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Soil amendment with biochar increases the competitive ability of legumes via increased potassium availability

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Abstract

Soil amendment with biochar is currently proposed as a management strategy to improve soil quality and enhance plant productivity. Relatively little is known about how biochar affects plant competition, although it has been suggested that it can increase the competitive ability of legumes. This study tested the impact of a biochar on the competitive ability of legumes through alterations to soil pH and/or nutrient availability. Biochar was produced from aboveground plant biomass from a species-rich semi-natural grassland pyrolysed at 400°C. In a greenhouse experiment, a legume (red clover, *Trifolium pratense* L.); a grass (red fescue, *Festuca rubra* L.); and a forb (plantain, *Plantago lanceolata* L.) were grown in (1) monocultures, (2) in a mixed culture of red fescue and red clover, and (3) in a mixture of all three species. Soil treatments included fertilization with nitrogen (N), potassium (K), phosphorus (P), or micronutrient fertilizer in the presence or absence of biochar; a pH-adjusted control soil; and a control (i.e. with no amendment). The competitive ability of red clover was quantified as the proportion of aboveground biomass of this species within the mixtures. Both biochar amendment and K fertilization significantly ($P < 0.001$) increased red clover biomass, and increased the competitive ability of red clover when grown with red fescue and plantain. Application of N fertilizer, irrespective of biochar amendment, resulted in significantly ($P < 0.001$) greater red fescue and plantain biomass and eliminated the competitive advantage of red clover. The biochar-mediated pH increase did not affect red clover biomass or its competitive ability. We conclude that biochar has a beneficial effect on red clover under N limiting conditions due to an increase in K availability. Our results suggest a potential role for biochar to maintain the proportion of forage legumes in agricultural pastures or semi-natural grasslands.

Key words

Biochar, *Trifolium pratense*, competition, potassium, nitrogen, intercropping

1. Introduction

Biochar is generally defined as biomass which is carbonised through heating in a low to zero oxygen environment and which is produced with the intention of application to soil (Lehmann and Joseph, 2009; Verheijen, et al., 2010; Sohi, 2012; Jeffery et al., 2013). Biochar has been promoted as improving soil quality and fertility. Application of biochar may increase crop production through several proposed mechanisms: providing a liming effect (Jeffery et al., 2011), increasing soil water-holding capacity (Karhu et al., 2011), enhancing water and nutrient uptake (Hunt et al., 2010) and delaying or reducing N leaching (Lehmann et al., 2003).

Next to recalcitrant carbon, macro and micro nutrients are the main components of most biochars (Lehmann et al., 2011), although, the amount and availability of nutrients varies based on the feedstock and pyrolysis conditions used. By providing additional nutrients to the soil, and consequently influencing plant nutrient uptake, biochar application may alter the competitive ability of particular plant species. In particular, leguminous species have been shown to benefit from biochar amendment (Rondon et al., 2007). For example, the addition of biochar, identical to the one used in the present study, to a species-rich grassland in the Netherlands resulted in a nearly threefold increase in the proportion and biomass of legumes (mainly red clover, *Trifolium pratense*) after one growing season (van de Voorde et al., *in press*).

Several mechanisms have been proposed to explain the enhanced competitive ability of legumes in the presence of biochar (Lehmann and Rondon, 2006). For example, N immobilization by the microbial community has been found after charcoal addition to a Ferralsol (Lehmann et al., 2003). Besides a reduction in available N, biochar-mediated increases in soil pH (Jeffery et al., 2011) could benefit legumes by stimulating biological nitrogen fixation (BNF) (Rondon et al., 2007) and thereby enhancing the competitive ability

of leguminous plants, particularly under N limiting conditions. Biochar addition may also increase the content of soil P, K, magnesium (Mg) and other nutrients, which in turn may also increase BNF (Rondon et al., 2007). It has been shown that, under K-limiting conditions, legume nodulation can be supressed (Sangakkara et al., 1996). Since grasses have a greater root mass density, they are effective competitors for K; potentially causing the proportion of legumes in a grass-legume mixed culture to decrease under K-limiting conditions (Mengel and Kirkby, 2001).

While these mechanisms have all been observed or proposed in separate studies, a coherent assessment of them within a single experiment is currently lacking. We hypothesised that the competitive advantage of legumes grown in sandy soils amended with carbonized material produced from a mixture of grassland species occurs due to 1) increased macronutrient availability from the biochar, 2) increased micronutrient availability from the biochar, and 3) a biochar-induced pH increase in the soil. We tested the hypothesized mechanisms for mixed and monocultures of red clover (*Trifolium pratense* L.), red fescue (*Festuca rubra* L.) and plantain (*Plantago lanceolata* L.) simultaneously in a greenhouse pot experiment.

2. Materials and methods

To test our hypotheses, we conducted a greenhouse pot experiment, composed of two sub-experiments. In Sub-experiment I we tested whether the performance of red clover, red fescue and plantain (in monocultures and a mixed culture of red clover + red fescue) was due to a pH or a nutrient effect. In Sub-experiment II, we tested the effects of micronutrients, as well as specific macronutrients, on the competitive ability of red clover in a three-species mixture (red clover + red fescue + plantain). The experiment was set up in a randomized block design and the sub-experiments were conducted simultaneously.

2.1. Soil and biochar characteristics

Soil was collected from a species-rich grassland, near Ede, the Netherlands (latitude +52° 3' 34.03", longitude +5° 45' 2.81"). This area is of glacial origin (Saalien ice age). The soil is classified as a Podzol (FAO 2007), with a coarse sand cover; the top 20 cm, the A horizon, texturally consist of 93.9% sand, 5.3% silt, and 3.4% clay, with an organic C content of 2.8%, which is described in detail in van der Putten et al. (2000) and see details, "Field 12" in van de Voorde et al. (2011). Soil was collected during Spring 2012 from the top 20 cm of the soil profile, air dried for 6 days (d), sieved to 8 mm and mixed. Biochar was produced from aboveground plant biomass collected from the same species-rich semi-natural grassland (van de Voorde et al., *in press)*. The grassland was dominated (> 65%) by *Lolium perenne*, *Bromus hordeaceus*, *Jacobaea vulgaris*, and *Holcus lanatus* (van de Voorde et al., 2011). As in van de Voorde et al. (in press), the natural grassland was mown in October 2010, and the dried cuttings were pyrolysed for 5 min at 400°C at Biogreen, ETIA, France using a Biogreen 130 pyrolyser with a continuous flow of 10 kg per hour. Mineral N, available K and P-PO_4 in biochar were photometrically determined in a 1:10 (w/v) 0.01 M $CaCl_2$ extract (Houba et al., 1986), and were found to be: 0.8 mg kg^{-1} (SE 0.03), 1620.8 mg kg^{-1} (SE 24.4) and 1.9 mg kg^{-1}(SE 0.02), respectively (van de Voorde et al., in press). Selected biochar characteristics are presented in Table 1. A pyrolysis GC/MS characterisation revealed that this biochar was devoid of fingerprints for labile C (e.g., levoglucosan; data not shown).

