pyromaize[b]	265 ± 11	ab	199 ± 21	ab	nd		15.1 ± 0.6	a
	Hydrowood[a]	323 ± 35	ab	224 ± 21	ab	66 ± 3	b	14.5 ± 0.4	a
	Hydromaize[b]	366 ± 68	a	334 ± 68	a	86 ± 1	a	13.7 ± 0.6	a
All soils	Control[a]	143 ± 47	c	84 ± 24	d	26 ± 3	d	16.0 ± 1.6	b
	Pyrowood[a]	206 ± 62	bc	111 ± 26	cd	37 ± 5	c	15.9 ± 1.7	b
	Pyromaize[b]	259 ± 67	b	129 ± 25	bc	nd		17.4 ± 1.8	a
	Hydrowood[a]	374 ± 109	a	155 ± 26	b	44 ± 9	b	15.5 ± 1.6	b
	Hydromaize[b]	408 ± 102	a	203 ± 52	a	72 ± 3	a	17.0 ± 2.0	a

[a]Ndfa was calculated by the natural abundance method.
[b]Ndfa was calculated by the dilution method.
[c]nd = not determinable.

All factors, *i.e.*, soil, carbonization method and feedstock, had a significant influence on plant nutrition (N, P, K, S). Addition of pyrochars and hydrochars generally increased plant nutrient concentration and content in comparison to the respective control (Tab. 5). Correlations between available nutrient concentration in soil and uptake into shoots were significant for P in CONMIN, K in all soils, and S in soil Caron. Shoot N concentrations did not correlate with available soil N (N_{min}) (Tab. 6), but both total soil N (N_{tot}) and N in soybean shoots were highest in the treatments with carbonized materials from the N-rich feedstock maize (Tab. 5).

3.3 Rhizobial symbiosis

3.3.1 Nodulation

Visual observation of soybean roots indicated a positive influence of all carbonized materials on nodule formation (Fig. 3). Nodule counts were higher in Pyromaize, Hydrowood, and Hydromaize in respect to control treatments (Tab. 4). The increase was far more pronounced in hydrochar amendments. The addition of hydrochars increased nodule DM (Fig. 2, Tab. 4), whereby Nodule DM per unit of plant biomass presented the same picture. Pyrochar and hydrochar produced from wooden feedstock had less effect on nodule DM than those produced from maize. Notably, nodule DM was correlated positively with S and negatively with N_{min} in soil, the only two parameters showing coherent correlations in all soils (Tab. 7, Fig. 4).

3.3.2 Estimates of nitrogen derived from atmosphere (Ndfa)

All factors (soil, carbonization, and feedstock) had a significant effect on Ndfa. Hydrowood increased Ndfa in soybean from 25% to 47% in the CONMIN soil and from 42% to 66% in the alkaline ToMa soil. In BIODYN, Hydrowood showed only a tendency to increase BNF (from 17% to 25% Ndfa) (Tab. 4). In the acid Caron soil the% Ndfa could not be determined for Hydrowood, delta values give a non-reasonable negative result, indicating inactive nodules or a lack of N transfer to the soybean shoots. Calculated across all soils Pyrowood improved BNF slightly (Tab. 4).

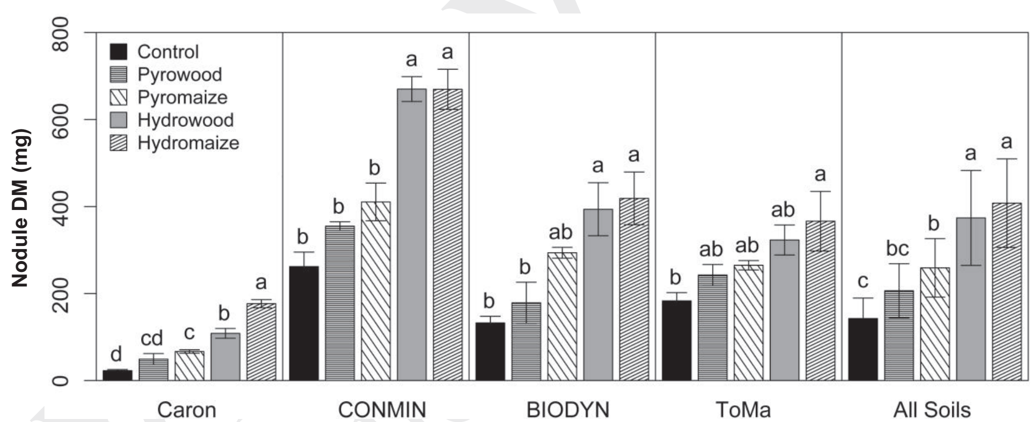

Figure 2: Nodule dry matter (DM) per pot as influenced by biochar amendments in four different soils and the mean over all soils. Soils without biochar addition served as control treatment. Significant differences of the mean are indicated by different capital letters ($p < 0.0001$). Error bars represent standard error of four replicates.

