Biochar application rate affects biological nitrogen fixation in red clover conditional on potassium availability.

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Abstract

Increased biological nitrogen fixation (BNF) by legumes has been reported following biochar application to soils, but the mechanisms behind this phenomenon remain poorly elucidated. We investigated the effects of different biochar application rates on BNF in red clover (*Trifolium pratense* L.) Red clover was grown in mono or mixed cultures with red fescue grass (*Festuca rubra* L.) and plantain (*Plantago lanceolata* L.) at a range of different biochar application rates (0, 10, 50 and 120 t ha^{-1}). In a separate experiment, nutrient effects of biochar on BNF were investigated using nitrogen, phosphorous and potassium (N, P, K) and micronutrient fertilisation using the same plant species.

Biochar addition increased BNF and biochar applied at a rate of 10 t ha^{-1} led to the highest rate of BNF. Total biomass also showed the greatest increase at this application rate. An application rate of 120 t ha^{-1} significantly decreased biomass production in both single and mixed cultures when compared to the control, with the greatest reduction occurring in red clover. Furthermore, BNF was significantly higher in pots in which red clover was grown in mixed cultures compared to monocultures. In the absence of biochar, K fertilization caused a significant increase in BNF. For N, P, and micronutrient fertilization, BNF did not significantly differ between treatments with and without biochar addition.

We conclude that different biochar applications rates lead to different effects in terms of BNF and biomass production. However, due to the high variety of biochar properties, different application rates should be investigated on a case specific basis to determine the optimum biochar application strategies.

1. Introduction

Biochar is charcoal which is made with the intention of applying it to soil. Biochar is often claimed to have several potential benefits, including carbon sequestration (Laird, 2008; Zimmermann et al., 2012); bioenergy generation (Laird, 2008; Lehmann, 2007); adsorbing organic and inorganic pollutants (Hale et al., 2011; Jiang, 2012) as well as improving soil fertility (Jeffery et al., 2011; Spokas et al., 2012).

Soil fertility effects have been explained in terms of inherent nutrient addition with biochar (Parvage et al., 2013) as well as by biochar-induced changes in soil physical, chemical or biological properties (Kookana et al., 2011; Oguntunde et al., 2008; Thies and Rilling, 2009). However, the mechanisms behind the observed yield effects remain unclear. Hypotheses for these effects include improved fertilizer use efficiency by reducing loss of nutrients through leaching (Blackwell et al., 2010; Laird et al., 2010) or increased nutrient availability due to increased microbial activity, such as arbuscular mycorrhizal fungi (AMF) (Warnock et al., 2007). Some studies also suggest that biochar addition to soil can enhance soil fertility through increased biological nitrogen fixation (BNF) when legumes are present (Nishio, 1996; Rondon et al., 2007). However, the mechanisms behind this effect also remain unclear.

Biological nitrogen fixation is estimated to contribute approximately 17.2×10^7 tonnes of nitrogen to soils globally each year (Ishizuka, 1992). Leguminous crops have been estimated to contribute approximately half of the global symbiotic BNF at an estimated 21.5×10^6 tonnes (Herridge et al., 2008). This demonstrates that BNF is an important ecosystem service for global agriculture and as such understanding the possible impacts of biochar application on this service is vital.

Different mechanisms for the observed effect of biochar on symbiotic BNF have been proposed. These include:

- Immobilisation of inorganic N, which is known to stimulate BNF (Rondon et al., 2007), (Bruun et al., 2011; Nelissen et al., 2012).
- Increased nodulation, which has been observed in white clover (*Trifolium repens*) (Rillig et al., 2010), soybean (*Glycine max*) (Ogawa and Okimori, 2010; Tagoe et al., 2008) and alfalfa (*Medicago sativa*) (George et al., 2012).
- Increased P bioavailability (Brewer et al., 2012; Nelson et al., 2011) which has been correlated with increased BNF in several legumes: soybean (Tagoe et al., 2008), common bean (*Phaseolus vulgaris*) (Rondon et al., 2007) and alfalfa (Nishio and Okano, 1991).
- Interactions between biochar and signalling for nodulation through adsorption of flavonoids and Nod-factors (Thies and Rilling, 2009).
- Increased pH, as claimed for the case of soybean (Ogawa and Okimori (2010).
- Introduction of macro and micro nutrients (Brewer et al., 2012; Major et al., 2010; Rondon et al. (2007), which may be beneficial to legumes. Further, as biochar application generally raises soil pH (Hass et al., 2012) micronutrient availability (e.g. Fe and Mn) can also be affected.

There is still a dearth of data on the effects of biochar application to soil on BNF in temperate regions. Furthermore, different effects have been reported with different application rates. For example, increasing biochar application rates has been found to increase BNF (Ogawa and Okimori, 2010; Rondon et al., 2007; Tagoe et al., 2008). However, reduced nodulation has also been reported with elevated application rates, even though nitrogenase activity remained unchanged (Quilliam et al., 2013). The mechanisms behind these changes in BNF remain largely hypothetical. Therefore, there is an urgent need to better understand the mechanisms by which biochar application affects BNF in order to allow robust predictions to

be made. Natural abundance ^{15}N analysis (Unkovich et al., 1994) provides an effective means of quantifying BNF and as such is a useful tool for investigating such mechanisms.

We formulated five hypotheses regarding the mechanism behind the effects of biochar on BNF in red clover (*Trifolium pratense* L.):

H1: Increasing application rates of biochar will increase BNF

H2: N application will negate the effect of biochar on BNF

H3, H4, H5: Fertilization with K (H3), P (H4), or micronutrient fertilizer (H5) will increase BNF to the same level as with biochar.

Two separate microcosm experiments were conducted to test these hypotheses by examining how biochar and fertilization affected plant growth of leguminous and non-leguminous plants.

2. Materials and Methods

2.1 Soil and biochar

The biochar was produced from aboveground plant biomass collected from a species-rich grassland in the nature reserve area De Mossel at Planken Wambuis, Ede, the Netherlands (+52° 3' 34.03", +5° 45' 2.81") via pyrolysis at 400°C. Soil was collected from the same location, air dried, homogenised by sieving through an 8 mm sieve and mixed thoroughly. Selected soil and biochar properties are presented in Table 1 and Table 2.

The homogenised soil was split between two experiments, described below. Pots filled with soil were incubated for 14 days under a polyethylene sheet to germinate weeds and to allow equilibration of the biochar-soil mixture. All seedlings that emerged from the seed bank were removed.

2.2 Experimental set-up

Two experiments were conducted to test the five hypotheses stated in the introduction (H1-H5). Experiment I investigated the effects of increasing biochar application rate on BNF

to test H1. Experiment II investigated the effect of individual fertilizer (N, P, K and micronutrients) in presence and absence of biochar on BNF to test H2-5. Both experiments were set up in a randomized complete block design with five replicates for each treatment.

2.2.1 Experiment I

The experiment was carried out from 23rd May to 18th July, 2012 in a greenhouse at Unifarm of Wageningen University, Wageningen, the Netherlands. The experiment included two factors, biochar application rate and plant species composition.

