# INTERACTIONS BETWEEN PLANT GROWTH PROMOTING MICROORGANISMS (PGPM) AND BIOCHAR

**BY:**

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# ABSTRACT

Rhizobia are frequently used in the agriculture sector to enhance legume growth and improve soil fertility. There is growing interest in utilizing biological nitrogen fixation as a means of increasing the potential for sustainable intensification of food production whilst simultaneously reducing environmental damage caused by overuse of chemical fertilisers. Biochar, a recalcitrant carbon-rich product of pyrolysis which may be added to soil as a fertilizer or as a soil improver, alters soil physico-chemical properties usually by acting as a liming agent, by increasing water holding capacity or by modifying cation exchange capacity. The effects of biochar on the soil microbial community are not fully understood. Therefore, the main aim of this investigation was to evaluate the effects of biochar on the *Rhizobium*-legume relationship and determine whether biochar could increase legume growth. To achieve this aim, a series of growth experiments were carried out under controlled conditions in which broad bean (*Vicia faba*) was grown with *Rhizobium leguminosarum* and the symbiosis tested against three concentrations of biochar applied as a soil amendment and with two different char particle sizes. Beans responded well to *Rhizobium* under char-free conditions but the effects of biochar on the symbiosis were variable and depended on char particle size, concentration and *Rhizobium* strain (commercial or indigenous). Powdered char inhibited plant growth when in the presence of the commercial rhizobia, but not with indigenous strains. This is an important finding since commercial inocula are commonly used in agronomic situations. Plant available soil nutrients were modified by biochar and surprisingly by an interaction between char concentration and the two rhizobia strains. When beans were co-cropped with wheat, beans

performed better when grown with powdered char than without. This is in contrast to the response of bean plants to powdered char in the absence of any competition. Since wheat was generally the superior competitor, powdered char amendment enabled the bean to take advantage of the N-limiting environment that powdered char created and perform better than in the soils that advantaged the wheat. The investigation highlighted the complexity of the system, but identified the importance of char particle size and *Rhizobium* strain selection.

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# 1 Introduction

Energy, global food security and climate change are three issues that dominate current scientific thinking. Thermal gasification of agricultural wastes and residues will facilitate energy production and simultaneously produce char, which is a recalcitrant carbon-based material showing promise as a soil improver (Müller-Stover *et al*., 2012) due to its ability to act as a liming agent, increase water holding capacity and modify cation exchange capacity (Warnock *et al*., 2007; Hansen *et al*., 2016). Furthermore, there is much interest in applying char to soils as a means of improving soil organic content and sequestering carbon because it may take thousands of years to degrade (Yang *et al*., 2016). It is not surprising that there is much interest in producing and utilising char. When char is applied to soils, it is often referred to as biochar; both terms will be used interchangeably within this thesis.

Feedstock used and production conditions (e.g. pyrolysis, gasification, torrefaction) will dictate the quality of char produced and its suitability for soil application in the agronomic context (see Albuequerque *et al*., 2016). Ideally, char properties could be modified for purpose, although guaranteeing good quality and consistent feedstock may be challenging. For example, changing pyrolysis temperature and retention time can alter pH due to removal of acid functional groups (Albuequerque *et al*., 2016).

Plant growth improvements have been demonstrated following char amendment although data are variable (Jeffery *et al*., 2011). The meta-analysis conducted by Jeffery *et al*. (2011) which considered findings across the globe, highlighted some interesting points; for example, biochar was most effective in medium and coarse textured soils, application rate does not make a difference to crop yield, it produces better results on acidic and neutral soils due to its liming effect and soybean and cowpea respond favourably to it. A key point also highlighted by these authors was that of biochar-related negative growth effects which have been reported in the literature.

Numerous reviews highlight the benefits of applying char to soils, yet there is an increasing awareness that there may be unintended consequences and further work is therefore required. Although char is recalcitrant it may still be degraded by soil microorganisms or undergo chemical oxidation, or physical breakdown (Cheng *et al*., 2006).

Since according to Jeffery *et al*. (2011), soybean and cowpea respond favourably to char application, it is logical to study the potential benefits of applying char to legume crops in an attempt to enhance efficiency of the symbiosis. Legumes are a staple food for humans (FAO, 2016) and in addition to growing them in monocultures, they are also grown within agronomic situations that encompass intercropping, forage crops and cover cropping, all aimed at improving nutrient-use-efficiency (Duchene *et al*., 2017).

Legumes fix atmospheric nitrogen and this makes them valuable in many agricultural settings where they may be intercropped or used as green manure as an alternative to chemical nitrogen fertilisers (Bedoussac *et al*., 2015). Estimates of nitrogen fixed through biological nitrogen fixation (BNF) are in the range of 139 million to 175 million tonnes of nitrogen. Approximately 44 million tonnes of N are fixed from arable land and a further 45 million tonnes from pasture (Zahran, 1999).

Application of rhizobia strains to enhance legume yields is an important approach in sustainable agriculture (Stajković *et al*., 2011) and there is growing interest in ways to increase nitrogen-use efficiency in arable systems and reduce reliance on chemical fertilisers which result in environmental degradation (Lu and Tian, 2017).

Some of the most important rhizobial species belong to the genus *Rhizobium* and the symbiosis between *Rhizobium leguminosarum* and broad bean (*Vicia faba*) is the mainstay of this thesis.

The rationale behind the work is predicated by a need to increase food production in a sustainable manner and simultaneously create a link within a food, energy and climate nexus.

**The main objectives of the work were to:**

- Determine the effects of biochar on the *Rhizobium*-legume symbiosis and establish whether there could be unintended consequences of using biochar in this context. Different char concentrations and particle sizes were tested. This objective was addressed in Chapter 2.

- Establish whether biochar modifies the competitive outcome between a legume and wheat. The aim was to simulate co-cropping. This objective was addressed in Chapter 3.

- Quantify whether biochar limits or enhances interactions within a tripartite system. In this case the partners were *Rhizobium leguminosarum*, *Vicia faba* and arbuscular mycorrhizal fungi. This objective was addressed in Chapter 4.

# 2 Interactions between biochar and *Rhizobium* during growth of broad bean (*Vicia faba*)

## 2.1 INTRODUCTION

Beans, including broad beans, are an important staple food crop for human consumption across the world and 2016 was designated International Year of Pulses (Food and Agriculture Organization of the United Nations, 2016). In nutritional terms, beans are an excellent protein source and are rich in minerals (especially iron and zinc) and also in vitamins (White and Broadley, 2009). Leguminous plants (including beans) can provide for their own nitrogen requirements through biological nitrogen fixation (BNF) in symbiosis with soil bacteria collectively known as rhizobia. These bacteria form root nodules on leguminous plants and convert atmospheric $N_2$ into ammonia which is subsequently assimilated into amino acids for biosynthesis. Application of effective rhizobial strains as biofertilizers to improve legume production is an important approach in sustainable agriculture (Stajković *et al*., 2011). There is growing interest in ways to increase nitrogen-use efficiency in arable systems, partly because in many developing countries N-fertilisers are unaffordable and in modern conventional agricultural systems, most applied N-fertiliser is lost, resulting in environmental problems such as soil degradation, water eutrophication and air pollution (see Lu and Tian, 2017 and references therein).

Some of the most important rhizobial species belong to the genus *Rhizobium* and the symbiosis between *Rhizobium leguminosarum* and broad bean (*Vicia faba*) is the focus of this Chapter. Although able to fix $N_2$, legumes will preferentially take up soil N if available because of the carbon costs to the plant, usually resulting in a mixture of soil-derived and atmospheric nitrogen in the total N mass of the plant (Peoples *et al*., 1995, 2009). Whilst fixation generally accounts for 50-70% of the total plant N, the amount of nitrogen fixed is related to the fitness of the plant (Bohlool *et al*., 1992) and to environmental stressors such as drought (Marino *et al*., 2007).

In addition to being cultivated as a staple food, legumes are often grown as cover crops in order to reduce nitrate losses from soils that would otherwise

remain bare over the winter and to simultaneously increase organic inputs into soils (Blesh, 2018). An alternative method of increasing soil carbon inputs and concurrently enhancing N retention is to amend soil with biochar. Biochar has been extensively studied with a view to increasing crop yield and soil fertility either directly or indirectly; for example, by modifying cation exchange capacity, pH or influencing water holding capacity (Lehmann and Joseph, 2009; Schomberg *et al*., 2012). It has been suggested that biochar addition to soil can enhance soil fertility through increased BNF when legumes are present (Nishio, 1996; Rondon *et al*., 2007), perhaps by enhancing the potential of the rhizobia-legume symbiosis (Kahindi *et al*., 1997; Thies and Rillig, 2009) due to altered soil properties following biochar application (Rondon *et al*., 2007). Biochar-related improved growth of soybean (*Glycine max*) (Tagoe *et al*., 2008; Suppadit *et al*., 2012) and of common bean (*Phaseolus vulgaris*) (Rondon *et al*., 2007) under field and greenhouse conditions were reported; specifically, increased number of nodules, plant height, nutrient uptake, yield and dry weight. Nevertheless, there is a great deal of variation in published data for crop performance (across a range of crops) and the most likely consistent explanation for improved plant growth is a liming effect in acidic soils and increased water holding capacity (see Jeffery *et al*., 2011 for a meta-analysis of published data). Compared to other crop species, limited attention has been paid to the influence of biochar on the important process of BNF, especially concerning the mechanisms of interaction between biochar and *Rhizobium* for improvement of growth and yield of leguminous plants.

Addition of biochar to soil will modify the soil C:N ratio. Some studies have demonstrated biochar-mediated increased nitrification rates (Nelissen *et al*., 2012), whilst others reported immobilisation of soil N by char amendment (Bruun *et al*., 2012). Any alteration in available N will potentially affect BNF and uptake of soil N by legumes and requires further study. Therefore, the **overall aim** of the experiment described in this Chapter was to determine whether soil amendment with char increased growth and nutrient uptake of broad bean when inoculated with a commercial rhizobia strain, or when left uninoculated. The experiment was a pot trial conducted under controlled conditions. Two char particles sizes were used, powdered char and 1 mm size particles. Two size particles were used because to date, particle size has largely been ignored, resulting in a knowledge gap that needs to be filled before char is applied on a

field-scale. However, Sasidharan *et al*. (2016) conducted a column experiment in which char of two sizes (>60 μm and 2 mm) was added to quartz and *Escherichia coli* cultures eluted through the column. The authors showed that recovery of *E. coli* was greater from the quartz column containing the larger char particles. Bacterial transport was thus lessened by the smaller char particles. Natural soils will be more complex than the quartz substrate used by Sasidharan *et al*. (2016); char-related changes in soil pH may influence bacterial retention by increasing electrostatic repulsion and therefore microbial transport within the soil. These interactions could affect microbial dynamics within the soil and effectiveness of the rhizobia.