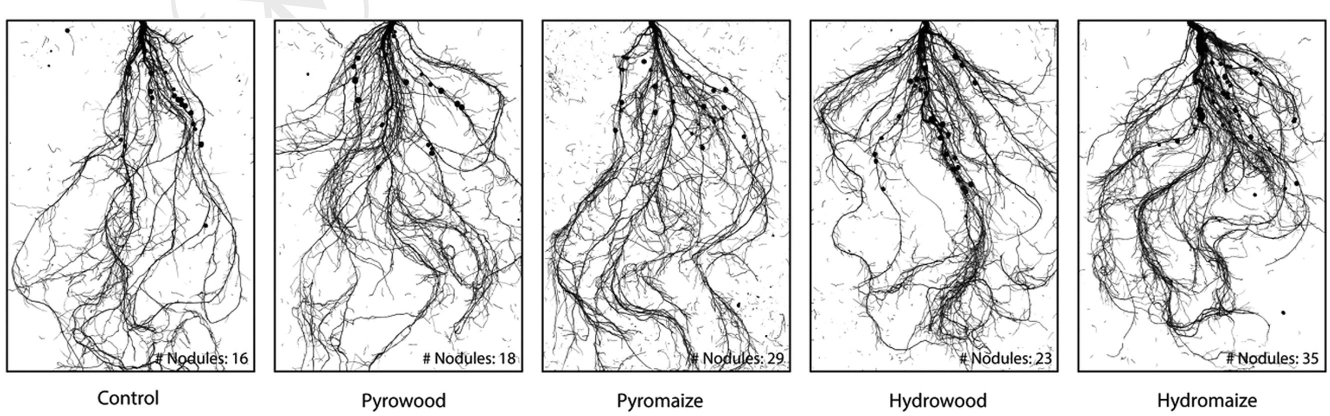

Figure 3: Soybean root scans of a typical soybean plant of the treatments control, Pyrowood, Pyromaize, Hydrowood, and Hydromaize in the CONMIN soil. Soybean root nodules, roundly shaped and of dark color, are visible. Their actual number is indicated bottom right of each root scan.

Table 5: Soybean shoot nutrient concentrations and contents for nitrogen, phosphorus, potassium, and sulfur (N, P, K, S) as influenced by the charred amendments applied to the four experimental soils. Soils without amendment served as control treatment. Values with the same letter for a given soil are not significantly different ($p < 0.05$). ± standard error between four replicates is displayed.