Five biochar application rates were used: 0 (control), 1, 10, 50 and 120 t ha^{-1}(w/w equivalents) of biochar. The biochar was incorporated into the top 10 cm. The rates were calculated as a soil: biochar ratio using an assumed bulk density of 1.3 g cm^{-3} and assuming that the biochar would have been mixed homogenously through the top 10 cm of the soil. This allowed the calculation of a soil: biochar ratio (w/w) comparable to biochar applied in the field. A soil:biochar mixture of the appropriate ratio for each application rate was then used for filling pots for each application rate. In order to maintain the same final potting volume for all treatments, the volume of soil was adjusted as needed in order to keep the total volume of the soil: biochar mixture and hence the potential rooting depth of the plants the same for all treatments. An amount of 1.75 kg soil-biochar mixture for 0, 1, 10 t ha^{-1}, 1.60 kg for 50 t ha^{-1} and 1.50 kg for 120 t ha^{-1} was loaded into pots of 15 cm diameter × 13 cm height.

Red clover (*T. pratense*) and red fescue grass (*F. rubra*), both being common grassland species in Northern Europe, were planted in single and mixed stands. The seeding densities were equivalent to 952 and 476 kg ha^{-1} for grass and clover in single stands, and equivalent to 476 and 238 kg ha^{-1} for grass and clover in two species mixed stands. Previous experiments have shown that these seedling densities produce fairly even biomass of the two species when grown in mixed stands. Plants were harvested once after 45 days.

The pots were maintained at 60% water filled pore space throughout the experimental period, and gravimetrically kept at constant moisture level on a daily basis. A germination layer of 200 g soil (no biochar) was added to the surface of each pot to reduce the potentially negative effect of biochar on germination of seeds (Rogovska et al., 2012).

2.2.2 Experiment II

Experiment II utilised the mesocosms described in Oram et al. (also published in this special issue) This experiment included nine soil treatments in mixed stands of red fescue grass (*Festuca rubra* L.), red clover (*Trifolium pratense* L.), and plantain (*Plantago lanceolata* L.). The soil treatments were biochar, N, P, K, micronutrients fertilization, biochar in combination with N, P, micronutrients and control (no amendment) (Table 1). The rate of fertilizer applications were N- 50 kg ha^{-1}; P- 30 kg ha^{-1}; K- 50 kg ha^{-1} equivalents and for micronutrients the rate was (B- 0.76 kg ha^{-1}; Mn- 0.78 kg ha^{-1}; Cu- 0.03 kg ha^{-1}; Zn- 0.78 kg ha^{-1}; Mo- 0.016 kg ha^{-1}). The rate of biochar was 10 t ha^{-1} assuming the top 10 cm as soil depth and a bulk density of 1.3 g cm^{-3}.

We used Mitscherlich pots (diameter: 19 cm, height: 22 cm; filled with 7.0 kg dry soil). Each pot had three layers: a bottom layer with 4.5 kg of soil, a middle layer with 1.5 kg of soil and a top layer (germination layer) with 1.0 kg of soil. Biochar was mixed thoroughly in both top and middle layer, resulting in a 10 cm biochar/soil layer, while fertilizers were mixed only with the middle layer consisting of 1.5 kg soil which was then covered with a top layer consisting of 1.0 kg of soil to function as a germination layer.

Two plant species were then sown; red fescue grass (*Festuca rubra* L.) and red clover (*Trifolium pratense* L.). The seeding densities were equivalent to 952 and 476 kg ha^{-1} for grass and clover in single stands and 476 and 238 kg ha^{-1} for grass and clover in the two species mixed stands, the same as for Exp. I. Pots were maintained at 60% water filled pore

space throughout the experimental period, by watering daily and reweighing. Every week, pots were re-randomized within blocks.

The effects of biochar application and nutrients on plant biomass are reported in more detail in Oram et al. (in this special issue). Here we report the effect of individual nutrients (N, 50 kg ha^{-1}; P, 30 kg ha^{-1}; K, 50 kg ha^{-1} equivalents) and micronutrients (B, 0.34 mg kg^{-1}; Mn, 0.35 mg kg^{-1}; Cu, 0.014 mg kg^{-1}; Zn, 0.35 mg kg^{-1}; Mo, 7.5 µg kg^{-1}) on BNF in the presence and absence of biochar using nine soil treatments in mixed stands of clover, grass and plantain. The soil treatments were biochar, N, P, K, micronutrients fertilization, biochar in combination with N, P, micronutrients and control (Table 1). Total aboveground biomass was harvested twice; after 28 days and after 56 days of growth. Data of the summed biomass are used.

2.3 Biomass measurements and nodule count

For both experiments, at harvest, aboveground biomass was sorted by hand to plant species. Roots of each pot were cleaned with running water and manually sorted according to species. Biomass was dried at 70°C for 72 hours and root and shoot dry weight was determined for each species. For the roots of Experiment I, the total number of nodules per g fresh weight of clover roots was determined for sub-samples of fresh root material. The number of nodules g^{-1} dry root mass was subsequently calculated.

2.3 Plant analysis

Dry plant material from both harvests was sorted per species, ground, and combined at a ratio based on percentage of total biomass harvested. Plant material was digested using a sulphuric acid (H_2SO_4), hydrogen peroxide (H_2O_2) and Selenium (Se) digestion (Temminghoff and Houba, 2004). N and P concentrations in the aboveground plant biomass were quantified by segmented flow analysis (SFA) with a Skalar 6 channel SFA analyser.

2.4 Soil analysis

Before sowing (Experiment II) and at final harvest (Experiments I and II), five random soil samples from each pot were collected using an auger (1.0 cm diameter). The soil samples were combined to obtain a representative sample of each pot. The combined soil samples were dried at 40°C for 72 hours and sieved to pass 5 mm before analysis. Soil pH ($CaCl_2$) was determined with a ratio of 1:10 (m/v) (ISO10390, 2005). Soil electrical conductivity (EC) was measured with demineralized water 1:5(m/v) (Houba et al. 2000).

2.5 Stable isotopes

Use of natural abundance ^{15}N analysis allows the identification of the source of nitrogen present in legumes, and the proportion that it derived from the atmosphere (i.e. through BNF) resp. from the soil N pool (Unkovich et al., 1994).

For ^{15}N and ^{13}C analysis ($\delta^{13}C$ analysis was undertaken as an indicator of salinity stress (Farquhar et al., 1989) which can occur due to salts in the ash which are added with the biochar), whole samples of dried aboveground biomass of each species from each pot were ground with a Cyclon Sample Mill. Subsamples (in case of Exp. II, samples from both the first and final harvest combined proportionately; see Oram et al., in this special issue, for further details) of the aboveground biomass were subsequently ball-milled. The milled samples were then dried at 105°C for 24 hours. A subsample of 3-4 mg was placed in a tin cup (8 mm × 5 mm) and the exact weight was recorded. The Stable Isotope Facility of UC-Davis, Davis, California, USA determined the $^{14/15}N$ and $^{12/13}C$ ratios using a continuous flow isotope ratio mass spectrometer (CF-IRMS, Europa Scientific, Crewe, UK).