The **specific objectives** of this experiment were:

(1) To determine if bean plant growth responds to soil char amendment.

(2) To quantify any growth effects resulting from addition of a commercial rhizobial inoculum.

(3) To establish whether char amendment affected the rhizobia-legume symbiosis.

The **main hypotheses** were:

(1) Addition of the commercial rhizobial inoculum would increase bean growth.

(2) Soil amendment with 1 mm size char would result in an additive growth effect beyond that of the rhizobial inoculum only, possibly due to increased soil fertility or to enhanced pH.

(3) Soil amendment with the powdered char would reduce rhizobial effectiveness due to bacterial retention and/or the powder 'clogging' soil pores.

(4) Soil amendment with a higher concentration of char would increase plant growth due to an indirect improvement in soil fertility through enhanced pH, or to a direct improvement in soil fertility.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Experimental approach

A pot experiment was conducted in which a dwarf variety of broad bean (*Vicia faba*) was grown in soil, or in soil amended with one of two size fractions of char each at two concentrations. A commercial *Rhizobium* inoculum (*Rhizobium leguminosarum* biovar *viciae*) donated by Legume Technology (Nottingham, UK) was added to half the pots. The intention was to compare plants with and without the symbionts, grown with or without char under controlled conditions in a growth room.

### 2.2.2 Soil and char preparation

The char used for this investigation was purchased from the BioRegional HomeGrown Company Ltd. The char consisted of mechanically chipped trunks and large branches of *Fraxinus excelsior*, *Fagus sylvatica* and *Quercus robur* that had been pyrolysed at 450$^{o}$C for 48h. The pH of the char was 9 and it contained negligible amounts of N.

Char was prepared by mashing and grinding lumps of charcoal and sieving to obtain a powder fraction (<0.7 mm) and a coarser fraction (1 mm). Sieved (2 mm) loam soil was mixed with Levington's Pot and Bedding Compost (2:1 soil:compost) and moisture content determined following oven drying subsamples of the soil/compost mixture at 105°C for 24h to enable calculation of the quantity of char to add on a dry weight equivalent basis. Soil/compost mixes (± char) were added to plastic plant pots (13.3 cm x 11.3 cm) prior to sowing seeds. The char concentrations used were calculated on a dry weight equivalent and each pot received either 7.5 g or 20 g of char, hereafter referred to as 2.5% and 7% char respectively. Char and soil/compost were thoroughly mixed. (Please note that the terms 'char' and 'biochar' are used interchangeably and mean the same thing.)

### 2.2.3 Experimental set up

The soil/compost mix was amended with char to give the following treatments:

(i) Soil/compost only (= control)

(ii) Soil/compost + 2.5% powdered char

(iii) Soil/compost + 7% powdered char

(iv) Soil/compost + 2.5% of 1 mm char

(v) Soil/compost + 7% of 1 mm char

Hereafter the soil/compost mix will be referred to as 'soil', or the 'no char' treatment. Eight pots of each char treatment were established, half of which received commercial *Rhizobium* inoculum and half remained uninoculated. This was applied by adding a 2 mL volume of inoculum to the planting hole prior to adding the bean seed. The inoculum carrier is peat and since the potting compost contained peat it was not necessary to add sterilised inoculum to the uninoculated pots. Two beans were sown in each pot and thinned after germination to give one plant per pot.

Therefore, each of the 5 treatments listed above (i-v) was duplicated to give one set with added *Rhizobium* and one set without added *Rhizobium*. There were 4 replicate pots per treatment.

The pots were placed in a growth room with 12 hours light-dark cycle with a daytime temperature of 20°C and a night-time temperature of 18°C. The plants were watered daily as required with deionized water. The experimental design was a fully factorial randomised block design with 4 replicate blocks. Plants were maintained to the flowering stage when they were harvested.

### 2.2.4 Harvest and sample processing

At the flowering stage, plants were carefully removed from the pots and roots and shoots separated. Roots were rinsed in tap water and blotted dry. Three root lengths were randomly selected from each root system and root length measured and number of nodules counted. These root portions were then oven dried (45$^{o}$C until consistent weight) so that the number of nodules could be expressed on a $cm^{-1}$, a $g^{-1}$ and a per whole root system basis. Thirty nodules were also randomly selected and removed from the remaining root system and oven dried. The rest of the root system was also oven dried and the various dry weights combined later to give the whole root biomass. It should be noted that

uninoculated plants were also nodulated, so this process was carried out for all plants. Shoots were also oven dried at 45°C until constant weight was reached.

Soil from each pot was homogenised and subsamples taken for chemical analyses that required fresh soil, whilst the remaining soil was oven dried at 105$^{o}$C.

#### *2.2.4.1 Exchangeable and available soil elements in fresh soil*

Exchangeable and available macro- and micro-nutrients in the soil were determined following extraction in 1 mol $L^{-1}$ ammonium nitrate ($NH_4NO_3$) and in Milli-Q water respectively. For both analyses, 2 g fresh soil (sieved and homogenised) were weighed into 50 mL centrifuge tubes. Eighteen mL of the appropriate extractant were added and the tubes laid horizontally on an orbital shaker (KS 500 Janke & Kunkel IKA Labortechnik) for one hour at 256-259 rpm and then centrifuged (Herml Z400) at 3000 rpm for 20 minutes. The extracts were then filtered (through Whatman No. 42 filter paper) into 30 mL Universal tubes and subsequently diluted 1:10 with 2% nitric acid ($HNO_3$) (1 mL sample:9 mL diluent). Following dilution, elemental concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS; Thermo-Fisher Scientific X-Series$^{II}$, Thermo Fisher Scientific Inc., Waltham, MA, USA). Data are only shown for the water extracts because anomalies were observed throughout data sets following ammonium nitrate extractions. The reason for this is unknown, but might be because the $NH_4NO_3$ concentration was insufficient for optimal elemental exchange in the presence of powdered char.

#### *2.2.4.2 Extractable nitrogen (TN) and organic carbon (TOC) in fresh soil*

Extractable nitrogen and organic carbon in the soils were determined following potassium sulphate (0.5 mol $L^{-1}$ $K_2SO_4$) extraction. Two g of fresh soil (well-mixed) from each pot were weighed into 50 mL centrifuge tubes. Ten mL of $K_2SO_4$ solution were added to the soil and tubes were shaken on an orbital shaker and centrifuged as described above (section 2.2.4.1). The extracts were then filtered as above and diluted with Milli-Q water (1:10) prior to

determination of extractable organic carbon and extractable total nitrogen using a Shimadzu TOC-V/TN analyser (Shimadzu Corporation, Koyoto, Japan).

#### *2.2.4.3 Soil pH*

Experimental soils were well mixed. Soil pH was determined on a soil:deionised water slurry mixture (1 part soil:2.5 parts deionised water) using a pH meter (pH 209 Hanna instruments).

#### *2.2.4.4 Soil dry weight and moisture content*

The remaining fresh soil from each experimental pot was oven dried at 105°C for 24 to 48 hours until constant weight was obtained. Soil fresh weight and dry weight of each sample were recorded. Soil moisture content was calculated using the equations below.

To calculate soil dry weight:

$$W_{DS} = (W_{DS} + W_C) - W_C$$

To calculate percentage of moisture content:

$$\% \text{ Soil moisture content} = \frac{(W_{FS} - W_{DS})}{W_{FS}} \times 100$$

Where, $W_C$ = weight of the cup, $W_{DS}$ = weight of dried soil, $W_{FS}$ = weights of fresh soil.

The oven dried soil was used for further analysis as described below.

#### *2.2.4.5 Total elemental analysis of dried soil*

Total elemental analysis of soil was determined following HF (hydrofluoric acid) digestion. Approximately, 0.2 g dried soil (ground and well-mixed; exact weight recorded) was weighed into perfluoroalkoxy (PFA) digestion vessels. The digestion was undertaken for three days. On the first day, 2 mL of 70% TAG $HNO_3$ and 1mL 60% $HClO_4$ were added to each sample. The samples were left

overnight. A 2.5 mL aliquot of 70% hydrofluoric acid (HF) was added to each of the samples on the second day. On the third day, 2.5 mL $HNO_3$ and 2.5 mL Milli-Q water were added to the samples. The samples were then left at 50°C for 1 hour in a 48-place Teflon-coated graphite block digester (Model A3, Analysco Ltd.). The block digester was turned off and the samples removed from the hot block. The samples were then made up to 50 mL with Milli-Q water in volumetric flasks and filtered if required. Approximately, 20 mL of each extract was poured into a Universal tube and diluted (1:10 with Milli-Q water). Elemental concentrations within the diluted samples were determined using ICP-MS.

#### *2.2.4.6 Total nitrogen (N) and carbon (C) analyses of dried soil*

Total soil carbon and nitrogen concentrations were determined on oven dried (ground) soil. Approximately 0.02 g of soil was placed into a capsule which was sealed and the material analysed by combustion at 900$^{o}$C in an Elemental Analyser (Flash EA1112, CE Instruments, UK). In this analysis, aspartic acid was used for bypass and standard and a reference peaty soil was used for QC.

### 2.2.5 Plant analyses

After weighing the dried roots and shoots, the material was milled using a centrifugal mill (ZM 200) at 12000 rpm speed or, in the case of the 30 root nodules, ground with liquid nitrogen using a mortar and pestle.

#### *2.2.5.1 Multi-elemental analyses*

Multi-elemental analyses were conducted on the shoot and root material following acid digestion using a closed vessel microwave heating system (MARS Xpress, CEM Microwave Technology Ltd., Buckingham, UK). Approximately, 0.2 g plant material was weighed (exact weight was recorded) into a digestion vessel. Six mL of concentrated $HNO_3$ (nitric acid) was carefully pipetted into each digestion vessel and the tops put on each tube and the caps screwed shut. Each digestion vessel was placed into a numbered position in the microwave rotor and digested at 1400 W for 15 minutes following a 40 minute ramp time.

When the process was completed, each sample was made to final volume of 20 mL and poured into a Universal tube. Prior to analysis using ICP-MS, the solutions were diluted 1:10 using Milli-Q water.

#### *2.2.5.2 Total nitrogen (N) and carbon (C) analyses*

Total carbon and nitrogen analyses were carried out on dried root and shoot subsamples and on the 30 nodules. Approximately 0.01 g of ground plant material was analysed as described in section 2.2.4.6, although in this case tomato leaves were used for QC.

### 2.2.6 Statistics

Data were analysed by two main methods, General Analysis of Variance and General Linear Regressions. The experimental design lends itself to ANOVA, but because nodules were present on all plants (including uninoculated plants), regressions were also performed to accommodate this because there was no longer a plus or minus *Rhizobium* treatment, but rather, a plus or minus added commercial inoculum treatment.