Soil	Treatment	Nutrient concentration in shoot								Nutrient content per shoot							
		N ($mg\ g^{-1}$)		P ($mg\ g^{-1}$)		K ($mg\ g^{-1}$)		S ($mg\ g^{-1}$)		N (mg)		P (mg)		K (mg)		S (mg)	
Caron	Control	26.1 ± 0.4	ab	0.9 ± 0.01	c	5.5 ± 0.2	c	1.2 ± 0.02	b	200 ± 5	bc	10.2 ± 0.2	b	62 ± 1	b	13.9 ± 0.1	b
	Pyrowood	24.8 ± 0.3	b	1.0 ± 0.03	c	8.4 ± 0.3	b	1.2 ± 0.02	b	188 ± 7	bc	11.1 ± 0.1	b	95 ± 1	b	13.9 ± 0.3	b
	Pyromaize	25.9 ± 0.3	ab	1.3 ± 0.1	ab	13.4 ± 0.3	a	1.4 ± 0.1	ab	241 ± 8	ab	17.1 ± 1.0	a	178 ± 7	a	19.3 ± 1.0	a
	Hydrowood	23.1 ± 0.6	b	1.1 ± 0.1	bc	6.0 ± 0.2	c	1.2 ± 0.1	b	170 ± 7	c	12.1 ± 0.5	b	66 ± 1	b	13.8 ± 0.6	b
	Hydromaize	28.9 ± 1.9	a	1.5 ± 0.1	a	13.9 ± 1.0	a	1.7 ± 0.1	a	265 ± 29	a	19.7 ± 2.0	a	181 ± 20	a	21.8 ± 2.4	a
CONMIN	Control	26.0 ± 1.0	c	1.6 ± 0.1	b	7.1 ± 0.2	d	1.2 ± 0.04	c	321 ± 3	c	29.9 ± 1.1	b	130 ± 3	c	21.9 ± 0.6	c
	Pyrowood	32.5 ± 1.4	ab	2.0 ± 0.2	ab	10.4 ± 0.4	c	1.3 ± 0.1	bc	402 ± 9	ab	37.5 ± 2.8	ab	200 ± 4	b	25.3 ± 1.2	bc
	Pyromaize	34.2 ± 1.4	a	2.2 ± 0.1	a	15.8 ± 0.3	a	1.7 ± 0.1	a	437 ± 34	a	43.1 ± 2.8	a	311 ± 20	a	33.9 ± 2.8	a
	Hydrowood	28.3 ± 1.2	bc	1.8 ± 0.1	b	7.6 ± 0.2	d	1.5 ± 0.1	abc	323 ± 15	bc	31.5 ± 1.4	b	137 ± 5	c	26.1 ± 1.1	bc
	Hydromaize	24.6 ± 0.3	c	1.8 ± 0.0	ab	12.1 ± 0.3	b	1.5 ± 0.1	ab	330 ± 13	bc	36.4 ± 1.2	ab	242 ± 6	b	30.8 ± 1.4	a-b
BIODYN	Control	26.0 ± 0.5	c	1.4 ± 0.0	b	6.3 ± 0.1	b	1.3 ± 0.02	c	346 ± 4	b	26.5 ± 0.4	c	120 ± 1	b	25.0 ± 0.4	b
	Pyrowood	30.6 ± 1.1	abc	1.7 ± 0.1	ab	9.1 ± 0.2	b	1.5 ± 0.04	abc	385 ± 21	b	30.9 ± 1.0	bc	167 ± 5	b	28.1 ± 0.8	b
	Pyromaize	35.2 ± 1.7	a	2.3 ± 0.3	a	16.4 ± 1.6	a	2.0 ± 0.2	ab	523 ± 22	a	48.7 ± 4.9	a	351 ± 31	a	42.4 ± 4.4	a
	Hydrowood	27.4 ± 1.0	bc	1.4 ± 0.04	b	6.9 ± 0.2	b	1.4 ± 0.04	bc	330 ± 9	b	26.0 ± 0.9	c	127 ± 2	b	26.6 ± 0.6	b
	Hydromaize	31.2 ± 1.2	ab	2.0 ± 0.2	ab	14.5 ± 1.5	a	2.1 ± 0.2	a	467 ± 26	a	42.8 ± 5.1	ab	311 ± 37	a	45.0 ± 5.4	a
ToMa	Control	29.5 ± 0.5	b	1.6 ± 0.04	a	10.4 ± 0.2	b	1.6 ± 0.04	a	244 ± 9	a	24.8 ± 0.8	a	157 ± 3	b	23.9 ± 0.6	a
	Pyrowood	31.3 ± 0.3	ab	2.0 ± 0.05	a	12.8 ± 0.1	ab	1.7 ± 0.03	a	241 ± 4	a	28.7 ± 1.0	a	188 ± 5	ab	25.1 ± 0.5	a
	Pyromaize	32.0 ± 1.5	ab	2.0 ± 0.1	a	15.5 ± 0.6	a	1.8 ± 0.1	a	263 ± 22	a	30.3 ± 2.7	a	235 ± 14	a	27.2 ± 2.8	a
	Hydrowood	31.2 ± 1.4	ab	1.9 ± 0.2	a	13.0 ± 1.1	ab	1.7 ± 0.1	a	221 ± 10	a	26.9 ± 3.1	a	189 ± 19	ab	25.3 ± 1.9	a
	Hydromaize	35.4 ± 0.3	a	1.9 ± 0.1	a	14.9 ± 0.9	a	1.8 ± 0.1	a	264 ± 11	a	26.1 ± 2.3	a	205 ± 19	ab	25.5 ± 2.7	a
All soils	Control	26.9 ± 1.0	b	1.4 ± 0.2	d	7.3 ± 1.0	d	1.3 ± 0.1	b	278 ± 31	cd	22.9 ± 3.9	c	117 ± 18	d	21.2 ± 2.3	b
	Pyrowood	29.7 ± 1.8	a	1.6 ± 0.2	bc	10.2 ± 0.9	c	1.4 ± 0.1	b	308 ± 50	bc	27.1 ± 5.2	bc	163 ± 21	c	23.1 ± 2.9	b
	Pyromaize	31.8 ± 2.2	a	1.9 ± 0.2	a	15.3 ± 1.0	a	1.7 ± 0.2	a	366 ± 65	a	34.8 ± 6.9	a	269 ± 39	a	30.7 ± 5.2	a
	Hydrowood	27.5 ± 1.8	b	1.5 ± 0.2	cd	8.4 ± 1.5	d	1.5 ± 0.1	b	261 ± 36	d	24.1 ± 4.1	c	130 ± 24	d	22.9 ± 2.9	b
	Hydromaize	30.0 ± 2.3	a	1.8 ± 0.1	ab	13.8 ± 1.1	b	1.8 ± 0.2	a	331 ± 47	b	31.3 ± 5.4	ab	235 ± 33	b	30.8 ± 5.4	a

Table 6: Correlations of nutrient contents of soybean plants (N, P, K, and S) with the corresponding nutrient available in soil, indicated by the direction (+/–) and the coefficient of determination *R*2. The cells are tinged according the correlation strength from 0 (white) to 1 (grey).[a]

Soil		N			P			K			S	
Caron	(–)	0.04	ns	(+)	0.10	ns	(+)	0.24	*	(+)	0.47	**
CONMIN	(+)	0.17	ns	(+)	0.56	***	(+)	0.32	**	(–)	0.00	ns
BIODYN	(+)	0.02	ns	(+)	0.19	ns	(+)	0.55	***	(+)	0.00	ns
ToMa	(+)	0.01	ns	(+)	0.16	ns	(+)	0.40	**	(–)	0.08	ns
All soils	(+)	0.04	ns	(+)	0.40	***	(+)	0.03	ns	(+)	0.03	ns

[a]ns = not significant; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

^{15}N content of wheat plants grown on Hydromaize was much higher than that of soybean. This can be attributed to the dilution by N derived from BNF. The ^{15}N dilution effect observed in the soybean plants of the Hydromaize treatment was equivalent to an average Ndfa of 72% (non-amended soil: 26%). Ndfa calculations were not feasible for soybean growing in soils amended with Pyromaize. The ^{15}N content in wheat and in soybean were similar.