The $\delta^{15}N$ was calculated using the equation of Peoples et al. (1989). This allowed calculation of the % of N derived from the atmosphere (%Ndfa; i.e. the portion of N obtained through BNF). Grass was used as reference plant in the experiment with grass and clover (Exp.-I), while the average $\delta^{15}N$ value of grass and plantain was used in experiment with

grass, clover and plantain mixed stands (Exp. II). The lowest $\delta^{15}N$ value (-1.958 for Exp. I and -2.778 for Exp. II) in clover was approximated to be the B value in each of the experiments (Eriksen and Høgh-jensen, 1998; Huss-Danell and Chaia, 2005). The amount of N fixed pot^{-1} was calculated using the equation of Handarson et al. (1987).

2.6 Statistical analysis

All statistical analyses were performed using SPSS (version 19: Scientific Graphing Software, SPSS Inc. Chicago, IL). Both soil treatment and plant species combination were included as fixed factors (two-way ANOVA) for Experiment I while soil treatment was used as factor for Experiment II (one-way ANOVA). Blocking was included as a random factor during analysis. Normality of data was checked with a Q-Q plot of residuals while Levene's test was used to check equal variance. Data of %Ndfa, number of nodules g^{-1} dry root, and EC in Exp. I were log-transformed to achieve a normal distribution of residuals. Individual comparisons were based on a Tukey HSD *post-hoc* test ($P \leq 0.05$).

3. Results

3.1 Experiment I: Biomass production at increasing biochar application rates

Both aboveground and total biomass production were significantly affected by biochar application rate, plant species combination and their interaction (all $P<0.01$). In all instances the largest increase in biomass in terms of both aboveground (Fig. 1a) and total biomass (Fig. 1b) occurred at 10 t ha^{-1} of biochar. At this rate, biomass was significantly higher than the control ($P<0.01$ for aboveground biomass, $P=0.02$ for total biomass production). Furthermore, the interaction between plant species and biochar application rate was significant ($P<0.01$) indicating a differential response of plant species to different biochar application rates. This can be seen in Fig. 1 where clover shows a stronger response to biochar application than *Festuca*. At 50 t ha^{-1}, none of the biomass results were significantly different from the control ($P>0.05$). At 120 t ha^{-1}, the biomass of clover, grass, and grass clover in mixed stands were

significantly reduced compared to the control ($P<0.01$). There were no significant differences in the relative yield total (RYT) or the ratio of clover to grass in the mixed stands under different biochar application rates ($P=0.42$ for RYT; $P=0.12$ for clover to grass ratio; data not shown).

As with biomass, both %Ndfa and total amount N fixed per pot increased significantly with applications of 10 t ha^{-1} (Fig. 2a and 2b; $P=0.03$ and $P<0.01$ respectively). The amount of N fixed per pot was lowest at the application rate of 120 t ha^{-1}. The %Ndfa did not change significantly above 10 t ha^{-1} up to the rate of 120 t ha^{-1} ($P>0.05$), the maximum rate used in this study. At 10 t ha^{-1}, the amount of N fixed per pot (average of both single and mixed stand) increased significantly by 117% ($P<0.01$), while %Ndfa increased significantly by16% (Fig. 2a and 2b) when compared to controls ($P<0.01$). The %Ndfa increased significantly when red clover was grown in mixed stands compared to the single stands (Fig 2a; $P<0.01$). However, the total amount of N fixed per pot was reduced in the mixed stands at all application rates when compared to red clover grown in single stands (Fig. 2b; $P<0.01$).

Nodule production per g dry weight of clover roots was significantly affected by both rate of biochar application, and plant species combination (Fig. 3; $P<0.01$ in all cases). The density of nodules in single stands increased by 72% at 10 t ha^{-1} and 80% at 50 t ha^{-1}. The density of nodules in the single stands (1641 g^{-1} root dry weight) was almost twice as high as in the mixed stand (834 g^{-1} root dry weight) ($P<0.01$).

Both plant N concentrations also increased significantly when biochar was applied at a rate of 120 t ha^{-1} ($P<0.01$; Fig. 4a). However, no significant effect was seen on plant P concentrations ($P=0.08$; Fig. 4b).

Soil electrical conductivity (EC) increased by 14 times at the highest biochar application of 120 tha^{-1} ($P<0.01$; data not shown). In the grass treatment, there was a significant increase in $\delta^{13}C$ (‰) with increasing application ($P<0.01$), while in case of clover

the $\delta^{13}C$ (‰) was not significantly affected (P=0.44), with values ranging from -31.49 to -31.45‰. Despite using a top layer of untreated soil, significantly fewer plants germinated in the 120 t ha^{-1} pots than at the lower application rates (P<0.05; data not shown).

At the highest application rate of 120 t ha^{-1}, we observed red colour development at the edges of the lower leaves in clover.

3.2 Experiment II.

Both the amount of N fixed per pot and the %Ndfa were significantly affected by the different fertilizer treatments in both the presence and absence of biochar (Fig. 5a and 5b; P<0.01 for %Ndfa and P=0.03 for N fixed pot^{-1}). Biochar addition at a rate of 10 t ha^{-1} did not lead to an increase in the amount of N fixed per pot or %Ndfa when compared to the control, phosphorus or micronutrient fertilization treatments (P>0.05).

K fertilization showed the strongest effect on the amount of N fixed per pot with an average, statistically significant, increase of 78% compared to the control (P<0.01). However, biochar in combination with N fertilizer reduced %Ndfa by 22% when compared to the control. Neither %Ndfa nor the amount of N fixed per pot were significantly different when the N, P, and micronutrient fertilization treatments were compared to respective biochar treatment combined with N, P, or micronutrient fertilization treatments (Fig. 5a and 5b).

4. Discussion

4.1 The effects of biochar application rate

Compared to the control, %Ndfa increased significantly up to the biochar application rate of 10 t $ha^{-1}$and remained at the same level with increasing application rates up to 120 t ha^{-1}, the maximum application rate used in this experiment. Therefore H1 (Increasing application rates of biochar will increase BNF) should be accepted but only up to a biochar application

rate of 10 t ha^{-1}. Above the rate of 10 t ha^{-1} no significant effects were seen on BNF compared to the control. This implies that the positive effect observed at 10 t ha^{-1} was negated by an as yet unidentified negative effect at rates above 10 t ha^{-1}. The lack of an increase in %Ndfa at a biochar application rate of 10 t ha^{-1} observed in Exp. II is likely an artefact of the increased rooting depth available for the plants owing to the larger pot size used which lead to an approximate 3 fold increase in the soil volume. As a consequence in Exp 2, a higher total amount of N was available in each pot and so N was less limiting. When N is not limiting BNF is often down regulated as it is a more energy intensive way of obtaining N than taking it up directly from the soil (Ingestad, 1980).