The factors used in analysis of the data were char size (soil only, powdered or 1 mm char), char concentration (0%, 2.5% or 7%) and *Rhizobium* inoculum (± commercial inoculum). Since the design was unbalanced a further factor 'soil or char' was included in the ANOVA model.

General Linear Models were used to determine relationships between a range of variables and the total number of root nodules and develop explanatory models for observations.

Data were analysed by Genstat version 17 (Lawes Agricultural Trust/VSN International, Hemel Hempstead, UK). Normality was tested by plotting residuals against expected normal quantiles and post hoc comparisons between means were based on least significant differences at the 0.05 probability level where appropriate.

## 2.3 RESULTS

A number of plants grown in soil amended with the 2.5% powdered char (with and without *Rhizobium* inoculum) failed to germinate, or died soon after germination. All factors were included in the analyses and missing values input as an asterisk (as recommended for Genstat) because there were sufficient surviving plants to warrant this. Although Genstat can calculate missing values, rather than risk biased interpretation of the data, the most complex model in ANOVA (char size x char conc. x *Rhizobium*) was ignored when significant and instead, the next most complex outcomes were used for interpreting the data where appropriate. These were 'char size x char concentration', 'char size x *Rhizobium* inoculation' and 'char concentration x *Rhizobium* inoculation'. This pooling ensured sufficient replication to yield unbiased analyses.

### 2.3.1 Root nodulation

All plants developed root nodules irrespective of whether the potting medium was amended with *Rhizobium leguminosarum* biovar *viciae* inoculum or not. Therefore, in the context of this Chapter, '+R*hizobium*' (R+) treatment refers to added inoculum, whilst '–*Rhizobium*' (R-) refers to pots that did not receive inoculum; the terms denote 'more' or 'less' rhizobia respectively, the former including commercial and native rhizobia and the latter just native rhizobia. This is important to note when reviewing the ANOVA output. In contrast, regressions were generally performed using total number of nodules per root system as a variable.

The original counts were carried out on subsamples of known root length and were later converted to nodules per gram of root following drying of the root material. When nodule counts were expressed on a per cm root length basis, *Rhizobium* inoculation resulted in a 24% increase in nodule number per cm root length, although this was not significant ($F_{1,13}$=3.38, $p$=0.089). However, the powdered char treatment significantly reduced nodule number $cm^{-1}$ root length (ANOVA: char size as a single factor, $F_{1,13}$=5.38, $p$=0.037, LSD=0.53; Fig. 2.1).

When expressed as nodule number per gram of root, *Rhizobium* amendment resulted in a significant increase in number of nodules $g^{-1}$ root (R+ = 1386 and

R- = 1033 nodules $g^{-1}$; ANOVA: *Rhizobium* inoculation as a single factor, $F_{1,12}$=9.84, $p$=0.009). Amending the soil with rhizobia inoculum therefore led to a 34% increase in the number of nodules $g^{-1}$ root, measured at the end of the experiment.

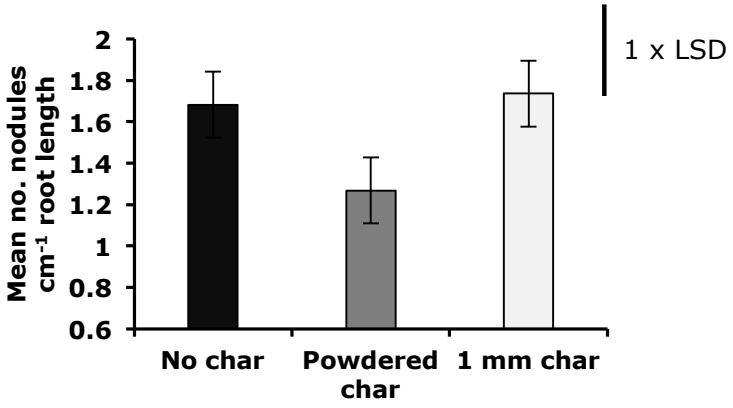



**Figure 2.1:** The mean number of rhizobia nodules $cm^{-1}$ root length from three randomly selected roots from within a whole plant root system (char size as a single factor, $p$=0.037).

The effect of char amendment was evident when considering the total number of nodules per whole root system. Fewer nodules were observed on roots grown in soil with the powdered char amendment (char size x *Rhizobium* inoculum interaction, $F_{1,12}$=9.14, $p$=0.011, LSD=2013) than on roots from any other treatment. Total nodule number was similar in roots from the soil treatment and the 1 mm char amended soil whether added *Rhizobium* was present or not (Fig. 2.2a). Char size as a single factor was significant and pooled means showed that powdered char lowered nodule number whilst the 1mm char and no char treatments yielded a similar degree of nodulation ($F_{1,12}$=9.32, $p$=0.010).

When considering the effect of char concentration x rhizobia inoculation (Fig. 2.2b), total number of nodules was similar across all char amendments (including no char) when additional *Rhizobium* had been added. In the absence of additional rhizobia, the 7% char amendment significantly reduced total nodule number compared to the 0% and 2.5% char additions (Fig. 2.2b). Char concentration as a single factor was only weakly significant ($F_{1,12}$=4.54, $p$=0.054, LSD=2013), but there appeared to be a trend towards lower total

nodule number with increasing char concentration (3663 nodules at 0% char and 2184 nodules at 7% char).

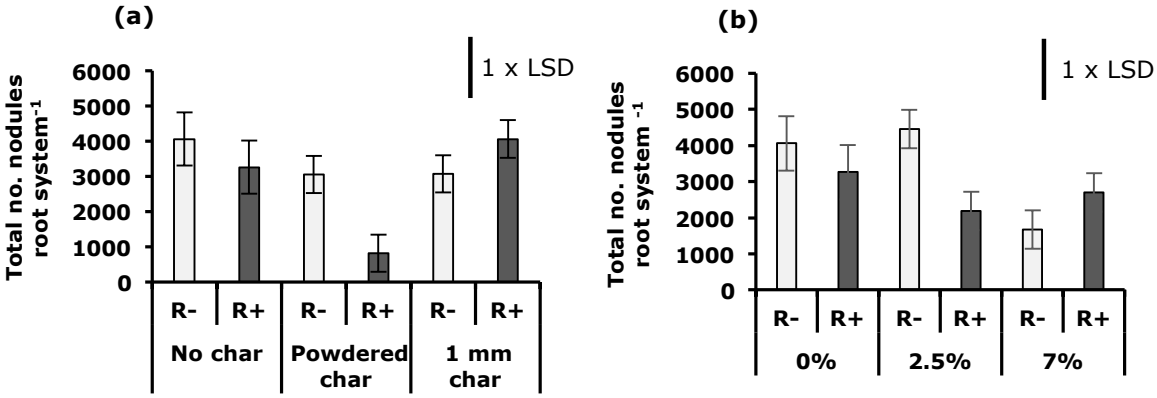



**Figure 2.2: (a)** Total number of root nodules per whole root system; char size x *Rhizobium* interaction, $p$=0.011. **(b)** Total number of root nodules per whole root system; char conc. x *Rhizobium* interaction, $p$=0.009. Individual standard errors are based on the pooled variance estimate from the ANOVA with 12 degrees of freedom. Vertical bars represent the LSD (5%).

### 2.3.2 Plant biomass

In the absence of additional *Rhizobium* inoculum (R-), char particle size did not affect whole plant dry weight. However, plants that were grown with powdered char and additional *Rhizobium* (R+) were significantly smaller than those grown with no char, or with the 1 mm char size fraction (ANOVA: char size x *Rhizobium* inoculum interaction, $F_{1,12}$ = 7.80, $p$=0.016, LSD=0.22; Fig. 2.3a). *Rhizobium* addition resulted in smaller plants when grown in soil without char and with powdered char, but not when grown with the 1 mm char size fraction.

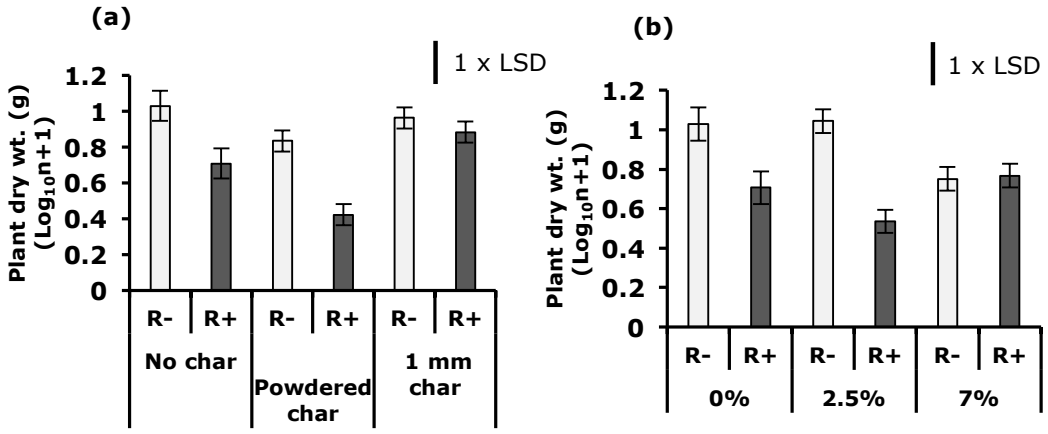



**Figure 2.3:** The effect of char amendment and additional *Rhizobium* inoculum on whole plant biomass. **(a)** Char size x *Rhizobium* inoculum interaction, $p$=0.016. **(b)** Char conc. x *Rhizobium* inoculum interaction, $p$<0.001. Individual standard errors are based on the pooled variance estimate from the ANOVA with 12 degrees of freedom. Vertical bars represent the LSD (5%).

When comparing plants grown in soil that received the 3 char concentrations (0%, 2.5% and 7%), *Rhizobium* amendment (R+) of soil containing 0% and 2.5% char reduced plant biomass relative to the corresponding R- treatments. This effect did not happen with the 7% char treatment. The R+ treatments across all three char amendments were not significantly different from one another. However, without additional *Rhizobium*, the 7% char amendment reduced plant biomass (ANOVA: char conc. x *Rhizobium* inoculum interaction, $F_{1,12}$=19.56, $p$<0.001, LSD=0.22; Fig. 2.3b). There was no char size x char concentration interaction. Root and shoot biomass followed a similar trend to that of the whole plant (Table 2.1).