There was a positive correlation between nodule DM and Ndfa in all soils, suggesting that nodules were active and able to fix atmospheric N (Tab. 7, Fig. 4). One of the most remarkable findings was that nodule dry matter and Ndfa showed a positive correlation with available S and a negative correlation with N_{min} in all soils. This indicates that alterations in these elements due to addition of carbonized materials give a clue on potential mechanisms governing increased biological N fixation. In addition, there was a strong positive correlation between Ndfa and available soil K (Tab. 8).

4 Discussion

4.1 Plant growth

In general, the pyrochar and hydrochar amendments did not lead to the expected increase in plant growth. While a clear effect on nodulation and N fixation was observed, plant growth was only slightly improved by pyrochar and hydrochar from maize feedstock. The following factors were identified to limit, most likely, plant growth under our experimental conditions: pH, P in acid and nutrient poor soil Caron, P in both DOK soils relative low in this element, and pH in alkaline soil ToMa (*Sinaj* et al., 2009). Depending on amendment, these growth-limiting factors were slightly worsened or improved by addition of carbonized organic materials. The pyrochar and hydrochar from maize with a higher nutrient content (N, P, K) than those from wood, led in general to improved soybean growth, whereas the other carbonized materials did not. Specifically Pyromaize led to a high increase of available K in all soils.

Increased biological N fixation in soils amended with hydrochar did not translate into enhanced plant N accumulation. Even though increased N contents in plants grown in Hydromaize amended soil along with increased nodule dry matter and plant biomass were observed, one would expect a stronger influence of the pronounced enhancement of BNF on plant N content and biomass. Limited plant growth in hydrochar treatments was already observed in earlier studies. *Bargmann* et al. (2014) recorded a light-green color of a legume growing in hydrochar along with increased biomass and N content. This is similar to *George* et al. (2012) who stated that phytotoxic components (organic acids, phenolic and fatty compounds) released by hydrochar may have harmed the plant. Previous studies on hydrochar soil application reported negative effects of hydrochar on germination and plant growth immediately after application (*Rillig* et al., 2010). We tried to avoid this in our experiment by applying the amendments two months prior to planting and indeed, no negative effects on seedlings were observed.

4.2 Nodulation and biological nitrogen fixation (BNF)

Both hydrochars induced an increase in nodule dry matter and number, whereas this effect was less pronounced in pyrochar treatments. The remarkable increase in nodule dry matter by a factor of up to 7.8, as observed in the acidic soil Caron after Hydromaize amendment, can be considered as soil specific, as for the other soils a factor of 2 to 5 were measured, which is in line with previous studies (*Ogawa* and *Okimori*, 2010; *George* et al., 2012).

Nodule dry matter was in general positively correlated with N derived from atmosphere (Ndfa), which indicates that nodules mature and function. Although in Pyromaize treatments root nodulation was not significantly different to hydrochar treatments, BNF could not be determined. As all other treatments, including the control, were supporting that BNF has been taken place, one could expect an enhanced BNF in the Pyromaize treatments, too. It became obvious that the dilution method for estimating BNF by (*Unkovich* et al., 2008) could not be applied to Pyromaize treatments. The most reasonable explanation is that only parts of the N introduced with the pyrochar were available to the plants. During wheat growth, the mineralizable pyrochar N was possibly depleted. Thus, wheat plants might have shown very low contributions of pyrochar N, leading to low signature differences between the soybean and the control wheat plants. Under such circumstances a reasonable estimation of BNF is not possible.

Table 7: Correlation of nodule dry matter with selected soil properties and total plant biomass indicated by the direction (+/–) and the coefficient of determination R^2. The cells are tinged according to correlation strength from 0 (white) to 1 (grey).[a]

Soil	pH	N_{tot}	N_{min}	P	K	S	Fe	Mn	Ndfa
Caron	(–) 0.04 ns	(+) 0.15 ns	(–) 0.67 ***	(+) 0.02 ns	(–) 0.00 ns	(+) 0.94 ***	(+) 0.05 ns	(+) 0.03 ns	(+) 0.89 ***
CONMIN	(–) 0.16 ns	(+) 0.13 ns	(–) 0.71 ***	(–) 0.14 ns	(–) 0.04 ns	(+) 0.44 **	(–) 0.03 ns	(+) 0.06 ns	(+) 0.60 ***
BIODYN	(–) 0.07 ns	(+) 0.19 ns	(–) 0.25 *	(–) 0.02 ns	(+) 0.01 ns	(+) 0.38 **	(+) 0.00 ns	(–) 0.02 ns	(+) 0.53 *
ToMa	(–) 0.14 ns	(+) 0.14 ns	(–) 0.22 *	(–) 0.01 ns	(–) 0.00 ns	(+) 0.37 **	(+) 0.02 ns	(–) 0.01 ns	(+) 0.43 **
All soils	(+) 0.01 ns	(–) 0.32 ***	(–) 0.17 ***	(+) 0.38 ***	(–) 0.01 ns	(+) 0.26 ***	(–) 0.25 ***	(–) 0.02 ns	(+) 0.33 ***

[a]ns = not significant; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Table 8: Correlation of atmosphere-derived nitrogen (Ndfa) with selected soil properties and total plant biomass, indicated by the direction (+/–) and the coefficient of determination R^2. The cells are tinged according to correlation strength from 0 (white) to 1 (grey).