Increased BNF in the presence of biochar has been reported before by a number of studies (Nishio, 1996; Quilliam et al., 2013; Rondon et al., 2007). In one study in which the common bean was grown, an increase in BNF was observed until 60 g biochar kg^{-1} soil (approximate equivalent to 78 t ha^{-1}, assuming bulk density 1.3 g cm^{-3}) (Rondon et al. 2007), while Tagoe et al. (2008) found an increased BNF in soybean even at 100 t ha^{-1}. This variation in results between studies may be the result of differences in biochar properties and soil nutrient status, or can be due to differential responses of plant species to biochar application. In contrast, in our study, the amount of N fixed per pot decreased at application rates above 10 t ha^{-1} and was not significantly different from the control at 50 t ha^{-1} and 120 t ha^{-1}. The amount of N fixed per pot (average of single and mixed stands) decreased by 52% at the highest application rate compared to the maximum N fixed per pot (achieved at a biochar application rate of 10 t ha^{-1}) This reduction was likely due to decreased biomass at higher application rates meaning less N fixed per plant as the plants were smaller.

Biochar application at increasing application rates led to an apparent optimum in both aboveground and total biomass production at 10 t ha^{-1} and subsequently declined at 120 t ha^{-1} in clover, grass, and clover-grass mixed stands compared to controls. Van de Voorde et al.

(2013) also found increased clover biomass in a plots with biochar applied at 10 t ha^{-1} in a field experiment. Other studies have also reported increased biomass production at higher application rates. These include: the common bean (*Phaseolus vulgaris* L.) at an application rate of 60 g kg^{-1} soil (approximate equivalent to 78 t ha^{-1}); white clover at an application rate of 50 t ha^{-1} (Quilliam et al., 2013) and lettuce (*Lactuca sativa*) at a biochar application rate of up to 10% w/w (approximately equivalent to 130 tha^{-1} assuming bulk density 1.3 g cm^{-3} and soil layer 10 cm) (Deenik et al., 2010). However, it is important to note that in these studies different types of biochar were used which can have very different physical and chemical properties (Jeffery et al. 2013).

4.2 Mechanisms

The decrease in biomass production that we observed at application rates of 120 t ha^{-1} can be attributed to reduced germination of plants as well as to a reduced growth of plants that did germinate. Other studies, such as Revell et al. (2012a) also reported reduced germination rates at the high application rates of biochar of 5% w/w (equivalent to approximately 75 t ha^{-1}).

Another factor which may be responsible for the reduced biomass and associated reduction in N-fixation per pot is salinity stress. Increases in soil salinity have been reported following biochar application to soil (Clay and Malo, 2012; Revell et al., 2012b). Further, salinity has been shown to negatively affect BNF (Figueiredo et al., 1999; Serraj et al., 1998). In our experiment, soil EC increased significantly by 14 times at application rates of 120 t ha^{-1} compared to the control.. However, the EC values did not exceed 0.6 dSm^{-1}, which is below the threshold EC of 1.5 dSm^{-1} at which negative effects have been reported for red clover (Maas, 1993). Therefore, salinity is unlikely to have affected growth or BNF in our experiment. In addition, the $\delta^{13}C$ (‰), an indicator of salinity stress (Farquhar et al., 1989;

van Groenigen and van Kessel, 2002), was not significantly higher at the high application rates, providing further evidence that salinity stress is unlikely to have been responsible for the observed result.

Biochar addition lead to increase in soil pH from 4.9 to 7.08 at the highest application rate of 120 t ha^{-1} (data not shown). While this pH change is likely to have been sufficient to affect nutrient availability in the soil, a pH of 7.08 is not usually detrimental to plant growth and so does not explain the negative effects on plant growth observed at the higher application rates.

Blackwell et al. (2010) reported that biochar application lead to reduced biomass production in wheat as a result of N and P immobilisation.. Similarly, reduced biomass production due to N immobilisation has been reported for sugar beet following applications of biochar at a rate of 10 t ha^{-1} (Gajić and Koch, 2012). In clover, we found a significant increase (9%) in shoot N concentration compared to the control following application of 120 t ha^{-1}, while P levels remained unchanged suggesting that immobilisation of N and P were not responsible for the reduced biomass in our experiment.

Rondon et al. (2007) claimed toxicity of biochar as one of the possible mechanisms for reduced biomass production in soils with biochar amendment. A phytotoxic effect of compounds released from biochar was also demonstrated by Gell et al. (2011) who observed immediate phytotoxicity in lettuce, radish (*Raphanus sativus* L.), and wheat (*Triticum aestivum* L.) after the application of biochar produced as a rest product of biodiesel and bioethanol production. Biochar contains compounds such as aliphatic and aromatic hydrocarbons including phenols which can be toxic at sufficient concentrations. The concentration of these chemicals may have been sufficiently high to cause phytotoxicity at the higher application rates used in our experiment. However, further work is needed to investigate this hypothesis.

We observed red colour development at the edges of the lower leaves in clover at the application rate of 120 t ha^{-1} which suggests some nutritional toxicity or deficiency occurred. In Exp. II the K concentration in the soil was significantly higher (192 mg kg^{-1} soil) at the biochar application of 10 t ha^{-1} compared to the control (29.10 mg kg^{-1} soil) (See Oram et al., 2013 for further details). Assuming linearity, the K concentration in Exp. 1 would have been more than 2.0 g kg^{-1} soil at the highest application rate. Therefore, K toxicity seems to be a potential mechanism which may have caused the growth reduction at the higher biochar applications in Exp. 1. However, evidence for K toxicity affecting plants is very limited and such a hypothesis requires further experiments to test it.

Fertilization with N did not significantly affect %Ndfa or the amount of N fixed per pot compared to addition of biochar alone. However, N combined with biochar led to a significant decrease in %Ndfa and N fixed per pot compared to the addition of biochar alone. This leads us to accept H2 that N application will negate the effect of biochar on BNF. Biochar in combination with N fertilizer decreased %Ndfa by 22% compared to the control treatment. Total soil N concentration did not change with biochar in combination with N fertilization compared to N fertilization alone although it was significantly different compared to the control. This suggests that N was available in the biochar to a degree such that when combined with N fertilizer there was sufficient N in the system leading to reduced BNF. Furthermore, we found a significant increase in shoot N concentration following biochar amendment of 120 t ha^{-1} in Exp. 1 suggesting that N availability increased in this treatment. These results contrast with those of Rondon et al. (2007) who reported that the supply of N from the soil decreased following biochar amendment, and these authors suggested that biochar application led to N immobilization. This decrease resulted in a lower foliar N concentration (in common bean), which was not completely compensated for by increased BNF in their study. These differences demonstrate that biochar effects can differ greatly

between studies and depend on the type of biochar and characteristics of the soil and plant species used.