*Rhizobium* application increased R:S ratio in plants grown in soil plus powdered char, but this effect was lost in plants grown in soil with the 1mm char (ANOVA: char size x *Rhizobium* inoculum interaction, $F_{1,12}$=8.31, $p$=0.014; LSD=0.12; Fig. 2.4a). The trend towards an R+ related increase in R:S ratio in plants grown in soil without char was not significant (based on LSD at 5%). The R:S ratio was generally unaffected by presence of the 1 mm particle size char irrespective of concentration (Fig. 2.4b). The higher amount (7%) of powdered char reduced R:S ratio compared to the 2.5% amendment, although there were no other significant differences (ANOVA: char size x char conc. interaction, $F_{1,12}$=6.80, $p$=0.023; LSD=0.10).

**Table 2.1:** Root and shoot dry weights of plants grown with or without additional *Rhizobium* inoculum and with different char size fractions (upper section) and concentrations (lower section). Data show a char size x *Rhizobium* inoculation interaction for both shoot and root (upper portion of table) and a char conc. x *Rhizobium* inoculum interaction (lower portion of table).

| **DWt. (g) $Log_{10}n+1$** | **None** | | **Powdered** | | **1 mm** | | **LSD** | $F_{1,12}$ | ***p*** |
|---|---|---|---|---|---|---|---|---|---|
| | **R-** | **R+** | **R-** | **R+** | **R-** | **R+** | | | |
| **Shoot** | 0.83 | 0.52 | 0.67 | 0.30 | 0.77 | 0.69 | 0.21 | 6.70 | 0.024 |
| **Root** | 0.69 | 0.45 | 0.53 | 0.24 | 0.64 | 0.57 | 0.18 | 5.04 | 0.044 |
| | | | | | | | | | |
| **DWt. (g) $Log_{10}n+1$** | **0%** | | **2.5%** | | **7%** | | **LSD** | $F_{1,12}$ | ***p*** |
| | **R-** | **R+** | **R-** | **R+** | **R-** | **R+** | | | |
| **Shoot** | 0.83 | 0.52 | 0.85 | 0.41 | 0.58 | 0.59 | 0.21 | 16.02 | 0.002 |
| **Root** | 0.69 | 0.45 | 0.70 | 0.30 | 0.46 | 0.48 | 0.18 | 16.05 | 0.002 |

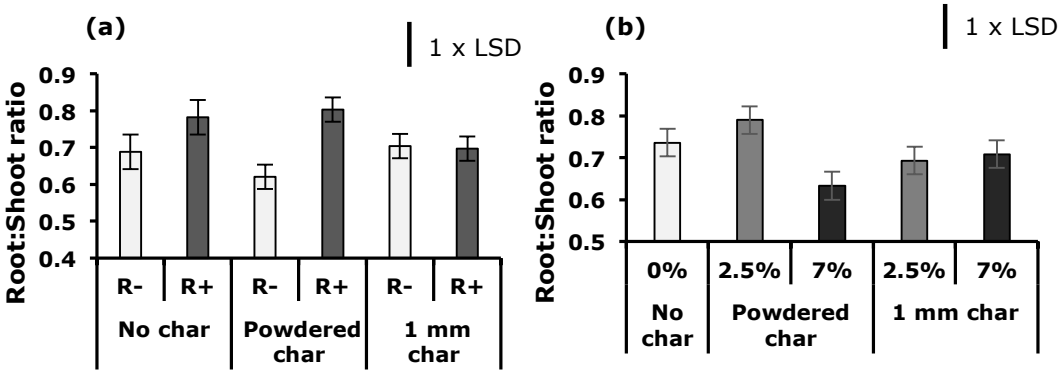



**Figure 2.4:** Root to shoot ratio affected by: **(a)** Char size x *Rhizobium* inoculum interaction, $p$=0.014 and **(b)** char size x char conc. interaction, $p$=0.023. Individual standard errors are based on the pooled variance estimate from the ANOVA with 12 degrees of freedom. Vertical bars represent average LSD (5%).

General Linear Models were utilised to determine relationships between root nodule number and a range of biomass parameters (Fig. 2.5a). Data were

pooled across char treatments and the effect of char groups was explored further (Fig. 2.5b).

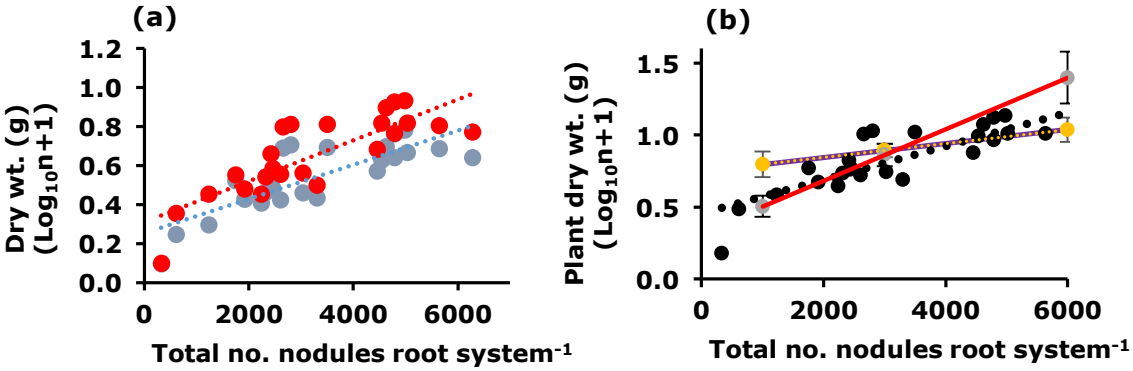



**Figure 2.5: (a)** Relationship between total number of nodules per root system and root (grey circles) and shoot (red circles) dry weight (linear regression, $p<0.001$ for both parameters). **(b)** Observed linear regression (black circles and black dotted line) and modelled predictions (± se) for changes between groups (red line = powdered char; purple/orange line = 1 mm char). The soil only (no char) prediction is not shown for clarity.

To determine if (and how) the relationship between total number of nodules and plant weight changed between the char treatments, a linear regression 'with groups' was carried out in which plant biomass was the response variate, total number of nodules per root system was the explanatory variate and char size (powdered, 1 mm or soil) was the 'group'. Plant dry weight was affected by powdered char (coefficient, -0.423; $t$= -2.77, $p$=0.012) and by an interaction between total number of nodules x powdered char (coefficient, 0.0001307; $t$=2.59, $p$=0.018) if the other variables were held constant. In this case, the reference class was the 1mm char treatment. The data suggest that when nodule numbers are low, powdered char results in smaller plants than those in soil with the 1 mm char. The model predicts that with high nodule numbers, powdered char would result in larger plants if all other variables were constant. However, the prediction relating to potential benefits of powdered char with high nodule numbers should be treated with caution, because the accumulated ANOVA indicated only a weak (and not significant) interaction (total nodule number x char size, $F_{2,19}$=3.34, $p$=0.057) whilst total number of nodules as a single factor was highly significant ($F_{1,19}$=27.54, $p<0.001$).

Nevertheless, within the powdered char treatment, plants with both the lowest and highest biomass were observed.

### 2.3.3 Time to germination and flowering

The number of days from sowing bean seeds to hypocotyl emergence was recorded. On average, it took 10 days from sowing to germination, but the range was from 7 to 18 days. Char size affected germination with an average time of 8.6 days for seeds sown in soil and in soil amended with the 1mm char fraction, whilst seeds sown in soil with powdered char took on average 13.6 days to germinate (ANOVA: char size as a single factor, $F_{1,14}$=44.30, $p$<0.001). Addition of *Rhizobium* inoculum did not affect speed of germination.

A significant relationship between the time taken for hypocotyl emergence and the total number of nodules was observed (regression: $F_{1,23}$=10.28, $p$=0.004; Fig. 2.6a). Since nodules were counted at the end of the experiment, rather than interpret *Rhizobium* presence (either added inoculum or indigenous strains) as a factor that quickened germination, it is more likely that rapid germination resulted in larger roots and by default, more successful colonisation and nodulation. This is supported by the significant relationship between plant biomass and speed of germination, where plants that germinated quickly were generally larger at harvest. This was evident for root and shoot biomass (regression: $p$<0.01; Fig. 2.6b) in addition to root weight (data not shown). It is further supported by the consistently poorer performance of plants grown with powdered char (e.g. Table 2.1). However, char particle size was not a significant factor when included in the regression model as an explanatory variable along with total number of nodules.

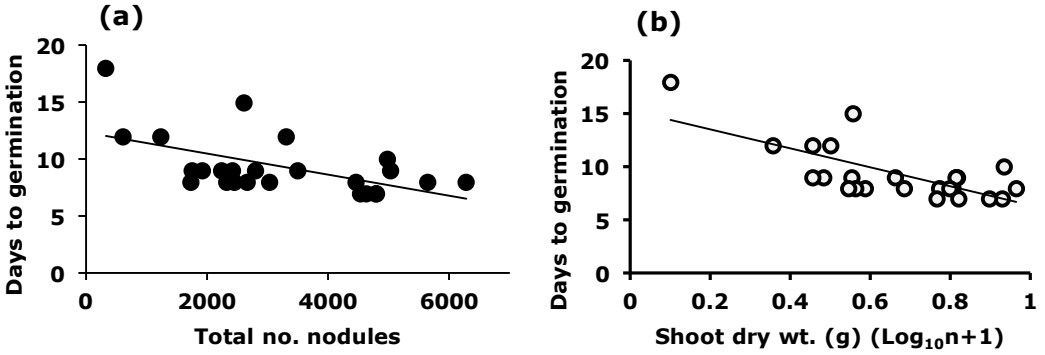


**Figure 2.6: (a)** Relationship between total number of root nodules and time to first appearance of the hypocotyl ($p$=0.004). **(b)** Relationship between shoot dry weight and days to germination ($p$<0.01).

The time taken from germination to appearance of the first flowers was, on average, 45 days. The key observation was that plants grown with the 2.5% powdered char amendment and added *Rhizobium* inoculum began flowering after 42.5 days, whilst plants within the same char treatment, but without supplementary *Rhizobium*, started flowering after 46 days, which was significantly later (ANOVA: char size x *Rhizobium* inoculation interaction, $F_{1,13}$ = 7.37, $p$=0.018, LSD=2.59). Plants grown with the other treatments all started flowering within 1-2 days of each other (data not shown). However, there was a significant trend between speed of germination and of flowering; plants that germinated slowly, flowered more quickly than those that germinated rapidly.

### 2.3.4 Foliar chemistry

Data for elemental analysis (macro- and micro-nutrients) of shoot material are given below. The key macronutrients quantified were: Carbon, nitrogen, phosphorus, potassium, sulfur, calcium and magnesium. The main micronutrients measured were: Iron, molybdenum, boron, copper, manganese, zinc, nickel, cobalt, aluminium and chromium.