2.2 Experimental setup

Sub-experiment I consisted of five soil treatments: a control treatment (C) with no amendment, a biochar amendment treatment (B) to test the effect of biochar addition on plant growth, and 3 additional treatments to test the effects of macronutrients and of pH

(Hypotheses 1 + 3): a pH treatment (liming: L), fertilization with macronutrients (F), and biochar and macronutrients (F+B). It included four plant treatments: red clover, red fescue, plantain monocultures and a red clover + red fescue mixture.

Sub-experiment II included the same soil treatments as Sub-experiment I. Seven additional soil treatments tested the effect of specific nutrients on plant growth: addition of N (N), P (P), K (K), or micronutrient fertilizer (Mic), as well as those treatments in combination with biochar (N+B, P+B, Mic+B respectively). Sub-experiment II consisted of only one plant treatment: a mixture of all three species.

Application rates were: Biochar (10 t ha^{-1}), lime (43 kg $CaMg(CO_3)_2$ ha^{-1}), N (calcium ammonium nitrate (CAN) 50 kg N ha^{-1}), P (triple super phosphate (TSP) 30 kg P ha^{-1}), K (patentkali, 50 kg K ha^{-1}), micronutrients (applied in a solution consisting of H_3BO_3 (794 g B ha^{-1}), $MnSO_4$ (803 g Mn ha^{-1}), $CuSO_4$ (32 g Cu ha^{-1}), $ZnSO_4$ (80 g Zn ha^{-1}), and $(NH_4)_6Mo_7O_{24}$ (17 g Mo ha^{-1})). The rate of lime that was needed to increase the soil pH to the same level as was induced by addition of 10 t ha^{-1} of biochar, was determined using a soil titration with $CaMg(CO_3)_2$ in a pilot experiment.

For both sub-experiments, Mitscherlich pots (diameter 19 cm, height: 22 cm) were filled with 7.0 kg dry weight (dw) equivalent of soil. Each pot contained three layers of soil (expressed in kg dw): a bottom layer of 4.5 kg untreated soil, a middle layer of 1.5 kg to which the treatments were applied, and an untreated top layer of 1.0 kg of soil. The upper two layers made up the top 10 cm of the pot. Fertilizers were added only to the middle layer to prevent seedling damage. Biochar or lime were added to the two uppermost layers of soil. The monoculture pots were sown on day 0 with 1.50 g red clover, 3.00 g red fescue or 0.75 g plantain. The two species mixed culture received 0.75 g red clover and 1.50 g red fescue, and the three species mixture 0.50 g red clover, 1.00 g red fescue, and 0.25 g plantain. Seeds were ordered from a commercial organic seed supplier (De Bolderik, Wervershoof, the

Netherlands). Under these conditions, an application rate of 10 t ha^{-1} biochar corresponds with 12.6 g kg $soil^{-1}$.

All treatments were replicated five times using a randomized block design with five blocks, resulting in 100 pots (5 soil treatments x 4 plant treatments x 5 replicates) in Sub-experiment I and 60 pots (12 soil treatments x 1 plant treatment x 5 replicates) in Sub-experiment II. The two sub-experiments were randomized together in a complete randomized block design in the greenhouse (day/night temperature (°C): 20/16; 60% relative humidity). The location of the blocks, as well as the location of the pots within the block were re-randomized weekly. Throughout the experiment, pots were watered gravimetrically with demineralized water to maintain them at 60% soil water holding capacity. Pots were weighed five times per week, and demineralized water was added when the pots lost more than 8% of their weight.

2.3. Biomass harvest and plant analysis

Aboveground biomass was clipped on days 26-30 and the experiment was harvested on days 54-58; one block per day. At the clipping and the harvest, aboveground biomass was cut to 2 cm, sorted according to species, dried (70°C, 24 h) and weighed. All results presented in this study are based on total aboveground (sum of the clipping and the harvest) biomass.

In treatments C, B, N+B, K, and N from Sub-experiment II, aboveground biomass was analysed for total N and K. Dry plant material, sorted per species from the clipping and the harvest was ground, and combined at a ratio based on percentage of total biomass gained. Plant material was digested using sulphuric acid (H_2SO_4), hydrogen peroxide (H_2O_2) and Selenium (Se) (Temminghoff and Houba, 2004). In the aboveground plant biomass, K concentration was analysed with flame atomic emission spectroscopy (F-AES) (Merck-Elex

6361 F-AES analyser, Merck, USA) and N concentration via segmented flow analysis (SFA; Skalar 6 channel SFA analyser, Skalar, Netherlands, 2003).

2.4. Soil sampling and chemical characteristics

Soil samples from the entire depth of the pot were taken at sowing (0 d) and harvest (54 d) using an auger (1.0 cm diameter, *c.* 15 g). Soil cores from each treatment taken prior to sowing were homogenised into one sample per treatment, in order to have a reference of the starting soil fertility and pH of each treatment. At harvest, soil samples were taken from each pot. All soil samples were dried at 40°C for 24 h and sieved to 2 mm.

Soil samples were extracted in a 1:10 (w/v) 0.01 M $CaCl_2$ (Houba et al. 2000). The pH of the soil suspension in $CaCl_2$ was measured using a pHM 92 pH meter (Radiometer Copenhagen, DK). The suspension was filtered through a R Quantum 0.45 µm syringe tip (Whatman, UK) , followed by measurement of total dissolved N (N-Nts), nitrate-N ($N\text{-}NO_3^-$ + NO_2^-), ammonium-N ($N\text{-}NH_4^+$) and phosphate-P ($P\text{-}PO_4^{3-}$) by SFA. Available K^+ was measured in the same extract using a inductively coupled plasma atomic emission spectroscopy (ICP-AES) with a Varian Vista Pro radial system analyser (Agilent, USA, 2001). Electrical conductivity (EC) was measured in a 1:5 soil: water (w/v) solution, after filtering, with a LF 300 Conductivity Meter (Wissenschaftlich-Technische-Werkastätten GmbH, Germany) (Houba et al., 2000).

2.5. Statistical analyses

Data were analysed using Genstat 14th edition, VSN International (Payne et al., 2008). Prior to data analyses, data were tested for normality using the Shapiro-Wilk test. To fulfil requirements of normality, data were log-transformed where necessary. In Sub-experiment I,

one-way ANOVAs were performed on each plant treatment separately, with soil treatment as a factor (C, B, L, F, B+F). In Sub-experiment II, a one-way ANOVA was conducted to determine significant differences in the effect of soil treatment (12 levels) on the three species mixture. In all mixed cultures, analyses were performed for total aboveground biomass per pot, and for each species separately. Final soil chemical data (sampled at harvest) were grouped per sub-experiment and analyzed using a two-way ANOVA in Sub-experiment I (5 levels for plant and soil treatment) and a one-way ANOVA in Sub-experiment II (12 levels for soil treatment). Individual comparisons, per plant species and culture, were based on a Bonferroni post-hoc test. All analyses included blocking as a factor. Significance was considered at the $P \leq 0.05$ significance level.