We found in Exp. 2 that the highest amount of fixed N per pot occurred in the treatments receiving K fertilization . Biochar addition has been found to increase soil K status in other experiments (e.g. Parvage et al., 2013; Spokas et al., 2012). Furthermore, Sangakkara et al. (1996) demonstrated that K availability can increase BNF. We also found that biochar increased K concentrations in the soil (See Oram et al., 2013 in this special issue for further details). However, a significant increase in both the amount of N fixed per pot and in %Ndfa compared to the control were only observed in the K treatment and not in the other biochar treatments even though biochar application also introduced large amounts of K to the soil (Oram et al., 2013). This suggests that following K fertilization, in the absence of biochar, clover gained a competitive advantage over the *Festuca* and *Plantago*. This is demonstrated by the increase in the amount of N fixed per pot which occurred due to the increase in clover biomass while the %ndfa did not significantly increase compared to the control. In contrast, in pots where biochar was added, *Festuca* and *Plantago* gained a competitive advantage over clover and this counteracted the advantage that clover gained from the increase in available K. This increase in competition led to a reduction in total N fixed per pot and in %Ndfa in all of the biochar treatments, so that N fixation and %Ndfa were not significantly different from the control any more. This suggests that increased K availability through biochar addition to soil, was the main factor affecting BNF and so we accept H3 that fertilization with K will increase BNF. However, the data further suggest that biochar addition to the soil increased the competitiveness of both *Festuca* and *Plantago*, or decreased the competitiveness of the clover compared to the addition of K alone. Potential mechanisms for these differential effects of K availability on competitiveness of plant species are discussed in more detail in Oram et al. (2013).

Increased P availability has been suggested to be responsible for increased BNF following biochar addition in some situations (Rondon et al., 2007; Tagoe et al., 2008). We found no significant effect of P fertilization on BNF. BNF did not increase significantly with a combination of biochar and P, although there was a significantly higher amount of P in soils with biochar with P fertilization compared to P fertilization alone. Furthermore, shoot P-concentrations of plants in our study were not significantly affected by biochar addition in our study. This suggests that the effects of biochar on BNF cannot be explained in terms of increased P availability in our study. Therefore we reject H4 that fertilization with P will increase BNF.

Micronutrient application, either alone or in combination with biochar did not result in an increase in BNF compared to the control. Therefore, it is unlikely that increased micronutrient availability was the mechanism driving increased BNF in our study and as such we reject H5 that micronutrient fertilizer will increase BNF. This contradicts the potential mechanism reported by Rondon et al. (2007) who stated that micronutrient availability (in particular B, and Mo) was the most likely factor leading to increased BNF following biochar addition.

It has been reported that biochar can enhance BNF by stimulating signalling for nodulation with adsorption of flavonoids and Nod factors (Thies and Rilling, 2009; Turner, 1955), although evidence for reduced nodulation has also been reported (Quilliam et al., 2013). In our experiment, root nodulation increased with biochar application rates of 10 t ha^{-1} in single stands and remained stable until 50 t ha^{-1} before declining at 120 t ha^{-1}. Similar results have also been reported by a number of authors (Ogawa and Okimori, 2010; Tagoe et al., 2008; Turner, 1955). Quilliam et al. (2013) reported a negative effect of biochar on nodulation in white clover with biochar addition. If this is the main mechanism by which biochar stimulates BNF, higher application rates should lead to increased nodulation, but this

was not observed in our study. This suggests that adsorption of flavonoids and Nod factors, and the stimulating role of this on nodulation, are unlikely to be main mechanisms by which biochar affects BNF.

5. Conclusions

Biochar application increased biomass of clover at a rate of 10 t ha^{-1} and reduced it at 120 t ha^{-1}. Biochar application at a rate of 10 t ha^{-1} or higher lead to statistically significant increases in BNF when compared to the control. This means that our study provides support for Hypothesis 1, that increasing application rates of biochar will increase BNF, although with the caveat that above 10 t ha^{-1} BNF did not further increase with increasing rates. Nodulation and BNF also increased significantly at an application rate of 10 t ha^{-1} and reduced at an application rate of 120 t ha^{-1}. The observed increases appeared to occur due to increased availability of K. Therefore, the results of our study lead us to accept the hypothesis that BNF increases with K fertilization and reject our hypotheses regarding an increase in BNF with biochar combined with P and micronutrients and a decrease in combination with N fertilization. Our results imply that the effects of biochar application on BNF may be short lived as K is very mobile in soil. Longer term experiments are needed to investigate the longer term effects of increased K availability on BNF following biochar application as well as its interaction with competition effects.

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Table 1. Selected soil characteristics of the base soil used for both Experiment I and II.

Soil Characteristics	
pH	5.24
N-NH_4 (mg N kg^{-1})	2.5
N-NO_3+NO_2 (mg N kg^{-1})	20.0
N-DON[1] (mg N kg^{-1})	4.0
P-PO_4 (mg P kg^{-1})	3.28
K^+ (mg K kg^{-1})	29.1
EC (dS m^{-1})	0.08

Table 2. The different factors, biochar application rates and fertilizer treatments used in the two experiments.

Factors	**Exp. I**	**Exp. II**
A. Soil Treatment	1. 0 (Control) 2. 1 t ha^{-1} biochar 3. 10 t ha^{-1} biochar 4. 50 t ha^{-1} biochar 5. 120 t ha^{-1} biochar	1 Biochar (10 t ha^{-1}) 2. N fertilizer (50 kg ha^{-1}) 3 P Fertilizer (30 kg ha^{-1}) 4 K fertilizer (50 kg ha^{-1}) 5 Micronutrient fertilizer (B – 794 g, Cu – 32.4 g; Mo 17.0 g; Mn – 803 g; Zn 79.7 g ha^{-1})
B. Plant species composition	1 Red clover (*Trifolium pratense)* 2. Grass (*Festuca rubra*) 3. Clover and grass mixed stands	1. Red clover (*Trifolium pratense),* Grass (*Festuca rubra*) and plantain (*Plantago lanceolata)* mixed stands

Table 3. Selected characteristics of the biochar produced at 400°C through slow pyrolysis from combined various grassland species

Biochar characteristics	
Volatile matter content	32.1% (S.E.= 1.89)
Ash	25.22% (S.E.= 5.01)
N	1.91% (S.E.= 0.09)
C	59.02% (S.E.= 1.35)
H	3.81 (S.E.= 0.06)
S	0.00% (S.E.= 0.0)
H/C	0.77 (S.E.= 0.03)
Mineral N	0.8 mg kg^{-1} (S.E. 0.03)
K	1620.8 mg kg^{-1} (S.E.= 24.4)
$P\text{-}PO_4$	1.9 mg kg^{-1} (S.E.= 0.02)

Table 4. Results of biochar application to soil at different application rates on clover grown in Exp. I.