Soil	pH	N_{tot}	N_{min}	P	K	S	Fe	Mn	Plant DM
Caron	(–) 0.08 ns	(+) 0.32 ns	(–) 0.83 ***	(+) 0.06 ns	(+) 0.69 ***	(+) 0.93 ***	(+) 0.12 ns	(+) 0.07 ns	(+) 0.43 *
CONMIN	(–) 0.01 ns	(+) 0.51 **	(–) 0.64 ***	(–) 0.03 ns	(+) 0.70 ***	(+) 0.64 ***	(–) 0.00 ns	(+) 0.09 ns	(+) 0.23 ns
BIODYN	(–) 0.15 ns	(+) 0.50 **	(–) 0.52 **	(+) 0.00 ns	(+) 0.42 **	(+) 0.23 ns	(+) 0.05 ns	(–) 0.08 ns	(+) 0.37 *
ToMa	(–) 0.46 **	(+) 0.32 *	(–) 0.61 ***	(–) 0.15 ns	(+) 0.49 **	(+) 0.77 ***	(+) 0.03 ns	(–) 0.01 ns	(–) 0.31 *
All soils	(+) 0.14 **	(+) 0.04 ns	(–) 0.59 ***	(+) 0.07 *	(+) 0.42 ***	(+) 0.41 ***	(–) 0.00 ns	(+) 0.14 **	(+) 0.00 ns

[a]ns = not significant; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

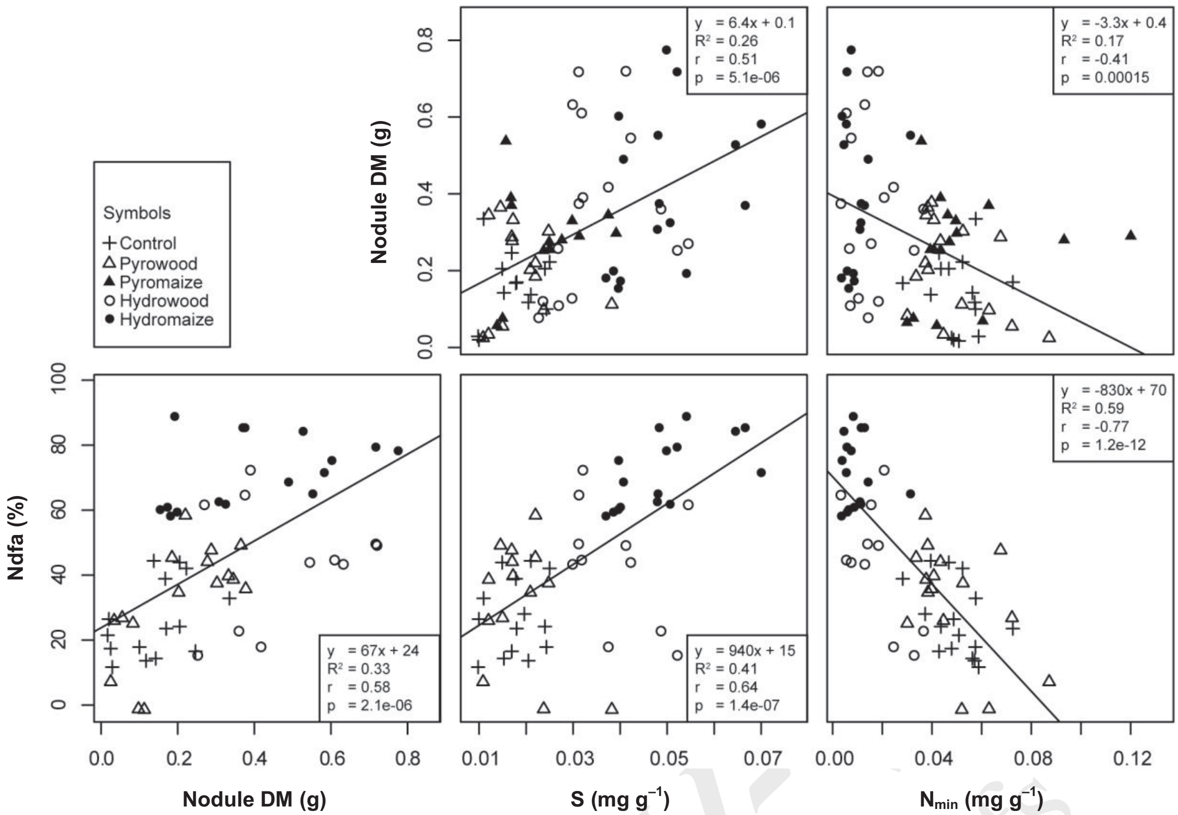

Figure 4: Correlations of nodule dry matter and nitrogen derived from atmosphere (Ndfa) with available N (N_{min}) and S in soil across all four soils. The correlation between nodule dry matter and Ndfa is also shown. Datapoints of the five treatments are indicated by different symbols. The parameters of the regression line as well as the coefficient of determination R^2 and Pearson's product-moment coefficient *r* are indicated