3. Results

3.1. Soil chemical characteristics

Chemical analyses of the soil at sowing (0 d) are shown in Table 2. Soil in the limed (L) treatment had the highest pH (5.56), along with pots receiving biochar (B) (5.55), compared to a soil pH of 5.24 in the control. Fertilized treatments (N, P, K, Mic, F) had an acidifying effect, which was counteracted by biochar addition when both fertilizer and biochar were added (N+B, P+B, Mic+B, F+B) (Table 2). Treatments with fertilizer were reflected in the nutrient analysis. Treatments with biochar showed increased levels of available K (Table 2).

Results of soil analyses at harvest can be found in Supplementary Information (Table S1a). In Sub-experiment I, the soil pH of the L, B, and F+B treatments were significantly ($P < 0.001$) higher than the control. The F+B treatment had significantly ($P < 0.001$) higher levels of $N\text{-}NO_3^- + NO_2^-$ than the C, B, L, or other fertilized treatments. Available P was highest when biochar and total fertilization were applied (Supplementary Information Table S1a).

In Sub-experiment II, treatments with lime or biochar had a significantly ($P < 0.001$) higher pH than the control treatment. Fertilized treatments were similar to the control in terms of pH (Table S1b). Addition of biochar with P fertilizer significantly ($P < 0.001$) increased extractable P-PO_4^{3-}. Similar to Sub-experiment I, levels of extractable P-PO_4^{3-} were significantly ($P < 0.001$) lower in the limed treatment compared to any other treatment. The N components, N-NH_4, N-NO_3, and dissolved organic nitrogen (N-DON) in the soil were not significantly influenced by soil treatments (Table S1b).

3.2. Plant biomass analyses

In Sub-experiment I, all three monocultures significantly ($P < 0.001$) differed between soil treatments in terms of aboveground biomass (Figure 1). Biochar addition (B) significantly increased aboveground biomass of all three monocultures as compared to the control treatment (C) ($P < 0.001$, Figure 1). Plant growth in the limed treatment was not significantly different from the control (Figure 1). The addition of fertilizer with and without biochar (F, F+B) significantly increased aboveground biomass of all three monocultures compared to the control. Plantain and red fescue aboveground biomass in both fertilizer (F, F+B) treatments was also significantly higher than in the biochar treatment ($P < 0.001$, Figure 1).

Aboveground biomass of the red clover + red fescue mixture was significantly higher in the B, F and F+B treatments, as compared to the control ($P < 0.001$, Figure 2). The limed treatment (L) did not differ from the control treatment (Figure 2). Biochar addition only stimulated red clover growth, whereas both fertilizer treatments stimulated only red fescue and plantain growth ($P < 0.001$, Figure 1).

In Sub-experiment II, total biomass of the 3-species mixed culture was significantly higher in all treatments that received biochar (B, F+B, Mic+B, N+B, P+B) and in the K fertilizer (K) and complete fertilizer (F) treatments, compared to the control ($P < 0.001$,

Figure 3). Red clover, when grown in the 3-species mixed culture had greater aboveground biomass in the biochar (B), biochar and micronutrient (Mic+B) and K fertilizer (K) treatments, whereas red fescue and plantain had greater biomass in both fertilizer treatments (F, F+B) and in both N fertilizer treatments (N, N+B) (Figure 3). Thus, red clover biomass was significantly higher in treatments that received additional K, in the absence of extra N. This is further underlined by a positive and significant relationship between the ratio of available K and total extractable N at the start of the experiment (values of available K and extractable N can be found in Table 2) and red clover biomass in the three species mixtures (Figure 3) ($R^2 = 0.47$; $P = 0.01$). Red fescue and plantain, on the other hand, performed better in treatments that received additional N (F, F+B, N+B, N).

The K fertilizer treatment resulted in a higher N concentration in red clover aboveground biomass when grown in combination with red fescue and plantain, compared to the control ($P < 0.001$) (Figure 4a). This was not the case for the red fescue or plantain. Although there was a significant effect of soil treatment on N concentration in red fescue and plantain aboveground biomass, ($P < 0.001$ and $P = 0.007$, respectively), the K fertilizer treatment did not increase N concentration in aboveground biomass of these two species. N concentration in red fescue was significantly ($P < 0.001$) higher when grown in the presence of N fertilizer with or without biochar (N, N+B). When biochar was applied, the K concentration of red clover, red fescue, and plantain was significantly ($P < 0.001$ for all species) increased, compared to treatments without biochar (Figure 4b).

3.3. Competition between plant species

Soil treatments in Sub-experiment I (C, L, F, B, F+B) had a significant ($P = 0.04$) effect on the composition of the red clover + red fescue mixed culture, in terms of aboveground biomass (data not shown), following the same trends as were observed in the three species

mix (Sub-experiment II, Figure 5). Biochar amendment increased the proportion of red clover from 69% of total aboveground biomass in the control, to 76% in the biochar treatment (Figure 2).

Composition of the three species mixture differed significantly between the soil treatments ($P < 0.001$). Addition of N fertilizer, regardless of the presence of biochar, decreased the proportion of red clover, and increased the proportion of plantain (Figure 5). However, in the absence of N fertilization, the treatments containing a K source (either through K fertilization or biochar addition) led to an increased proportion of red clover (Table 3).

4. Discussion

The biochar used in the current study improved soil available K, which mediated an increase in the competitive ability of a legume when grown with a grass and a forb under N limiting conditions.

We found that an increase in K availability, either through biochar addition or K fertilization, significantly ($P < 0.001$) increased red clover biomass (Table 3). In the absence of N addition, biochar increased the proportion of red clover when grown with red fescue and plantain (Figure 5). This supports our first hypothesis, that biochar increases the competitive ability of a legume by mediating availability of macronutrients- specifically, increasing K availability. This demonstrates that one mechanism underlying the biochar-induced increase in red clover biomass and competitive ability is an increase in K availability. Neither the biomass nor the competitive ability of red clover was significantly altered due to an increase in micronutrient availability or an increase in pH in this specific field soil. However, this does not rule out the possibility that pH-induced increases in micronutrient availability could be

the mechanism behind altered competition dynamics in more acidic soils with lower micronutrient availability, as was hypothesized by Rondon et al. (2007).

4.1. Biomass and competition: K and N

Increases in legume biomass when grown on biochar-amended soil have been documented previously (Rondon et al., 2007). In the present study, biochar application substantially increased available K in the soil at the beginning of the experiment, even exceeding concentrations in the treatments that received K fertilizer by 3-4 fold (Table 2). This led to a significant increase in red clover biomass in the 3-species mixed culture in those treatments where K was applied (either as biochar or as fertilizer) and where no N fertilizer was applied (Table 3). The root system of red clover is shorter, thicker, and less branched than grasses, potentially putting it at a disadvantage in terms of nutrient uptake (Evans, 1977; Høgh-Jensen, 2003). Therefore, under low K conditions, the proportion of legumes in a mixed culture may be expected to decrease as the legumes are outcompeted for K acquisition (Mengel and Kirkby, 2001).