	Aboveground biomass			Total biomass			Number of Nodules		% Ndfa		N fixed per pot	
Biochar Application Rate	Clover	Grass	Clover + Grass	Clover	Grass	Clover + Grass	Clover	Clover+ Grass	Clover	Clover+ Grass	Clover	Clover+ Grass
0	4.11 (±0.56)	3.97 (±0.04)	3.98 (± 0.18)	5.11 (± 0.69)	8.2 (± 0.21)	7.18(±0.27)	1336 (± 278)	852 (± 84)	35.85 (± 3.62)	67 (± 2.83)	31.49 (± 7.37)	13.17 (± 2.87)
1	5.7 (±0.25)	4.178 (± 0.17)	4.654 (±0.21)	7.01 (± 0.29)	8.76 (± 0.39)	7.19 (± 0.16)	1644 (± 314)	971 (± 164)	41.21 (± 2.64)	66.25 (± 1.28)	55.21 (± 9.82)	14.45 (± 2.32)
10	6.27 (±0.31)	4.45 (± 0.12)	5.10 (± 0.25)	7.45 (± 0.32)	10.73 (± 0.79)	7.63 (± 0.16)	2223 (± 4240	1488 (± 214)	47.88 (± 1.34)	75.73 (± 1.47)	71.91 (± 13.69)	25.15 (± 3.59)
50	4.06 (±0.26)	3.70 (±0.15)	3.81 (± 0.11)	4.79 (± 0.32)	6.31 (± 0.41)	5.83 (± 0.320	2403 (±736)	588 (± 132)	45.89 (± 3.01)	74.95 (± 6.18)	49.82 (± 11.31)	12.27 (± 2.87)
120	0.76 (±0.20)	2.68 (± 0.15)	1.80 (± 0.25)	0.90 (±0.24)	4.21 (± 0.37)	2.89 (±0.51)	604 (±231)	276 (± 88)	49.51 (± 2.59)	70.36 (± 9.51)	13.87 (± 3.98)	7.74 (± 2.84)

Figure 1. Mean ±SE aboveground (a) and total (b) biomass production of clover, grass and mixed stands of both species in soil amended with different biochar application rates (t ha^{-1}), n=5; (Exp. I)

Figure 2. Mean±SE percentage N derived from the atmosphere (%Ndfa) (a) and amount of N fixed pot^{-1} (b) in clover in monoculture and in a mixed culture of clover and grass in soil amended under different biochar application rates (t ha^{-1}), n=5; (Exp. I)

Figure 3. Mean±SE nodule production (g^{-1}root dry weight) in clover in single stands of clover and mixed stands of clover and grass under different biochar application rates, n=5; (Exp. I)

Figure 4. Mean±SE nitrogen (a) and phosphorous (b) shoot content from clover grown in soil/biochar mixtures at 0, 10 and 120 t ha^{-1} biochar application rate equivalents, n=5. Bars indicating the same letter were not significantly different (P>0.05) (Exp. I)

Figure 5. Mean±SE percentage N derived from the atmosphere (%Ndfa) (a) and amount of N fixed pot^{-1}(b) by clover in 3 species mixtures under different fertilization treatments: Biochar (B), Control (C), K fertilization (K), N fertilization (N), N fertilization with biochar (N+B), P fertilization (P), P fertilization with biochar (P+B), Micronutrient (Mic),Micronutrients with biochar treatment (Mic+ B), n=5. Bars indicating the same letter were not significantly different (p>0.05) (Exp. II)

Fig 1a

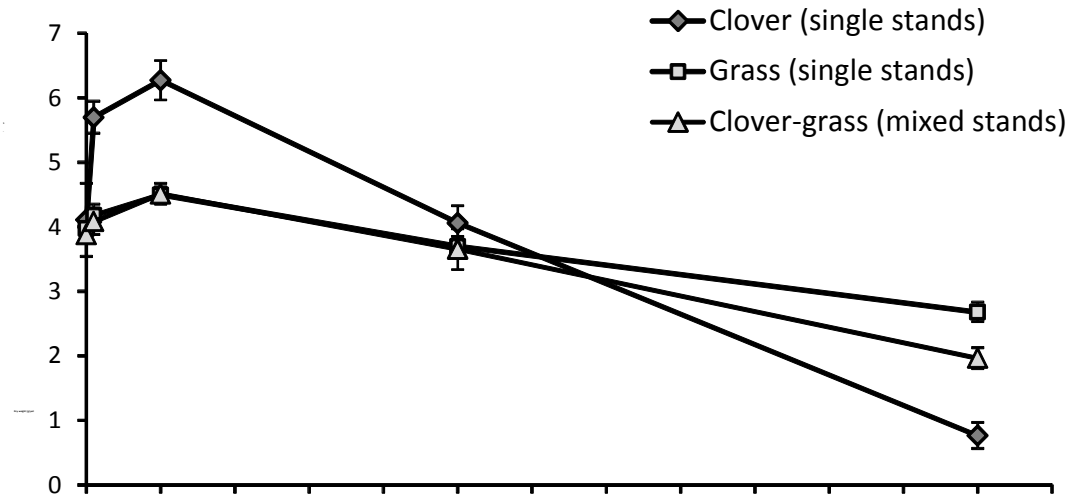
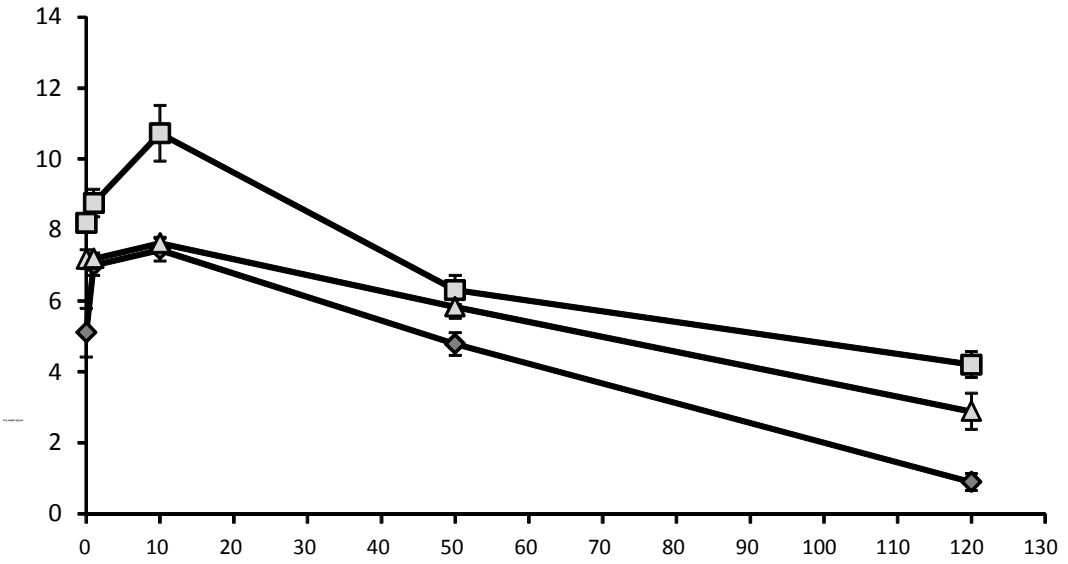


Fig 1b



Biochar application rate (t ha^{-1})