Nodule dry matter and Ndfa did correlate with two soil properties: positively with available S and negatively with available N. This suggests that both elements might play a crucial role for the increase in nodulation and BNF. S availability increased after Pyromaize and both hydrochars, and N availability decreased after hydrochar amendment. In soybean and other legume crops S is commonly applied as a fertilizer to increase yields, BNF and nodule formation (*Scherer* and *Lange*, 1996; *Miransari* and *Smith*, 2007; *Scherer*, 2008). S availability is known to have a strong influence on BNF, as it responds much earlier to S deficiency than photosynthesis (*Scherer* et al., 2008). This may be related to the fact that S is a key element in subunits of the nitrogenase complex (Fe-S, Mo-Fe-S), the enzyme which catalyzes the reduction of elemental N_2 to NH_3 (*Fisher* and *Newton*, 2002). Indeed, in a pot experiment with *Medicago sativa, Scherer* and *Lange* (1996) found that nitrogenase activity increased with increasing S supply. Generally, S accumulates in pyrochar because it requires higher temperatures to volatize (above 375°C) than C and N (*DeLuca* et al., 2009). A high increase in S availability after pyrochar addition was reported previously by (*Uchimiya* et al., 2010). Nutrient concentrations depend on feedstock as well as on production factors, time and temperature for pyrolysis and procedure for hydrothermal carbonization (*Libra* et al., 2011). In our case, pyrochar and hydrochar made of maize feedstock showed significantly higher S content than those made from wood. Hydrochars are particularly enriched in S because the production process includes an acidification step with sulfuric acid (H_2SO_4).

A strong decrease in N availability after hydrochar amendments was observed, but not after pyrochar amendments. *Bargmann* et al. (2014) reported a similar observation. The negative correlation between N availability and nodule dry matter is supported by a bulk of evidence of increased nodulation under N limiting conditions (*Spaink*, 2000; *Sadowsky*, 2005; *Voisin* et al., 2010). We hypothesize that in our study hydrochar amendment along with increased S supply induced a feedback loop *via* low N availability in the soil solution (*Voisin* et al., 2010), which may have caused the observed high increase in nodulation and BNF. Available N may either be bound to added carbonized materials or may have been immobilized by microorganisms which were stimulated by increased dissolved organic carbon, as soils are often limited in energy for microbial growth. However, earlier studies mostly found no decrease in N availability after pyrochar amendment (*Jones* et al., 2011; *Anderson* et al., 2014), but cases in which pyrochar immobilizes inorganic N are also recorded (*Rondon* et al., 2007; *Biederman* and *Harpole*, 2013).

Increased soil S availability may explain the observed increase in nodulation and BNF in general, and the reduced N availability especially for hydrochar treated soils. One point that remains unclear is the consequence of enhanced K availabilities in our study. *Mia* et al. (2014) tested different nutrients and found that fertilization with K alone led to an increase in BNF. They suggest that the elevated K availability after pyrochar amendments is responsible for observed increases in BNF. In our study, there was a correlation between soil K availability and BNF, but not between K availability and nodulation. Thus, we assume that the influence of K was inconsistent and/or of little importance, because of the already sufficient K levels in the tested soils.

5 Conclusions

The current study provides new insights into how biochar amendment promotes soybean growth either directly through a fertilizing effect or indirectly through enhanced root nodulation and biological N fixation. However, the complexity of the underlying processes that occur simultaneously make it difficult to assign clear causal relationships. It was found that the carbonized amendments stimulated nodulation and BNF in soybean but not necessarily soybean biomass. Soybean growth remained soil dependent and was enhanced in soils amended with carbonized materials made from maize, which were richer in N, P, and K than those from wood. The encountered increase in soil pH neither affected plant growth nor root nodulation. Root nodulation and BNF correlated positively with available soil S and negatively with available soil N. As a possible underlying mechanism, we hypothesize that higher amounts of available S and reduced N availability lead to an enhanced stimulation of nodule formation and BNF of soybean plants. Hydrochars showed a stronger effect on nodule formation and BNF than pyrochar by influencing S and N simultaneously. Detailed mechanisms by which pyrochar and hydrochar increase nodulation and BNF remain to be elucidated. In further experiments the effect of hydrochar amendments should be tested at different time points before and during soybean growth, and additional controls in regard to nutrient addition, such as S and mineral N, should be performed.

Carbonized organic materials have a potential to increase plant N-uptake by biological N fixation of leguminous crops, specifically hydrochar, because it substantially reduces N in soil solution initially due to consecutive mineralization of nutrients. In view of the use of limited fossil resources for synthetic N production, N fertilizer use could be reduced by improving the N fixation of leguminous crops. Specifically hydrochar, showing greater potential than pyrochar, could play an important role in future legume crop management.

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References

Anderson, C. R., *Hamonts, K.*, *Clough, T. J.*, *Condron, L. M.* (2014): Biochar does not affect soil N-transformations or microbial community structure under ruminant urine patches but does alter relative proportions of nitrogen cycling bacteria. *Agr. Ecosyst. Environ.* 191, 63–72.

Bamminger, C., *Marschner, B.*, *Jüschke, E.* (2014): An incubation study on the stability and biological effects of pyrogenic and hydrothermal biochar in two soils. *Eur. J. Soil Sci.* 65, 72–82.

Bargmann, I., *Rillig, M. C.*, *Kruse, A.*, *Greef, J. M.*, *Kücke, M.* (2014): Effects of hydrochar application on the dynamics of soluble nitrogen in soils and on plant availability. *J. Plant Nutr. Soil Sci.* 177, 48–58.