#### *2.3.4.1 Foliar macronutrients*

Carbon concentration was lower in leaves from plants grown in soil amended with 2.5% powdered char than in leaves from plants grown in unamended soil, or in soil treated with either concentration of the 1 mm char (Fig. 2.7a). Percentage C was higher in leaves from plants grown with both concentrations of 1 mm size char than in leaves from plants grown in soil only (ANOVA: char size x char conc. interaction, $F_{1,13}$=6.71, $p$=0.022, LSD=1.38). *Rhizobium* inoculation did not affect leaf [C] or leaf [N].

However, there was a significant relationship between the total number of nodules per root system and leaf %C, which is a reminder that all roots were nodulated whether inoculated or not. Greater numbers of nodules were related to higher leaf C (GLM: $F_{1,23}$=4.67, $p$=0.041) when nodule number was the sole explanatory variable (Fig. 2.7c), although nodule number only explained 13.3% of the variation. When the regression model included char size, 45.3% of the variation was accounted for (GLM: nodule number $F_{1,19}$=4.84, $p$=0.014; char size, $F_{2,19}$=4.84, $p$=0.020; nodule number x char size interaction, $F_{2,19}$=3.90, $p$=0.038). Estimated means of leaf C (%) were predicted for each size class of char (Fig. 2.7c). The estimated coefficient was significant for the soil only treatment (GLM: $t$=-3.50, $p$=0.002 and for the nodule number x soil interaction (GLM: $t$=2.79, $p$=0.012). The reference value was derived from the 1 mm char size fraction and the significant coefficient for soil denotes the difference between the two char classes (no char and 1mm fraction). The negative coefficient for the soil variable means that %C will be less than 0% if the predictor variables are set to 0. Since it is illogical to have 0% C in leaves of *Rhizobium*-free plants, the negative coefficient is probably a result of the strong relationship between the independent variable (root nodule number) and the dependent variable (%C). Increasing nodule numbers when plants were grown in soil resulted in a predicted steep increase in leaf C, whilst char amendment dampened the response to increasing nodule number (Fig. 2.7c).

Plants grown with 1 mm size char, irrespective of char concentration, had higher leaf %N than plants grown with powdered char or in unamended soil (Fig. 2.7b). There was no significant difference in leaf N concentration between plants grown in soil only, or with powdered char (ANOVA: char size as a single factor, $F_{1,13}$ = 27.5, $p$<0.001, LSD=0.57; Fig. 2.7c).

Leaf N (%) increased with increasing nodule number (GLM: $F_{1,21}$=8.05, $p$=0.010) and according to char size (GLM: $F_{2,21}$=3.52, $p$=0.048) but there was no interaction between the two explanatory variables. The estimated coefficient was only significant for powdered char (GLM: $t$=-2.29, $p$=0.033; Fig. 2.7d). The reference value within the model was the 1 mm char size treatment, therefore leaf N responded differently to nodulation when plants were growing in soil with powdered char than when in soil with the 1 mm char fraction.

The remaining macronutrients (Ca, Mg, P, K and S) all significantly responded to a char size x char concentration interaction.

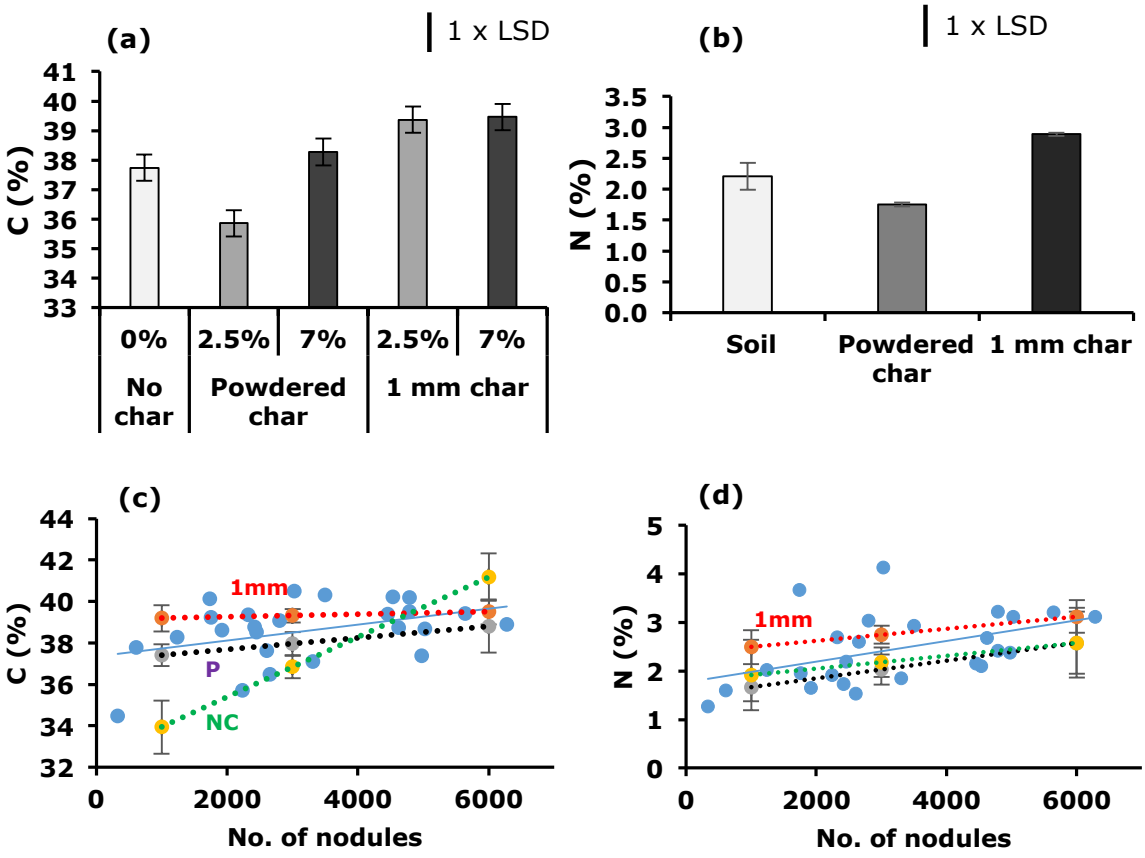



**Figure 2.7: (a)** Leaf carbon (%) (char size x char conc., $p$=0.022) and **(b)** leaf N (%) (char size as a single factor, $p$<0.001). Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bars represent the LSD (5%). **(c)** Relationship between leaf C (%) and total number of nodules showing observed data (blue markers and trend line) and predictions for char groups (NC, no char; P, powdered char; 1 mm, the 1 mm size fraction). Standard errors are for predictions at three given points (1000, 3000 & 6000 nodules per root). **(d)** Relationship between leaf N (%) and total number of nodules showing observed and predicted values as described for (c).

Ca concentrations were reduced by additional *Rhizobium* inoculum, although this effect was not observed in plants grown with the 7% char amendment. With 7% char, the R- treatment resulted in leaves with lower Ca concentrations

that those observed in the other two R- treatments (ANOVA: char conc. x *Rhizobium* interaction, $F_{1,13}$ = 5.06, $p$=0.042, LSD=15.5; Fig. 2.8a).

In the absence of additional *Rhizobium* inoculum, leaf [K] was highest in leaves from plants grown in soil with the 7% char amendment compared to plants grown without char, or with the 2.5% char concentration. *Rhizobium* inoculation increased leaf [K] at 0% and 2.5% char amendment, but had no effect on K concentrations in leaves of plants grown with 7% char. Leaf [K] was similar in all plants irrespective of char concentration when grown with additional *Rhizobium* inoculum (char conc. x *Rhizobium* inoculation, $F_{1,13}$=12.00, $p$=0.004, LSD= 7.3; Fig. 2.8b).

*Rhizobium* inoculation significantly reduced leaf [Mg] in plants grown with the 0% and 2.5% char treatments, but had no effect on leaf [Mg] in plants subjected to 7% char. Across the three char concentrations, *Rhizobium* inoculation did not alter leaf [K] which remained similar in all R+ treatments. However, in the absence of additional *Rhizobium*, leaf [Mg] was significantly lower in leaves of plants grown with the 7% char amendment compared to the 0% and 2.5% char treatments. There was no difference in leaf [Mg] between the uninoculated 0% and 2.5% char amendments (char conc. x *Rhizobium* inoculation, $F_{1,13}$=5.92, $p$=0.030, LSD= 2.6; Fig. 2.8c).

Additional *Rhizobium* significantly increased leaf [P] compared to uninoculated plants (R-) when grown with 0% and 2.5% char. Added rhizobia did not have this effect when plants were grown at the highest char concentration (char conc. x *Rhizobium* inoculation, $F_{1,13}$=4.87, $p$=0.046, LSD= 1.5; Fig. 2.8d). The trend towards higher leaf [P] with increasing soil char concentration in uninoculated plants (R-) was not significant (based on LSD). Similarly, the apparent decrease in leaf [P] in leaves from plants grown in the R+/7% treatment was not significant. The key observation from these data is that *Rhizobium* inoculation lowered leaf Ca and Mg concentrations and increased leaf [K] and [P].

Mean leaf S concentration was significantly higher in plants grown with the 2.5% char/R+ treatment than in all others, which remained similar irrespective of treatment. Since one of the values in the data set for that treatment was much greater than all the others (4.8 g $kg^{-1}$ compared to an average of 2.8 g

$kg^{-1}$ for all other treatments) it is likely that this skewed the analysis resulting in a Type I error. Nevertheless, *Rhizobium* inoculation (as a single factor) enhanced leaf [S] from 2.79 g $kg^{-1}$ in the absence of additional *Rhizobium* to 3.70 mg $kg^{-1}$ when present ($p$<0.001). Char amendment (pooled across all char treatments) also increased leaf [S] from 2.62 g $kg^{-1}$ when plants were grown in soil to 3.40 g $kg^{-1}$ when soil was amended with char ($p$<0.001).