Fig 2a

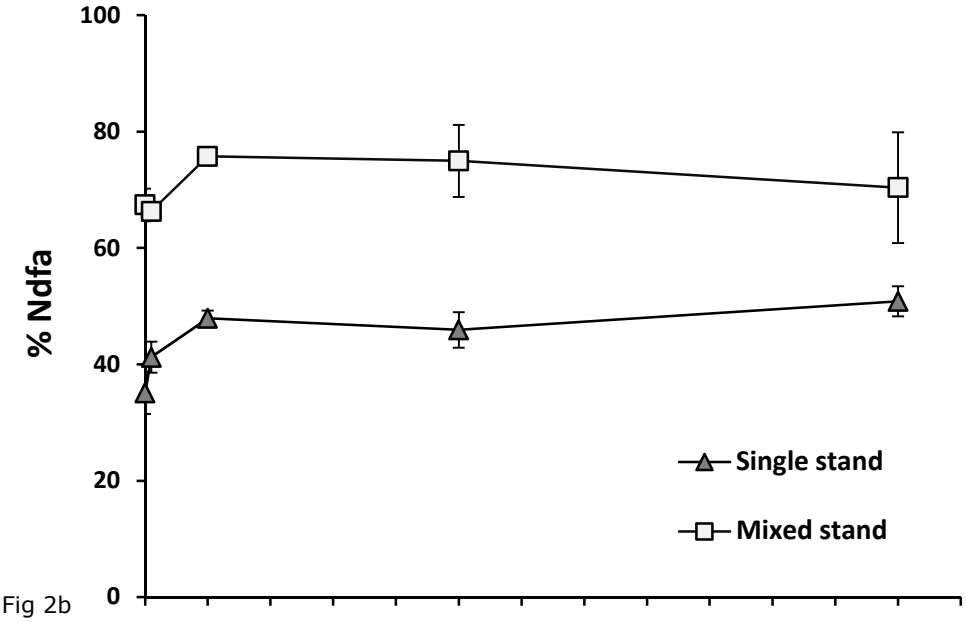

100
80
60
40
20
0
% Ndfa
Single stand
Mixed stand

Fig 2b

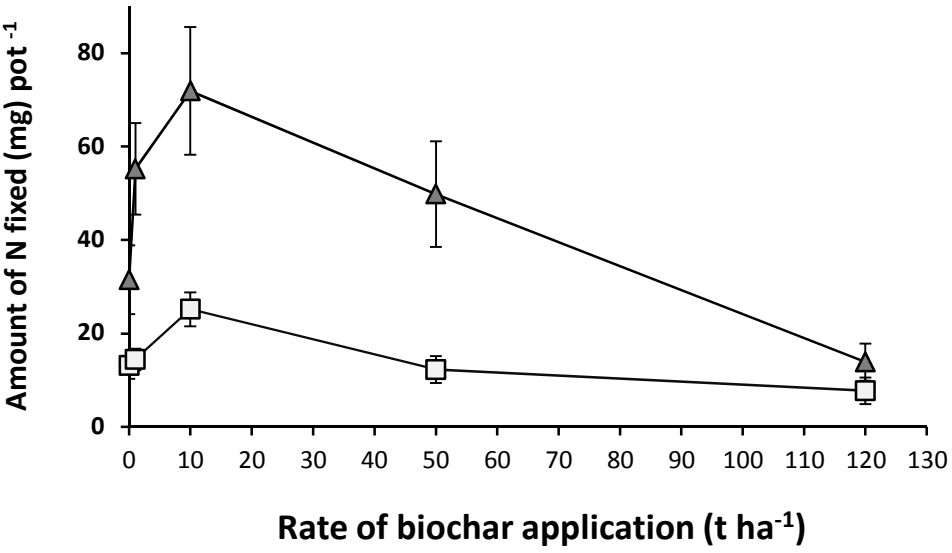

Amount of N fixed (mg) pot -1
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Rate of biochar application (t ha-1)

Fig 3

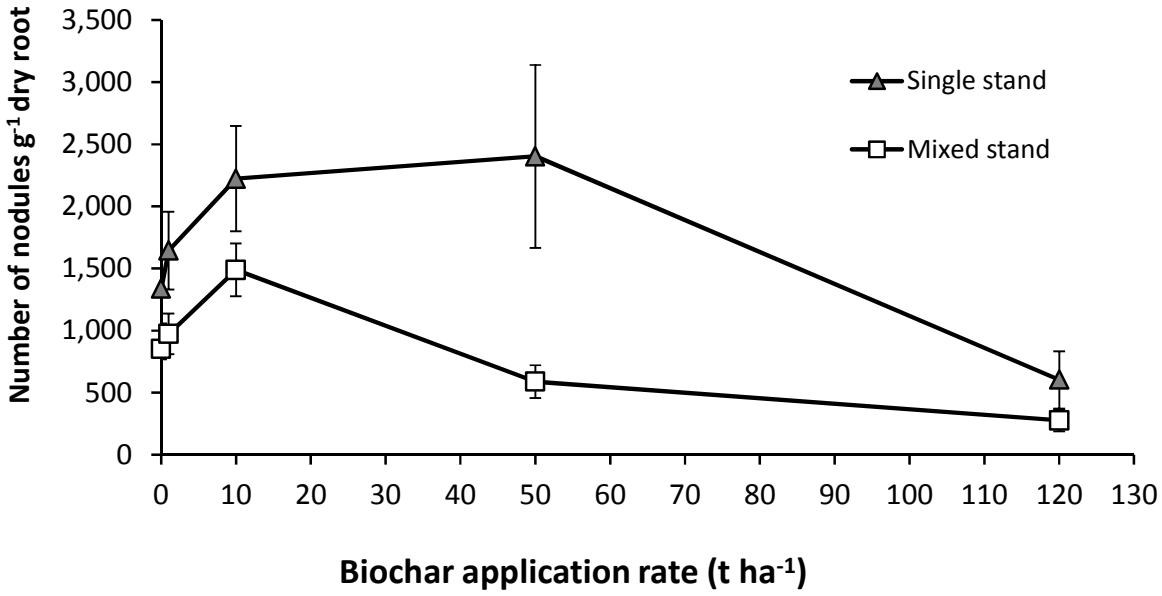

Number of nodules g-1 dry root
3,500
3,000
2,500
2,000
1,500
1,000
500
0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Biochar application rate (t ha-1)
Single stand
Mixed stand