Bever, J. D., *Dickie, I. A.*, *Facelli, E.*, *Facelli, J. M.*, *Klironomos, J.*, *Moora, M.*, *Rillig, M. C.*, *Stock, W. D.*, *Tibbett, M.*, *Zobel, M.* (2010): Rooting theories of plant community ecology in microbial interactions. *Trends Ecol. Evol.* 25, 468–478.

Biederman, L. A., *Harpole, W. S.* (2013): Biochar and its effects on plant productivity and nutrient cycling: a meta-analysis. *Global Change Biol. Bioenerg.* 5, 202–214.

Brownsort, P. A., *Mašek, O.* (2011): Small-scale Continuous Pyrolysis Apparatus: capabilities and initial results. 3rd UKBRC Annual Conference on "Biochar 2011", May 25th 2011, Edinburgh, UK.

DeLuca, T. H., *MacKenzie, M. D.*, *Gundale, M. J.* (2009): Biochar Effects on Soil Nutrient Transformations, in Lehmann, J., Joseph, S. (ed.): Biochar for Environmental Management: Science and Technology. Earthscan, London, UK, pp. 251–270.

Fisher, K., *Newton, W. E.* (2002): Nitrogen Fixation—a General Overview, in Leigh, G. J. (ed.): Nitrogen Fixation at the Millennium. Elsevier, Amsterdam, The Netherlands, pp. 1–34.

George, C., *Wagner, M.*, *Kücke, M.*, *Rillig, M. C.* (2012): Divergent consequences of hydrochar in the plant–soil system: arbuscular mycorrhiza, nodulation, plant growth and soil aggregation effects. *Appl. Soil Ecol.* 59, 68–72.

Jones, D. L., *Edwards-Jones, G.*, *Murphy, D. V.* (2011): Biochar mediated alterations in herbicide breakdown and leaching in soil. *Soil Biol. Biochem.* 43, 804–813.

Kondorosi, E., *Mergaert, P.*, *Kereszt, A.* (2013): A paradigm for endosymbiotic life: cell differentiation of Rhizobium bacteria provoked by host plant factors. *Annu. Rev.Microbiol.* 67, 611–628.

Lehmann, J. (2007): Bio-energy in the black. *Front. Ecol. Environ.* 5, 381–387.

Lehmann, J., *Rillig, M. C.*, *Thies, J.*, *Masiello, C. A.*, *Hockaday, W. C.*, *Crowley, D.* (2011): Biochar effects on soil biota—a review. *Soil Biol. Biochem.* 43, 1812–1836.

Libra, J. A., *Ro, K. S.*, *Kammann, C.*, *Funke, A.*, *Berge, N. D.*, *Neubauer, Y.*, *Titirici, M.-M.*, *Fühner, C.*, *Bens, O.*, *Kern, J.*, *Emmerich, K. H.* (2011): Hydrothermal carbonization of biomass residuals: a comparative review of the chemistry, processes and applications of wet and dry pyrolysis. *Biofuels* 2, 71–106.

Mäder, P., *Fliessbach, A.*, *Dubois, D.*, *Gunst, L.*, *Fried, P.*, *Niggli, U.* (2002): Soil fertility and biodiversity in organic farming. *Science* 296, 1694–1697.

Mia, S., *van Groenigen, J. W.*, *van de Voorde, T. F. J.*, *Oram, N. J.*, *Bezemer, T. M.*, *Mommer, L.*, *Jeffery, S.* (2014): Biochar application rate affects biological nitrogen fixation in red clover conditional on potassium availability. *Agr. Ecosyst. Environ.* 191, 83–91.

Miransari, M., *Smith, D. L.* (2007): Overcoming the stressful effects of salinity and acidity on soybean nodulation and yields using signal molecule genistein under field conditions. *J. Plant Nutr.* 30, 1967–1992.

Nguyen, T. T. N., *Xu, C.-Y.*, *Tahmasbian, I.*, *Che, R.*, *Xu, Z.*, *Zhou, X.*, *Wallace, H. M.*, *Bai, S. H.* (2017): Effects of biochar on soil available inorganic nitrogen: a review and meta-analysis. *Geoderma* 288, 79–96.

Nutman, P. S. (1952): Host factors influencing infection and nodule development in leguminous plants. *Proc. Roy. Soc. Lond. B Biol. Sci.* 139, 176–185.

Oberson, A., *Nanzer, S.*, *Bosshard, C.*, *Dubois, D.*, *Mäder, P.*, *Frossard, E.* (2007): Symbiotic N_2 fixation by soybean in organic and conventional cropping systems estimated by ^{15}N dilution and ^{15}N natural abundance. *Plant Soil* 290, 69–83.

Ogawa, M., *Okimori, Y.* (2010): Pioneering works in biochar research, Japan. *Soil Res.* 48, 489–500.

Oram, N. J., *van de Voorde, T. F. J.*, *Ouwehand, G.-J.*, *Bezemer, T. M.*, *Mommer, L.*, *Jeffery, S.*, *Van Groenigen, J. W.* (2014): Soil amendment with biochar increases the competitive ability of legumes via increased potassium availability. *Agr. Ecosyst. Environ.* 191, 92–98.