In our study, the increased competitive ability of red clover when grown in biochar amended soil disappeared when N fertilization was applied (Figure 5). This is in line with a previous study, which showed that grasses are superior competitors, compared to clover, in N-rich environments (Torssell et al., 2007). Under N-rich conditions, legumes' ability to fix N ceases to be a competitive advantage. Therefore, in the present study, the biochar-mediated increase in competitive ability is facilitated by both the alleviation of K deficiency and the limitation of N, as the biochar used in the present study did not add substantial N to the soil (Table 2). Treatments with biochar and N fertilizer (N+B, F+B) resulted in less available N than the N-fertilized treatments without biochar (F, N), despite the fact that the same amount of CAN was added in each (Table 2). In this N limited environment, addition of K favored

legume growth over the growth of grasses or forbs, resulting in an increased competitive ability of red clover. It should be noted that a possible effect of P on plant competition in our experiment was unlikely, as the soil has a relatively high P status due to previous agricultural activity (Bezemer et al., 2006; Van de Voorde et al., 2010). This was confirmed by the results of the P fertilized treatment (P), and the combination of biochar and P, (P+B) (Figure 3). In soils deficient in P, plant competition for this nutrient is likely to play a role in plant competition next to N and K.

Legume biomass and N uptake are sensitive to changes in K availability (Blaser and Brady, 1950). In the present study, red clover N concentration was significantly higher when fertilized with K (Figure 4a). However, N concentration decreased following biochar amendment, despite the biochar mediated increase in soil K. Similarly, Rondon et al. (2007) found that biochar addition did not increase N uptake in common bean. Nitrogen mineralization and net nitrification can decrease with biochar addition (Dempster et al., 2012). Biochar, consisting of charred secondary forest material, has been reported to increase plant K uptake and foliar K concentration in cowpea (Lehmann et al., 2003). Biochar produced from *Eucalyptus deglupta* increased K uptake in common bean (Rondon et al., 2007). In the present study, addition of biochar produced from grassland species also yielded increased concentrations of K in legume (red clover) biomass (Figure 4b), although it was derived from different feedstock.

4.2. Biomass and competition: micronutrients and pH

Contrary to our hypotheses which stated that biochar would increase the competitive ability of red clover due to an increase in micronutrient availability (2) and/or due to an increase in pH (3), a biochar mediated increase in pH or micronutrients did not increase the biomass or competitive ability of red clover (Figures 1a-c, 3). Previous studies have found

that wood-derived biochar amendment both increased soil pH (Oguntunde et al., 2004; Rondon et al., 2007), and stimulated BNF in common bean (Rondon et al., 2007). However, in that work BNF increased at biochar application rates that had yet to induce significant increases in soil pH (Rondon et al., 2007). Moreover, that study was conducted in the tropics on an acid Oxisol, where increases in pH may bring the soil into a pH range more suitable for plant growth, and decrease the toxicity of metals such as Al. When soil pH was below 6, a biochar mediated-pH increase of 0.1 to greater than 2.0 units resulted in significantly improved crop productivity (Jeffery et al., 2011). In the present study, biochar increased the pH by 0.22 units from 4.99 to 5.21. Although optimal red clover growth occurs in soils with a pH of 6.0-7.0, satisfactory yields can still be achieved in the 5.0-6.0 pH range (Smith et al., 1985). This could explain the absence of a pH effect on biomass.

Increases in BNF have been attributed to the increased availability of micronutrients such as B and Mo (Rondon et al., 2007). Mo is an essential nutrient for rhizobia; B is essential for establishment of the symbiosis. Deficiencies in either can compromise nodule development and function (O'Hara, 2001). However, in the present study, the addition of micronutrients did not increase BNF or legume biomass; perhaps because the soil already contained adequate levels of these nutrients. A more in-depth analyses of the effect of biochar on BNF and an overview of the responsible mechanisms in this greenhouse experiment can be found in Mia et al. (this issue).

5. Conclusions

This study focused on the mechanisms that underlie red clover's apparent competitive advantage in the presence of a biochar derived from pyrolised aboveground biomass of grassland species. We showed that an increase in K, in the absence of N addition, led to a higher proportion of red clover and increased biomass. Understanding why certain species

flourish in grasslands can contribute to designing a pasture or grassland management practices to meet agriculture goals - providing high nutrient fodder for animals and/or reducing the need for N inputs and, thus, their associated economic and environmental implications. Legumes such as red clover, have great value in terms of grassland quality as well as environmental sustainability. Soil amendments that increase the competitive ability of legumes could be used to increase their fitness and proportion in pastures. In order to fully understand how soil amendments such as biochar influence the competitive dynamics and, thus, the composition of the grasslands, further research needs to be conducted. Our results provide insight into the mechanisms behind observed effects of a biochar on legumes. Furthermore, they suggest a potential role for biochar to maintain the proportion of forage legumes in agricultural pastures or semi-natural grasslands.

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List of table and figure captions

Figure 1. Mean cumulative aboveground biomass (clipped biomass + biomass from the final harvest) ± SE of red clover, red fescue, and plantain grown in monocultures. Different letters denote significant differences in total aboveground biomass per pot between soil treatments per plant species, based on within plant species comparisons using a bonferroni post-hoc test ($P < 0.05$).

Figure 2. Mean cumulative aboveground biomass (clipped biomass + biomass from the final harvest) ± SE of red clover + red fescue grown in a mixed culture. Different capital letters denote significant differences in total aboveground biomass per pot between soil treatments, and different lower case letters denote significant differences per plant species, based on within plant species comparisons using a bonferroni post-hoc test ($P < 0.05$).

Figure 3. Mean cumulative aboveground biomass (clipped biomass + biomass from the final harvest) ± SE of red clover + red fescue + plantain grown in a mixed culture. Different capital letters denote significant differences in total aboveground biomass per pot between soil treatments, and different lower case letters denote significant differences per plant species, based on within plant species comparisons using a bonferroni post-hoc test ($P < 0.05$).

Figure 4. Mean ± SE concentration of (**a**) N and (**b**) K (mg g biomass^{-1}) in red clover, red fescue, and plantain aboveground biomass when grown together in a 3-species mixed culture. Different letters denote significant differences between soil treatments per plant species, based on a bonferroni post-hoc test, n = 75 (**a**) $P < 0.001$ for all plant species, and (**b**) $P < 0.001$ for red clover and red fescue, $P = 0.007$ for plantain.

Figure 5. Proportion of red clover, red fescue, and plantain in the three species mixed cultures. Different letters denote significant differences between soil treatments within plant species based on a bonferroni post-hoc test, n = 35.

Tables

Table 1. Chemical characteristics of the biochar used in the present study. Biochar was produced at 400°C through slow pyrolysis from combined various grassland species.