Fig 4a

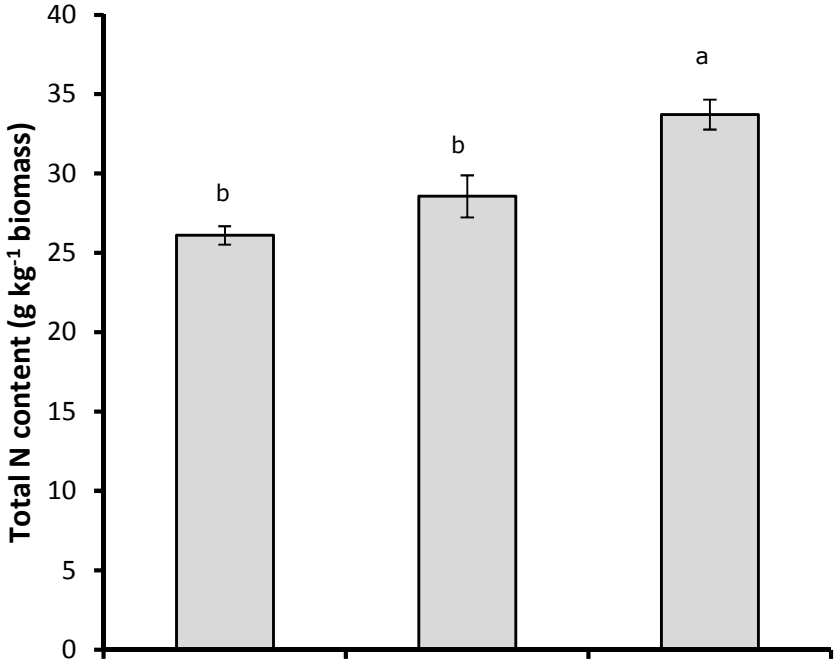

40
35
30
25
20
15
10
5
0
Total N content (g kg-1 biomass)
b
b
a

Fig 4b

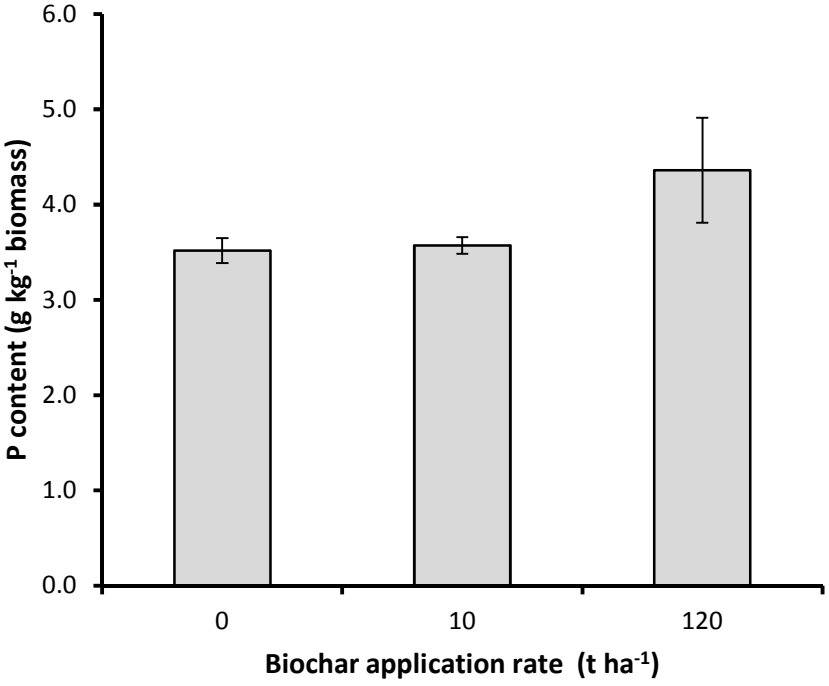

6.0
5.0
4.0
3.0
2.0
1.0
0.0
P content (g kg-1 biomass)
0
10
120
Biochar application rate (t ha-1)

Fig 5a

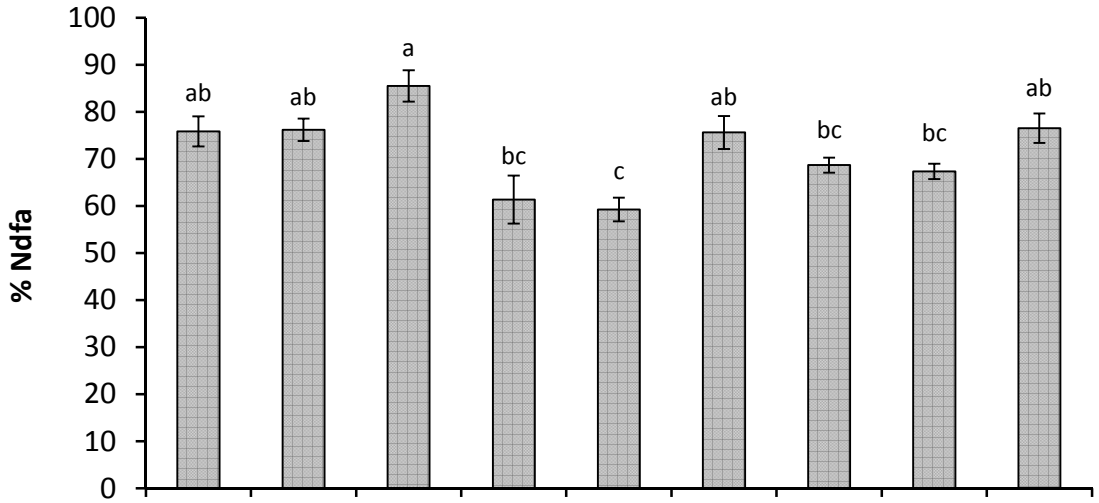
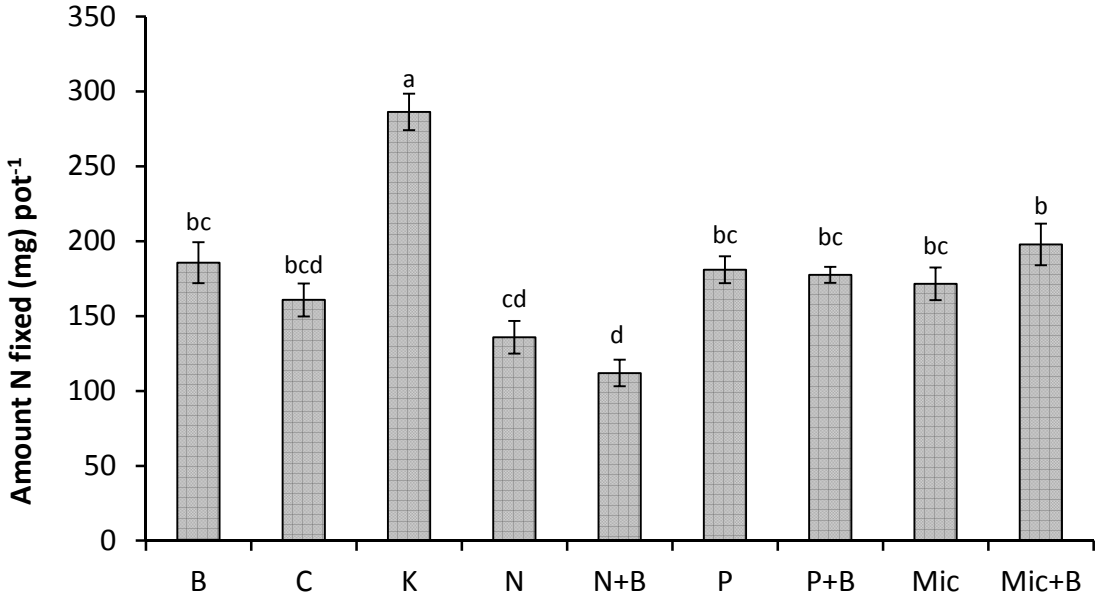

% Ndfa
100
90
80
70
60
50
40
30
20
10
0
ab
ab
a
bc
c
ab
bc
bc
ab

Fig 5b


Amount N fixed (mg) pot-1
350
300
250
200
150
100
50
0
bc
bcd
a
cd
d
bc
bc
bc
b
B
C
K
N
N+B
P
P+B
Mic
Mic+B