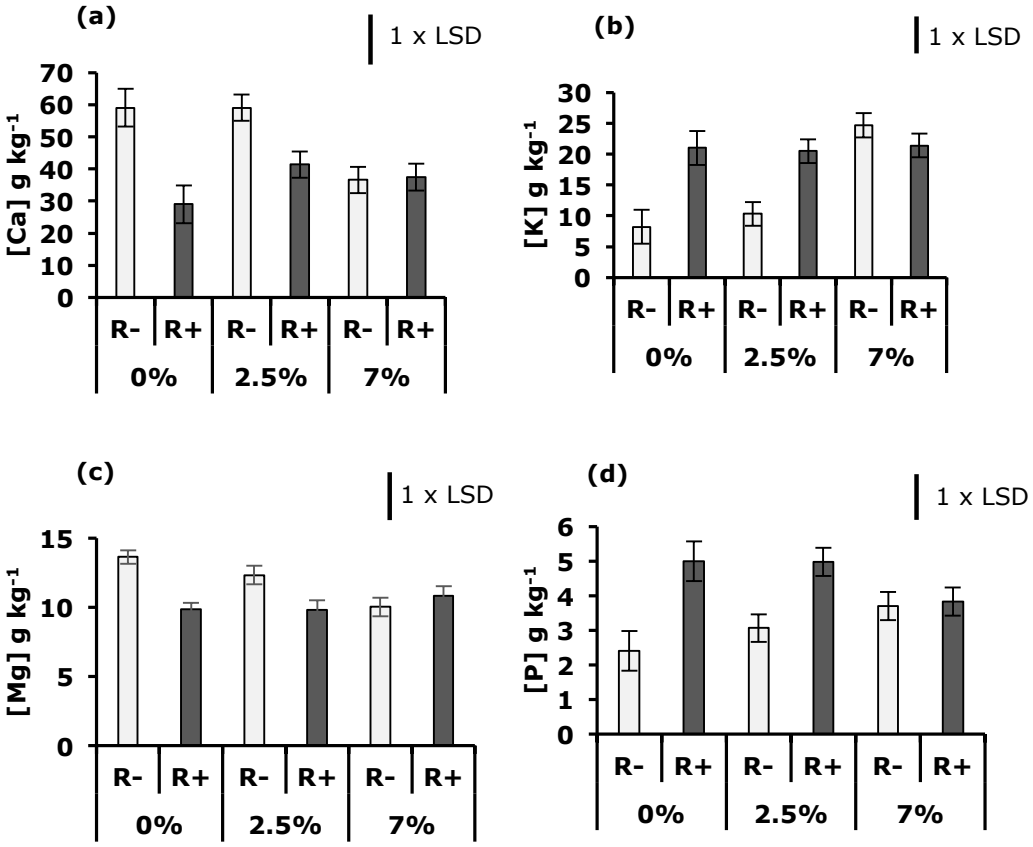



**Figure 2.8:** Macronutrient concentrations **(a)** Ca, **(b)** K, **(c)** Mg and **(d)** P in leaves of plants grown with three char concentrations with or without added *Rhizobium* inoculum. Data show a char conc. x *Rhizobium* inoculation interaction for **(a)** calcium, $p$=0.042; **(b)** potassium, $p$=0.004; **(c)** magnesium, $p$=0.030 and **(d)** phosphorus, $p$=0.046. Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bars represent the LSD (5%).

Char particle size affected leaf P, Mg and K concentrations irrespective of char amount or *Rhizobium* inoculation (Fig. 2.9). Leaf [K] was significantly higher in

plants grown with powdered char compared to those from the no char and 1 mm char treatments (ANOVA: char size as a single factor, $F_{1,13}$=15.95, $p$=0.002, LSD=5.2). In contrast, leaf [Mg] was significantly lower in plants grown in the soil amended with powdered char (char size as a single factor, $F_{1,13}$=15.44, $p$=0.002, LSD=1.8). The 1 mm char size fraction resulted in lower leaf [P] compared to that in plants grown in soil or with 2.5% char (ANOVA: char size as a single factor, $F_{1,13}$=14.29, $p$=0.002, LSD=1.1).

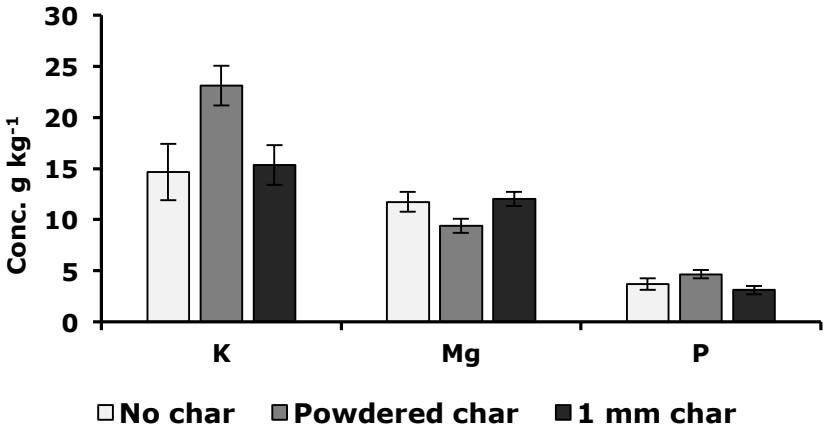



**Figure 2.9:** Potassium, magnesium and phosphorus concentrations in bean leaves when grown in soil with char size as a significant single factor. Data are pooled across each concentration. K, $p$=0.002; Mg, $p$=0.002; P, $p$=0.002. Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom.

### *2.3.4.2 Foliar micronutrients*

Foliar micronutrients were affected by *Rhizobium* inoculum, char concentration and char size to different degrees.

Molybdenum concentration was increased by the highest soil char amendment in the absence of additional *Rhizobium* inoculum. Other than an enhancement of leaf [Mo] by *Rhizobium* addition when plants were grown with 2.5% char, *Rhizobium* supplementation did not affect [Mo] relative to the plants not receiving the amendment (ANOVA: char conc. x *Rhizobium* inoculum interaction, $F_{1,13}$=11.04, $p$=0.005, LSD= 18.1; Fig. 2.10a).

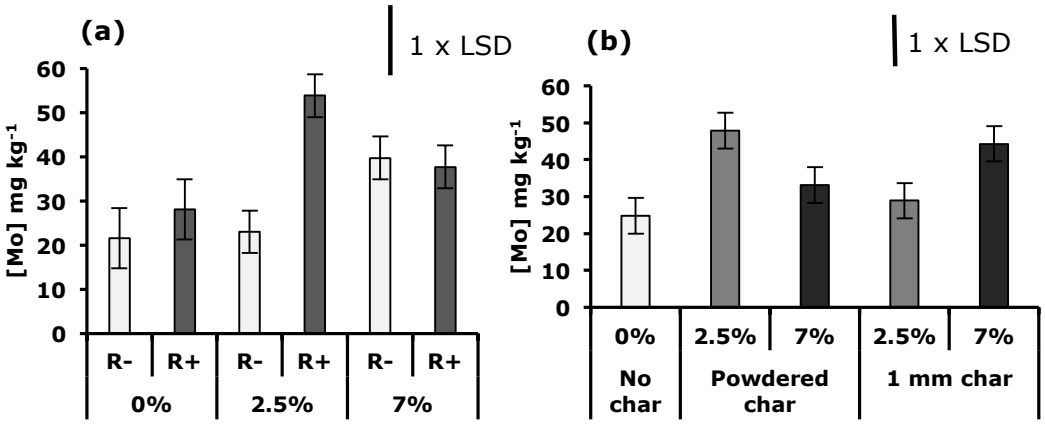



**Figure 2.10:** Leaf Mo concentration showing **(a)** char concentration x *Rhizobium* inoculation interaction, $p$=0.005 and **(b)** char size x char concentration interaction, $p$=0.008. Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bars represent the LSD (5%).

The effect of char concentration on leaf [Mo] was different depending on whether the char was in powdered form or as 1 mm particles. Increased concentration of the 1 mm char led to significantly enhanced leaf [Mo]. In contrast, the apparent decrease in [Mo] with the powdered char is not quite significant (based on the LSD). Neither the 2.5% amendment of the 1 mm char, nor the 7% addition of powdered char enhanced leaf [Mo] over that of plants grown in soil only (ANOVA: char size x char conc. interaction, $F_{1,13}$ = 9.71, $p$=0.008, LSD=14.8; Fig. 2.10b). Char addition as a single factor increased leaf [Mo] from 24.8 mg $kg^{-1}$ in soil-grown plants to 38.6 mg $kg^{-1}$ in plants grown with char (char treatments pooled) (ANOVA: $F_{1,13}$ = 6.47, $p$=0.025, LSD=11.7).

Foliar [Zn] was unaffected by *Rhizobium* supplementation when plants were grown in soil without char. However, additional *Rhizobium* significantly reduced foliar [Zn] when plants were grown with 2.5% char and increased it when plants were subjected to 7% char (ANOVA: char conc. x *Rhizobium* inoculation interaction, $F_{1,13}$=18.6, $p$<0.001, LSD=39.3; Fig. 2.11a). When compared to plants grown in soil only, leaf [Zn] was lowered by the highest char application rate (7%) when applied as a powder, but not when applied as large particles (ANOVA: char size x char conc. interaction, $F_{1,13}$=6.21, $p$=0.027, LSD=32.1; Fig. 2.11b).

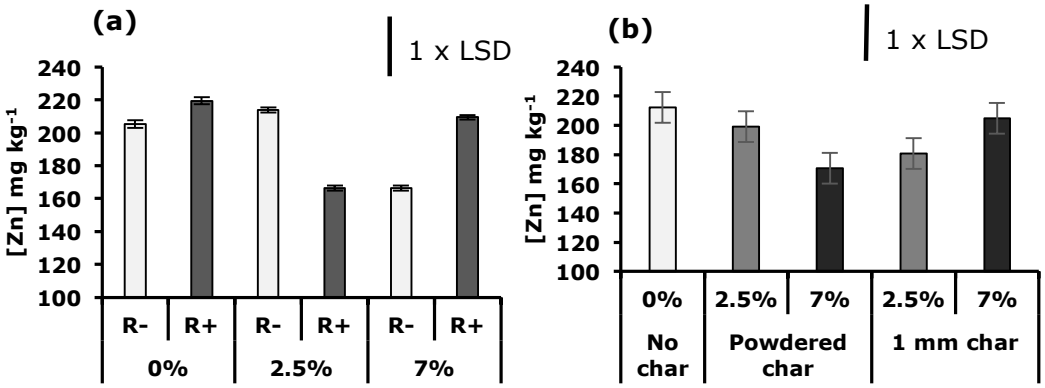



**Figure 2.11:** Leaf Zn concentration showing **(a)** char concentration x *Rhizobium* inoculation interaction, $p<0.001$ and **(b)** char size x char concentration interaction, $p=0.027$. Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bars represent the LSD (5%).

The key observation with other micronutrients (B, Fe and Ni) that responded to a char size x char conc. interaction, was that the highest amendment (7%) of the powdered char reduced leaf concentration of each element, whilst the equivalent concentration of the 1 mm char did not (Fig. 2.12). This pattern was also observed with Mo and Zn (Figs 2.10b and 2.11b).