	Mean ± SE		
Volatile matter content[1]	32.10 %	±	1.89
Ash[1]	25.22 %	±	5.01
N[2]	1.91 %	±	0.09
C[2]	59.02 %	±	0.78
H[2]	3.81 %	±	0.06
S	0.00 %	±	0.0
Atomic H/C	0.77	±	0.03
pH[3]	7.41	±	0.01
$CaCO_3$ equivalent value	8.68%		

[1]Ash and volatile organic matter content were determined according to ASTM D 1762-84 (2007).

[2] Carbon (C), hydrogen (H) and nitrogen (N) were determined using a Thermo Scientific FLASH 2000 Organic Elemental Analyzer.

[3] pH was measured in a 1:10 (w/v) biochar : water suspension using a pHM 92 meter (Radiometer Copenhagen, DK).

Table 2. Soil chemical properties of the treatments under study at start of the greenhouse experiment (sowing, day 0).

Treatment	**pH**	**N-NH_4** (mg N kg^{-1})	**N-NO_3+NO_2** (mg N kg^{-1})	**N-DON[1]** (mg N kg^{-1})	**P-PO_4** (mg P kg^{-1})	**K^+** (mg K kg^{-1})	**EC[2]** (dS m^{-1})
Control (C)	5.24	2.5	20.0	4.0	3.28	29.1	0.08
Lime (L)	5.56	2.9	25.0	4.8	2.39	27.0	0.07
N fertilizer (N)	4.96	3.8	53.4	4.9	3.59	30.8	0.14
P fertilizer (P)	5.00	2.4	26.3	4.5	4.57	30.0	0.08
K fertilizer (K)	5.07	2.5	24.8	4.8	3.31	50.2	0.10
Micronutrient fertilizer (Mic)	5.00	2.7	18.5	4.7	3.49	28.1	0.08
Total fertilization (F)	4.99	5.2	53.4	4.2	4.90	61.0	0.21
Biochar (B)	5.55	2.6	20.6	5.5	3.77	192.0	0.10
Biochar + N fertilizer (N+B)	5.44	7.3	38.0	6.2	3.89	182.8	0.14
Biochar + P fertilizer (P+B)	5.48	2.7	16.3	6.2	5.10	198.0	0.12
Micronutrient fertilizer + biochar (Mic+B)	5.42	3.5	18.3	6.1	3.79	194.0	0.13
Total fertilization + biochar (F+B)	5.51	9.4	34.8	5.5	4.77	205.3	0.18

[1]DON- dissolved organic nitrogen

[2]EC- electrical conductivity

Table 3. Mean cumulative aboveground biomass ± SE of red clover, red fescue, and plantain grown in monocultures and a 3-species mixed culture with N addition (+ N), or with K and no N fertilization (+ K, - N). Significance between the groups was determined with a two-sided T-test.

	Monoculture (g biomass pot^{-1})			**Three species mixed culture** (g biomass pot^{-1})		
	Red clover	Red fescue	Plantain	Red clover	Red fescue	Plantain
+ N	21.13	12.99	13.04	8.00	5.72	4.00
+ K, - N	20.37	10.23	8.38	9.87	4.17	2.19
P	0.44	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Figures