Quilliam, R. S., *DeLuca, T. H.*, *Jones, D. L.* (2013): Biochar application reduces nodulation but increases nitrogenase activity in clover. *Plant Soil* 366, 83–92.

Reckenholz, A. F. (1999): Referenzmethoden der Eidgenössischen landwirtschaftlichen Forschungsanstalten, Band 1: Bodenuntersuchung zur Düngeberatung. Eidg. Forschungsanstalten FALRAC-FAW, Zurich, Switzerland.

Rillig, M. C., *Wagner, M.*, *Salem, M.*, *Antunes, P. M.*, *George, C.*, *Ramke, H.-G.*, *Titirici, M.-M.*, *Antonietti, M.* (2010): Material derived from hydrothermal carbonization: effects on plant growth and arbuscular mycorrhiza. *Appl. Soil Ecol.* 45, 238–242.

Rondon, M. A., *Lehmann, J.*, *Ramírez, J.*, *Hurtado, M.* (2007): Biological nitrogen fixation by common beans (*Phaseolus vulgaris* L.) increases with bio-char additions. *Biol. Fert. Soils* 43, 699–708.

Sadowsky, M. J. (2005): Soil Stress Factors Influencing Symbiotic Nitrogen Fixation: Nitrogen Fixation, in Werner, D., Newton, W. E. (eds.): Agriculture, Forestry, Ecology, and the Environment. Springer, Dordrecht, The Netherlands, pp. 89–112.

Salvagiotti, F., *Cassman, K. G.*, *Specht, J. E.*, *Walters, D. T.*, *Weiss, A.*, *Dobermann, A.* (2008): Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. *Field Crop. Res.* 108, 1–13.

Scherer, H. W. (2008): Impact of sulfur on N_2 fixation of legumes, in Khan, N., Singh, S., Umar, S. (eds.): Sulfur Assimilation and Abiotic Stress in Plants. Springer, Berlin, Germany, pp. 43–54.

Scherer, H. W., *Lange, A.* (1996): N_2 fixation and growth of legumes as affected by sulphur fertilization. *Biol. Fert. Soils* 23, 449–453.

Scherer, H. W., *Pacyna, S.*, *Spoth, K. R.*, *Schulz, M.* (2008): Low levels of ferredoxin, ATP and leghemoglobin contribute to limited N_2 fixation of peas (*Pisum sativum* L.) and alfalfa (*Medicago sativa* L.) under S deficiency conditions. *Biol. Fert. Soils* 44, 909–916.

Schimmelpfennig, S., *Glaser, B.* (2012): One step forward toward characterization: some important material properties to distinguish biochars. *J. Environ. Qual.* 41, 1001–1013.

Sinaj, S., *Richner, W.*, *Flisch, R.*, *Charles, R.* (2009): GRUDAF 2009–Grundlagen für die Düngung im Acker-und Futterbau. *Agrarforschung Schweiz* 16, 1–100.

Spaink, H. P. (2000): Root nodulation and infection factors produced by rhizobial bacteria. *Annu. Rev. Microbiol.* 54, 257–288.

Taylor, R. W., *Williams, M. L.*, *Sistani, K. R.* (1991): N_2 fixation by soybean-Bradyrhizobium combinations under acidity, low P and high Al stresses. *Plant Soil* 131, 293–300.

Turner, E. R. (1955): The effect of certain adsorbents on the nodulation of clover plants. *Ann. Bot.* 19, 149–160.

Uchimiya, M., *Lima, I. M.*, *Klasson, K. T.*, *Wartelle, L. H.* (2010): Contaminant immobilization and nutrient release by biochar soil amendment: roles of natural organic matter. *Chemosphere* 80, 935–940.

Unkovich, M., *Herridge, D.*, *Peoples, M.*, *Cadisch, G.*, *Boddey, B.*, *Giller, K.*, *Alves, B.*, *Chalk, P.* (2008): Measuring Plant-Associated Nitrogen Fixation in Agricultural Systems. Australian Centre for International Agricultural Research (ACIAR), Canberra, Australia.

van de Voorde, T. F. J., *Bezemer, T. M.*, *Van Groenigen, J. W.*, *Jeffery, S.*, *Mommer, L.* (2014): Soil biochar amendment in a nature restoration area: effects on plant productivity and community composition. *Ecol. Appl.* 24, 1167–1177.

Vantsis, J. T., *Bond, G.* (1950): The effect of charcoal on the growth of leguminous plants in sand culture. *Ann. Appl. Biol.* 37, 159–168.

Verheijen, F., *Jeffery, S.*, *Bastos, A.*, *van der Velde, M.*, *Diafas, I.* (2010): Biochar Application to Soils. Office for Official Publications of the European Communities, Luxembourg City, Luxembourg.

Voisin, A.-S., *Munier-Jolain, N.*, *Salon, C.* (2010): The nodulation process is tightly adjusted to plant growth. An analysis using environmentally and genetically induced variation of nodule number and biomass in pea. *Plant Soil* 337, 399–412.

Werner, R. A., *Brand, W. A.* (2001): Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Commun. Mass Sp.* 15, 501–519.

Zahran, H. H. (1999): Rhizobium–legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 63, 968–989.