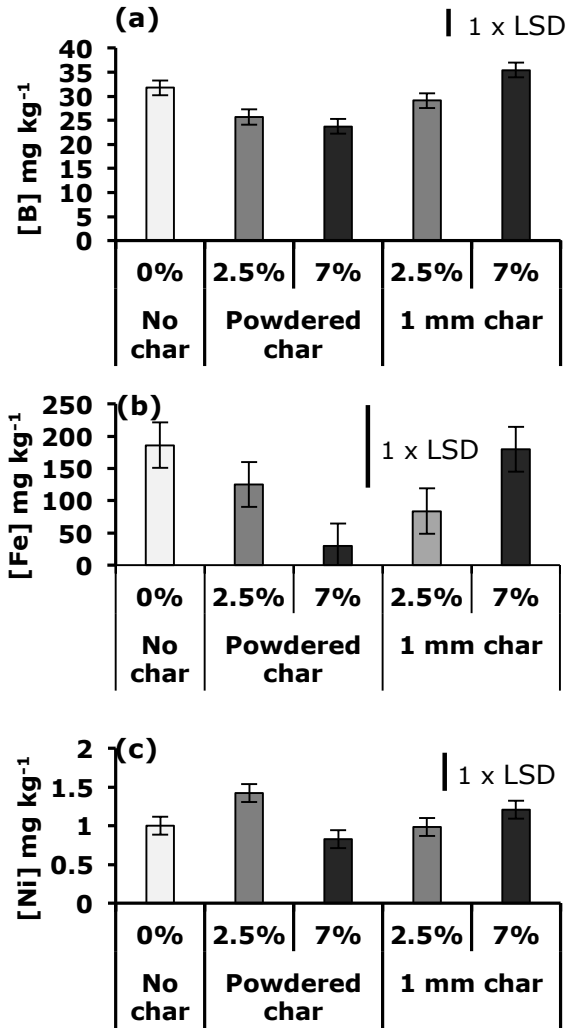



**Figure 2.12:** The effect of char particle size x char concentration on: **(a)** Boron ($F_{1,13}$=7.17, $p$=0.019, LSD=4.8), **(b)** iron ($F_{1,13}$=7.40, $p$=0.017, LSD=106.8) and **(c)** nickel ($F_{1,13}$=12.74, $p$=0.003, LSD=0.35) leaf concentration. Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bars represent the LSD (5%).

Inoculation with supplementary *Rhizobium* (as a single factor) resulted in significantly lower foliar Co and Cu and increased Cr and Mn (Table 2.2).

Aluminium concentration in leaves was affected by both char concentration ($p$=0.001) and char size ($p$=0.027) where the 2.5% char amendment and the powdered char significantly increased foliar Al by 65% and 41% respectively.

**Table 2.2:** Effect of *Rhizobium* inoculum as a single factor on leaf concentration of four essential plant micronutrients.

| mg $kg^{-1}$ | R- | R+ | *SE | p |
|---|---|---|---|---|
| **Co** | 0.64 | 0.44 | 0.052 | 0.018 |
| **Cr** | 0.20 | 0.91 | 0.043 | <0.001 |
| **Cu** | 4.13 | 3.46 | 0.214 | 0.018 |
| **Mn** | 218 | 142 | 23.59 | 0.046 |

* Standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom.

### 2.3.5 Root chemistry

Data for elemental analysis (macro- and micro-nutrients) of root material (with associated nodules) are given below. The key macro- and micro-nutrients quantified were the same as for shoots. The term 'root' refers to root + nodules. However, subsamples of nodules were also taken and C and N concentrations measured.

#### *2.3.5.1 Root macronutrients*

Root C (%) was significantly lower in plants grown in soil amended with powdered char (39.4%) compared to those in soil supplemented with the 1 mm size char (41.7%). C concentration in soil-grown roots (40.8%) was not significantly different from that of the other two treatments (ANOVA: char size as a single factor, $F_{1,13}$=14.66, $p$=0.002, LSD=1.57).

*Rhizobium* inoculation reduced root %C in plants grown in 0% and 2.5% char (size fractions pooled) compared to plants without added *Rhizobium*. However, this was only significant for the 2.5% char treatment. Inoculation did not affect %C in roots grown with 7% char compared to the uninoculated plants from the same char treatment. However, none of the *Rhizobium* treatments was significantly different across the char concentrations and the interaction is driven by the lower %C in R- plants in the 7% char treatment (ANOVA: char conc. x *Rhizobium* inoculum interaction, $F_{1,13}$=8.86, $p$=0.011, LSD=2.22; Fig. 2.13).

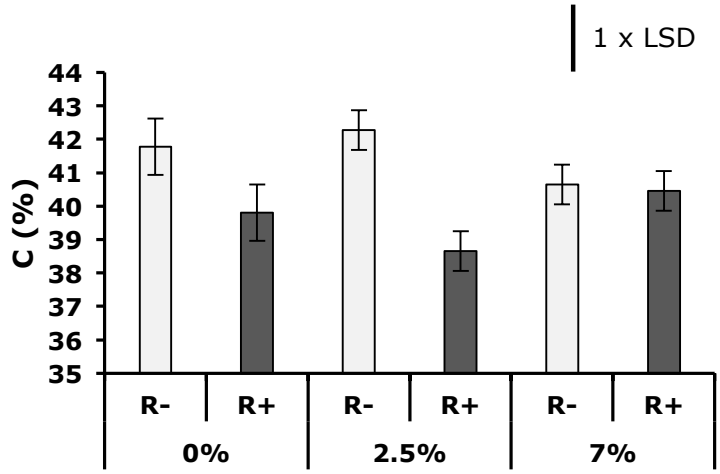



**Figure 2.13:** Root carbon (%) (char conc. x *Rhizobium* inoculation, $p$=0.011). Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%).

Nodule %C was increased by the powdered char/R- treatment compared to all other treatments. Percentage C was also higher in nodules taken from the 1 mm char/R+ treatment compared to the R+ /no char treatment (ANOVA: char x *Rhizobium* inoculation interaction, $F_{1,13}$=24.05, $p$<0.001, LSD=6.6; Fig. 2.14a). The presence of powdered char in soil also significantly increased nodule N content in the absence of supplementary *Rhizobium* (ANOVA: char x *Rhizobium* inoculation interaction, $F_{1,13}$=12.0, $p$=0.004, LSD=0.74; Fig. 2.14b). The apparent increase in %N in nodules from the R+/1 mm char treatment was not significant. No other elements were quantified within nodules.

There was no significant linear relationship between total nodule number and nodule C content. However, when char size was fitted in an accumulated model (GLM: total nodule number ($F_{1,21}$=3.32, $p$=0.083) + char size ($F_{2,21}$=6.81, $p$=0.005) then 36.7% of the variance in the data was explained. There was no interaction and the parallel lines relating to the predictions show this (Fig. 2.14c). When treatment categories were separated out, %C within nodules was greater with increasing numbers of nodules. The regression coefficients were significant for nodule number ($t$=3.03, $p$=0.006), soil only ($t$=-2.32, $p$=0.031) and soil + powdered char ($t$=2.14, $p$=0.044). The 1mm char was set as the reference class.

Root N (grand mean, 2.3%) was unaffected by any treatment.

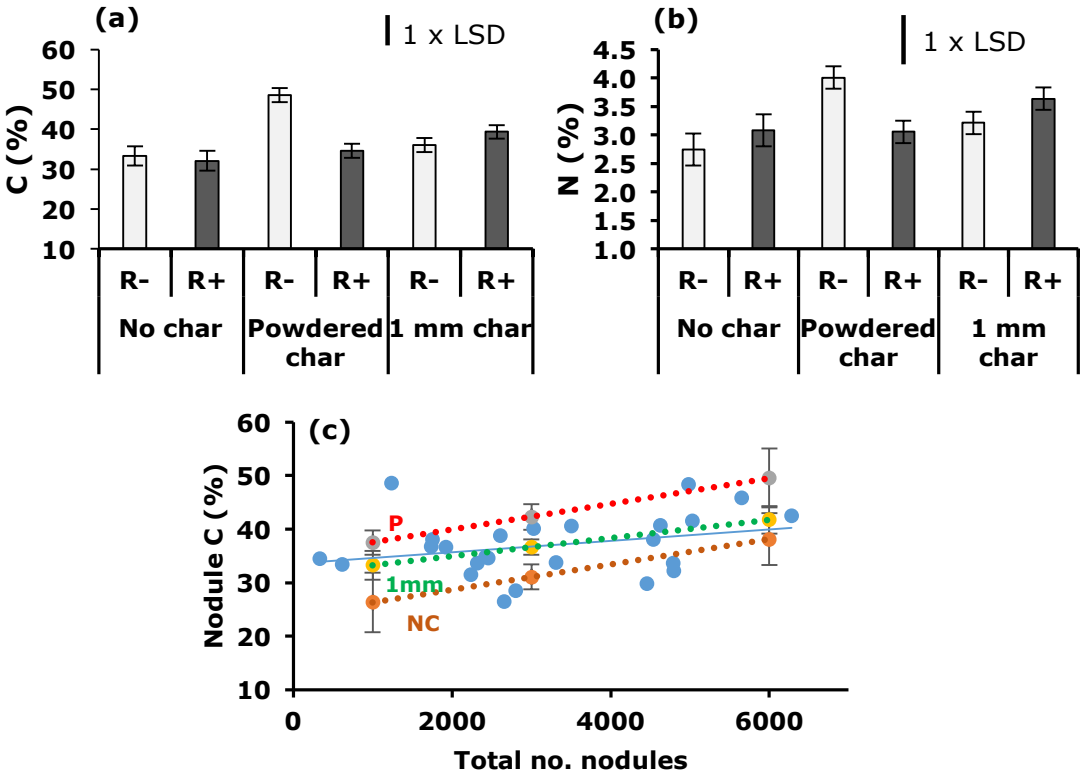



**Figure 2.14: (a)** Nodule %C and **(b)** nodule %N. (Char size x *Rhizobium* inoculation, $p<0.001$ for %C and $p=0.004$ for %N). Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%). **(c)** Relationship between total number of root nodules and nodule %C with observed and predicted data for char size groups, powdered (P), 1 mm size fraction and no char/soil only (NC). (GLM model: Nodules (NS) + char size ($p=0.005$))

*Rhizobium* inoculation as a single factor significantly increased the concentration of root P, K and S, but decreased Ca and Mg concentrations. Only Ca, Mg and K concentrations were affected by *Rhizobium* as a single factor and not as part of a *Rhizobium* x char interaction (Table 2.3). Char size as a single factor affected root [Ca] and [K], with a lower [Ca] in roots grown with the powdered char amendment compared to the 1mm char treatment (soil, 6.0 g $kg^{-1}$; powdered char, 5.6 g $kg^{-1}$; 1 mm fraction, 6.4 g $kg^{-1}$; $p=0.011$, LSD= 0.6). Root [K] was also reduced by the presence of powdered char (soil, 17.9

g $kg^{-1}$; powdered char, 24.4 g $kg^{-1}$; 1 mm fraction, 17.4 g $kg^{-1}$; $p$=0.028, LSD = 6.1).