Figure 1

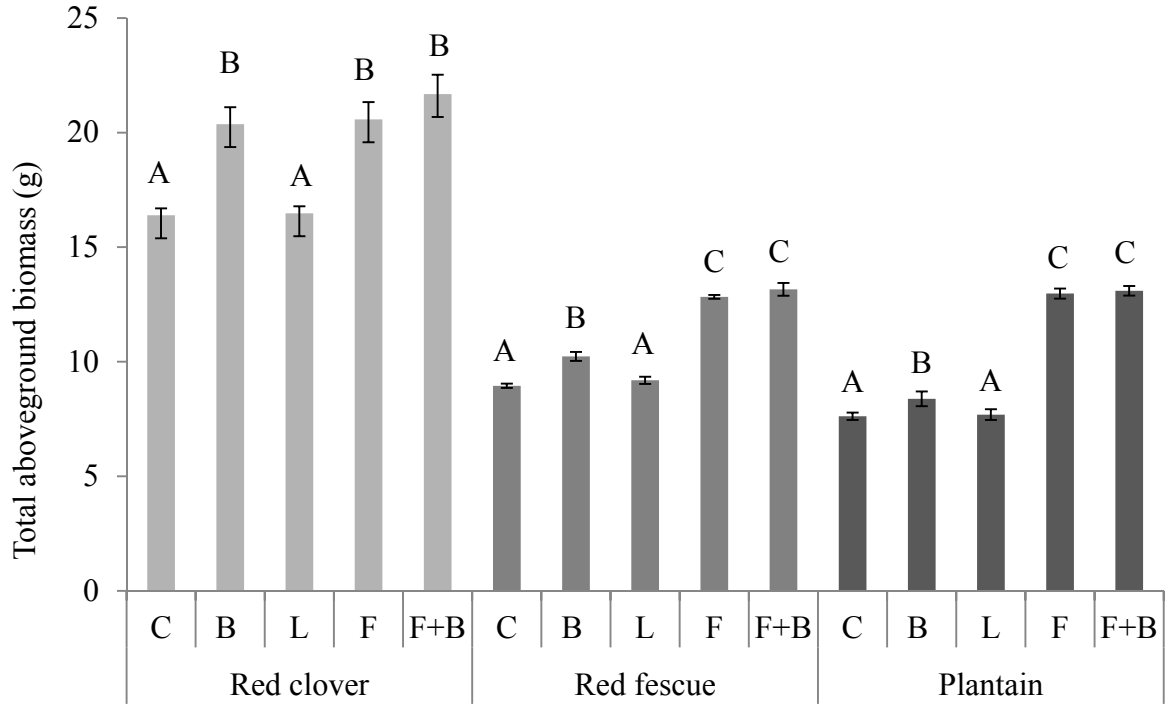


Figure 2

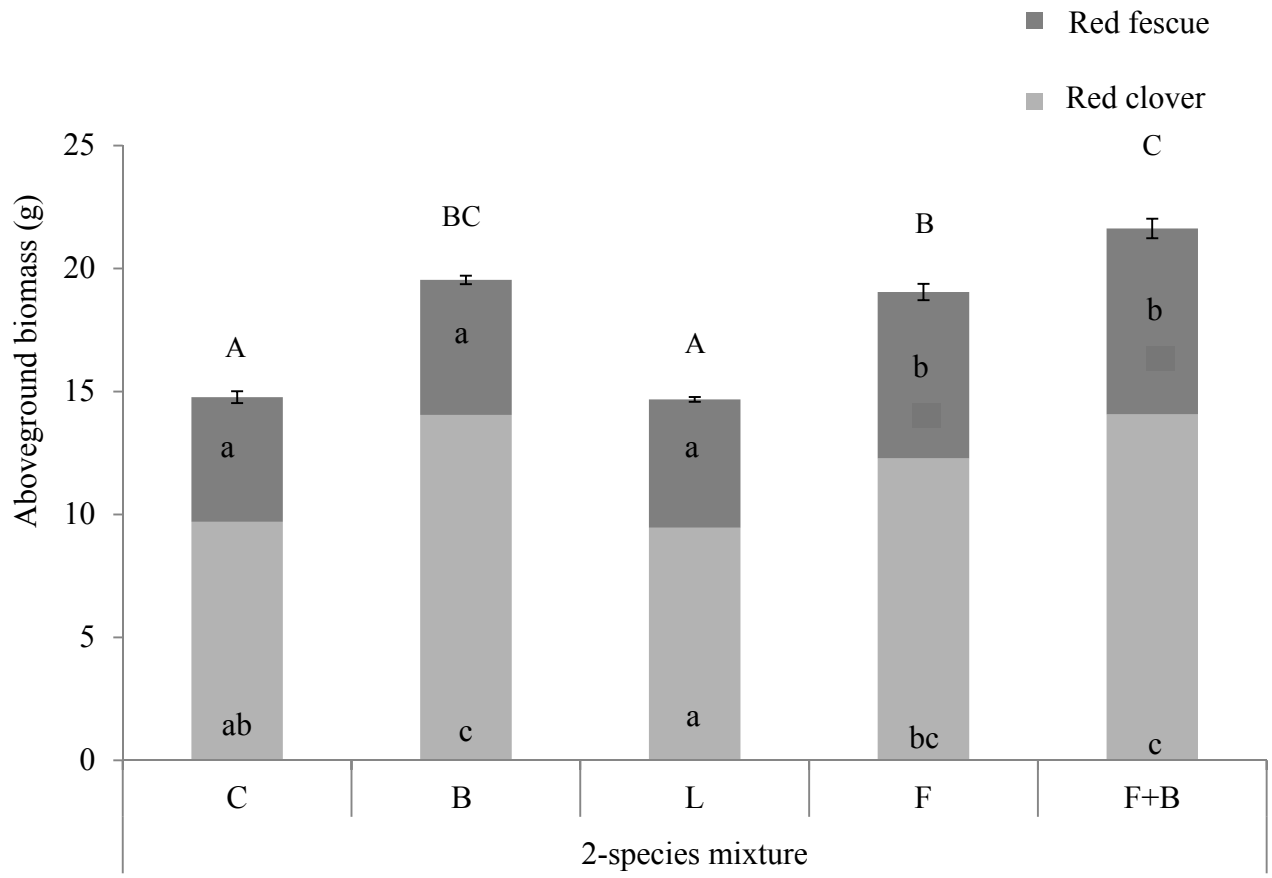

Red fescue
Red clover
Aboveground biomass (g)
25
20
15
10
5
0
A
BC
A
B
C
a
a
a
b
b
ab
c
a
bc
c
C
B
L
F
F+B
2-species mixture

Figure 3

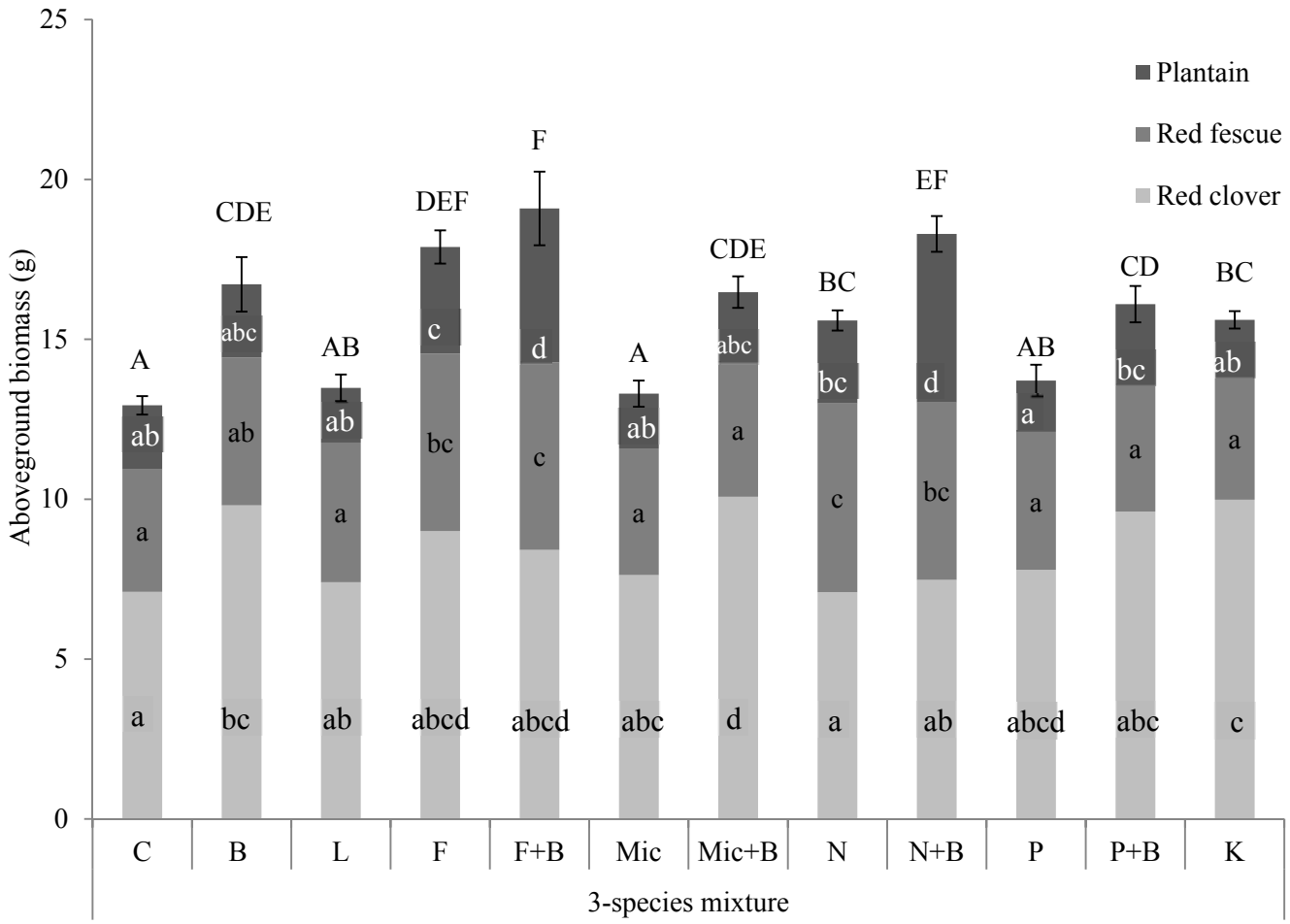
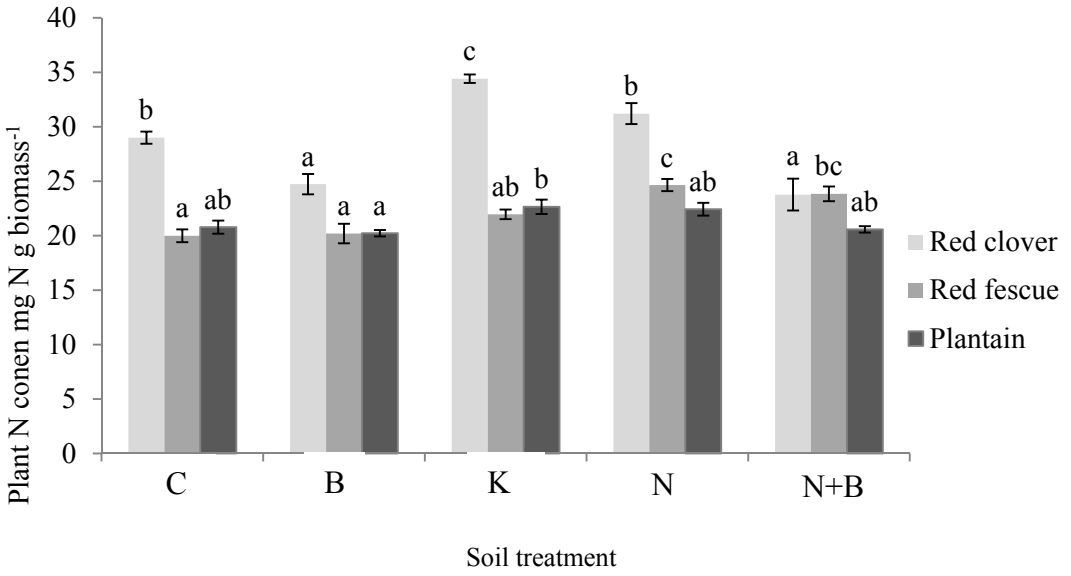

Aboveground biomass (g)
25
20
15
10
5
0
Plantain
Red fescue
Red clover
A
CDE
AB
DEF
F
A
CDE
BC
EF
AB
CD
BC
ab
abc
ab
c
d
ab
abc
bc
d
a
bc
ab
a
ab
bc
c
a
c
bc
a
a
a
a
a
a
a
a
a
a
bc
ab
abcd
abcd
abc
d
a
ab
abcd
abc
c
C
B
L
F
F+B
Mic
Mic+B
N
N+B
P
P+B
K
3-species mixture

Figure 4

(a)


Plant N conen mg N g biomass-1
40
35
30
25
20
15
10
5
0
b
a
ab
a
a
a
c
ab
b
b
c
ab
a
bc
ab
C
B
K
N
N+B
Soil treatment
Red clover
Red fescue
Plantain

(b)

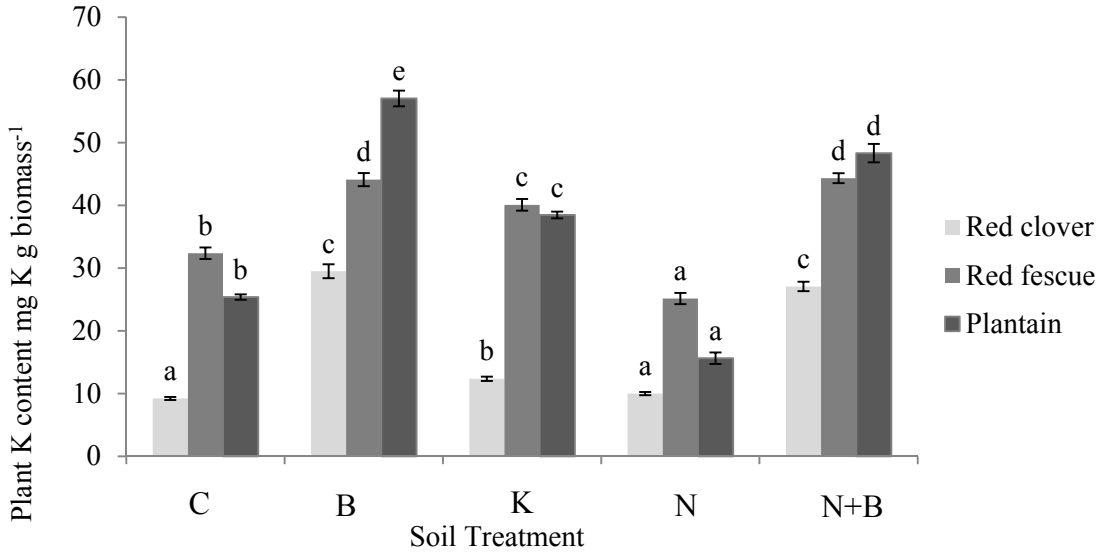
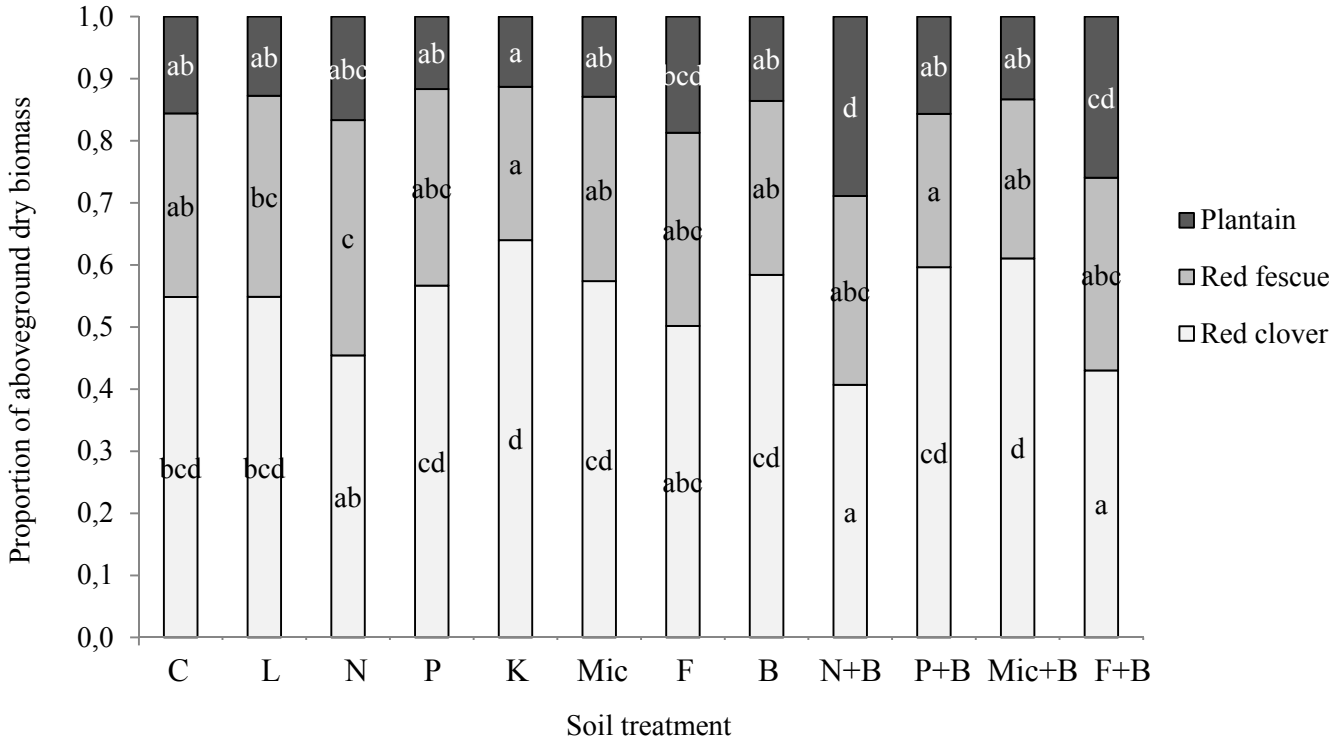

Plant K content mg K g biomass-1
70
60
50
40
30
20
10
0
a
b
b
c
d
e
b
c
c
a
a
a
c
d
d
C
B
K
N
N+B
Soil Treatment
Red clover
Red fescue
Plantain

Figure 5


Proportion of aboveground dry biomass
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
C
L
N
P
K
Mic
F
B
N+B
P+B
Mic+B
F+B
Soil treatment
Plantain
Red fescue
Red clover

Supplementary Tables

Table S1. Mean ± SE available soil chemical parameters measured at harvest in **(a)** sub-experiment I and **(b)** sub-experiment II. The *P* value depicts the results of **(a)** a two-way ANOVA with plant and soil treatment as factors, and **(b)** a one-way ANOVA with soil treatments; for each soil nutrient analysis separately.

Supplementary information; Table S1a

Nutrient	Plant Treatment	Soil Treatment															
		C			L			F			B			F+B			*P* value
pH	Red clover	4.94	±	0.03	5.30	±	0.04	4.87	±	0.06	5.10	±	0.07	5.09	±	0.03	soil < 0.001
	Red fescue	4.99	±	0.07	5.37	±	0.04	5.05	±	0.02	5.38	±	0.07	5.23	±	0.11	plant = 0.001
	Plantain	4.98	±	0.05	5.41	±	0.05	4.95	±	0.03	5.22	±	0.05	5.17	±	0.06	
	Red clover + red fescue	5.03	±	0.01	5.30	±	0.05	4.94	±	0.02	5.18	±	0.03	5.24	±	0.03	
$P\text{-}PO_4$ (mg $P\text{-}PO_4$ kg soil^{-1})	Red clover	3.28	±	0.05	2.68	±	0.04	4.06	±	0.09	4.21	±	0.08	5.12	±	0.17	soil*plant < 0.001
	Red fescue	3.31	±	0.28	2.56	±	0.07	3.63	±	0.10	3.30	±	0.03	3.83	±	0.07	
	Plantain	3.24	±	0.05	2.59	±	0.10	3.94	±	0.20	3.94	±	0.04	4.91	±	0.14	
	Red clover + red fescue	3.05	±	0.04	2.57	±	0.04	3.86	±	0.08	3.93	±	0.05	4.29	±	0.07	
$N\text{-}NO_3^-$ (mg $N\text{-}NO_3^-$ kg soil^{-1})	Red clover	1.30	±	0.30	1.64	±	0.41	1.90	±	0.64	1.87	±	0.27	3.93	±	1.95	soil < 0.001
	Red fescue	0.28	±	0.11	0.58	±	0.17	0.36	±	0.10	0.48	±	0.11	1.86	±	0.66	plant < 0.001
	Plantain	0.36	±	0.12	0.54	±	0.18	0.67	±	0.26	0.70	±	0.29	2.48	±	1.15	
	Red clover + red fescue	0.50	±	0.17	0.60	±	0.21	0.74	±	0.27	0.60	±	0.14	0.88	±	0.31	
N-Nts (mg N-Nts kg soil^{-1})	Red clover	14.86	±	1.98	15.75	±	1.64	15.93	±	1.75	17.31	±	2.07	17.35	±	1.83	soil = nsd
	Red fescue	11.98	±	2.20	11.40	±	2.07	13.58	±	2.85	13.57	±	2.98	15.64	±	2.70	plant = 0.02
	Plantain	11.58	±	2.22	12.57	±	2.33	14.63	±	3.26	13.46	±	3.72	15.23	±	2.51	
	Red clover + red fescue	13.77	±	2.50	12.20	±	1.54	16.04	±	2.53	13.32	±	2.21	13.83	±	1.93	
$N\text{-}NH_4^+$ (mg $N\text{-}NH_4^+$ kg soil^{-1})	Red clover	7.25	±	2.09	7.65	±	2.18	7.63	±	2.20	8.20	±	2.18	6.63	±	1.66	nsd
	Red fescue	6.82	±	2.26	6.20	±	2.08	7.82	±	2.61	8.05	±	2.84	8.10	±	2.78	
	Plantain	6.14	±	2.07	6.74	±	2.31	8.49	±	2.94	7.22	±	3.47	6.94	±	2.49	
	Red clover + red fescue	7.47	±	2.45	5.79	±	1.44	8.96	±	2.76	7.03	±	2.04	6.40	±	1.69	

Table S1b

	pH	P-PO_4 (mg P kg $soil^{-1}$)	N-NO_3^- (mg N-NO_3- kg $soil^{-1}$)	N-Nts (mg N-Nts kg $soil^{-1}$)	N-NH_4^+ (mg N-NH_4^+ kg $soil^{-1}$)
C	5.03 ± 0.04	3.18 ± 0.06	0.42 ± 0.18	12.75 ± 1.79	6.55 ± 1.75
L	5.39 ± 0.09	2.52 ± 0.15	0.52 ± 0.15	15.74 ± 3.25	9.78 ± 3.33
N	4.96 ± 0.03	3.25 ± 0.12	0.50 ± 0.15	15.77 ± 2.57	8.63 ± 2.72
P	5.01 ± 0.01	3.91 ± 0.02	0.40 ± 0.15	14.00 ± 2.46	7.72 ± 2.42
K	4.94 ± 0.02	3.20 ± 0.04	0.44 ± 0.15	14.05 ± 2.58	7.58 ± 2.43
F	4.97 ± 0.01	3.87 ± 0.10	0.38 ± 0.11	12.24 ± 2.19	5.50 ± 1.77
B	5.16 ± 0.05	3.86 ± 0.07	0.52 ± 0.10	16.58 ± 3.70	8.95 ± 3.24
N+B	5.21 ± 0.02	3.87 ± 0.08	1.10 ± 0.36	1.13 ± 1.84	5.57 ± 1.17
P+B	5.20 ± 0.02	4.66 ± 0.08	0.58 ± 0.16	11.88 ± 1.16	5.14 ± 1.11
Mic+B	5.14 ± 0.05	3.92 ± 0.12	0.66 ± 0.13	16.99 ± 3.24	9.59 ± 3.28
F+B	5.17 ± 0.06	4.39 ± 0.06	0.82 ± 0.42	12.24 ± 2.19	6.16 ± 2.27
P value	< 0.001	< 0.001	nsd	nsd	nsd