Root [P] and [S] were both increased by R+/no char and R+/2.5% char treatments, but this effect was not observed in roots subjected to the 1 mm char fraction. However, R+/1mm char lowered [P] and [S] compared to that in roots from the R+/2.5% char treatment. Since the trends for root P concentration (ANOVA: char conc. x *Rhizobium* amendment interaction, $F_{1,13}$=18.32, $p$<0.001) and root S concentration (ANOVA: char conc. x *Rhizobium* inoculation interaction, $F_{1,13}$=19.95, $p$<0.001, LSD=1.8) are the same, only data for [S] are shown (Fig. 2.15a).

**Table 2.3:** Effect of *Rhizobium* inoculum as a single factor on root concentration of three essential plant micronutrients.

| g $kg^{-1}$ | R- | R+ | *SE | *p* |
|---|---|---|---|---|
| **Ca** | 6.30 | 5.72 | 0.16 | 0.026 |
| **K** | 16.08 | 24.48 | 1.79 | 0.005 |
| **Mg** | 8.12 | 5.93 | 2.65 | <0.001 |

* Standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom.

Roots subjected to the 1 mm size fraction, ± added *Rhizobium*, had less S than roots from any other treatment except for those grown in R- soil (no char). Overall, the highest [S] was observed in roots from the powdered char + additional *Rhizobium* treatment (ANOVA: char size x *Rhizobium* inoculation interaction, $F_{1,13}$=5.36, $p$=0.038, LSD=1.8; Fig. 2.15b).

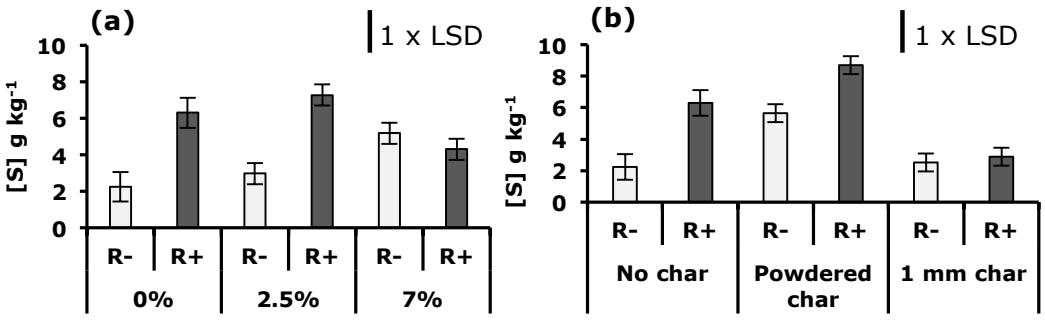



**Figure 2.15:** Root S concentrations showing: **(a)** Char conc. x *Rhizobium* inoculation interaction, $p<0.001$ and **(b)** char size x *Rhizobium* inoculation interaction, $p=0.038$). Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%).

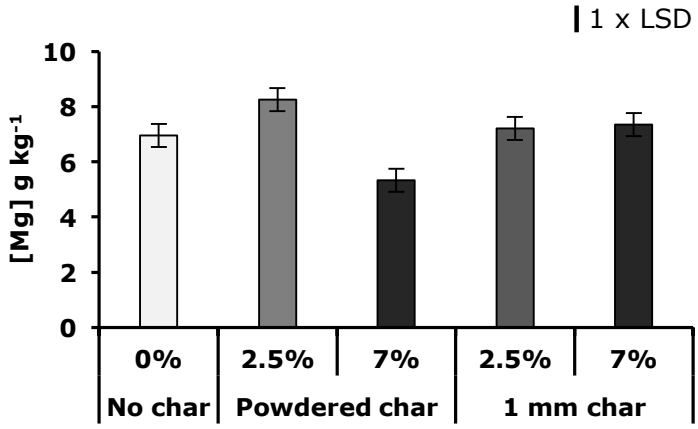



**Figure 2.16:** Root Mg concentration (char size x char conc. interaction, $p=0.003$). Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%).

A significantly greater [Mg] was recorded from the roots grown with 2.5% powdered char than from those grown in soil only, although this was not significantly different from the 1 mm char treatment. The 7% powdered char amendment had a detrimental effect, lowering [Mg] to below concentrations observed in roots from any other treatment (char size x char conc. interaction, $F_{1,13}=13.34$, $p=0.003$, LSD=1.28; Fig. 2.16).

### *2.3.5.2 Root micronutrients*

Only root [Cu] was unaffected by either char or *Rhizobium* amendments and neither [Ni] or [Cr] responded to *Rhizobium* addition. All other micronutrients measured responded to *Rhizobium* amendment as a single factor (Table 2.4). Mo and Zn concentrations were affected by *Rhizobium* x char interactions, whilst Co, Cr, Fe and Ni responded to char amendment as a single factor, whether by concentration (Ni), particle size (Co, Fe and Zn) or by char presence (size and concentration pooled) (Cr).

**Table 2.4:** Effect of *Rhizobium* inoculum as a single factor on root concentration of five essential plant micronutrients.

| mg $kg^{-1}$ | R- | R+ | *SE | *p* |
|---|---|---|---|---|
| **Al** | 315 | 239 | 20.43 | 0.022 |
| **B** | 13.4 | 14.6 | 0.26 | 0.004 |
| **Co** | 0.7 | 0.5 | 0.04 | 0.009 |
| **Fe** | 595 | 455 | 64.50 | 0.031 |
| **Mn** | 129 | 83 | 8.27 | 0.002 |

* Standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom.

Root [Mo] was significantly increased by the 2.5% powdered char and the 7% 1 mm char amendments compared to that of roots grown in soil without char. The 7% 1 mm char and the 2.5% powdered char applications resulted in the highest Mo concentration (ANOVA: char size x char conc., $F_{1,13}$= 5.86, $p$=0.031, LSD=13.4; Fig. 2.17a).

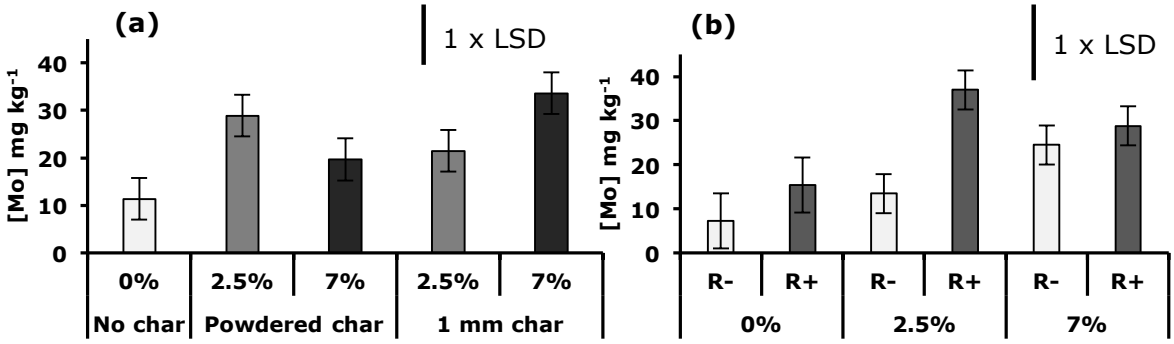



**Figure 2.17:** Root Mo concentration. **(a)** Char size x char conc. interaction, $p$=0.031. **(b)** Char conc. x *Rhizobium* inoculation, $p$=0.048. Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%).

Root [Mo] was significantly increased by the R+/2.5% char treatment, but not by R+ when applied to soil only, or to the 7% char concentration (ANOVA: char conc. x *Rhizobium* inoculation, $F_{1,13}$=4.77, $p$=0.048, LSD=16.5; Fig. 2.17b).

In the absence of additional *Rhizobium*, the 7% char treatment led to a higher root [Zn] concentration than that of the other R- treatments. *Rhizobium* inoculation led to significantly higher [Zn] in roots from soil and 2.5% char treatments but not from the 7% char treatment (ANOVA: char conc. x *Rhizobium* inoculation, $F_{1,13}$=13.73, $p$=0.003, LSD=9.9; Fig. 2.18).

Zinc concentration in roots was significantly higher (by 9 mg $kg^{-1}$) when plants were grown in powdered char relative to those grown with the 1 mm size fraction or in soil only ($F_{1,13}$=14.06, $p$=0.002).

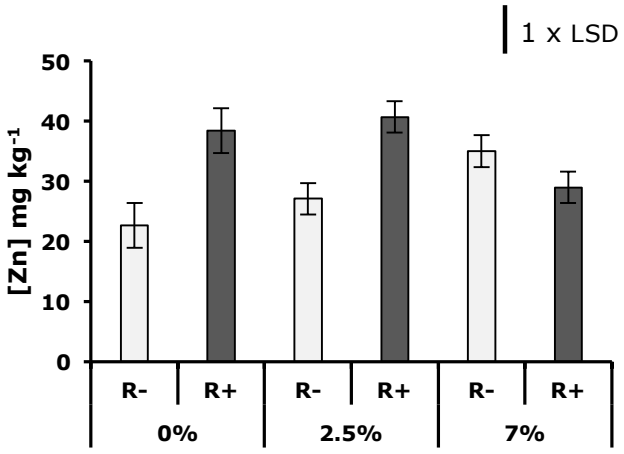



**Figure 2.18:** Root Zn concentration (char conc. x *Rhizobium* inoculation, $p$=0.003). Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%).

The presence of char (particle size and concentration combined) significantly lowered root [Cr] ($p$=0.019), whilst powdered char reduced root [Co] and [Fe] (char size as a single factor, $p$=0.020 and $p$=0.003 respectively) and 7% char concentration (as a single factor) reduced [Ni] compared to the other soil treatments ($p$=0.030).

### 2.3.6 Soil chemistry

#### *2.3.6.1 Water extractable elemental concentrations*

*Rhizobium* and char amendments altered the water extractable concentrations of both macro- and micro-nutrients in the potting medium as single factors and as interactions. Of the char treatments, particle size had an effect on water extractable concentrations of most elements quantified except for B, Cu, Mo and Zn. However, B, Cu and Zn responded to char concentration as a single factor and Mo was affected by a char concentration x *Rhizobium* inoculation interaction and a char concentration x char size interaction. Only extractable C was not significantly different between treatments. Extractable N was significantly lower in the powdered char treated soil (68.8 mg $kg^{-1}$) than in the soil without char (94.8 mg $kg^{-1}$) or that with the 1 mm size fraction (98.8 mg

$kg^{-1}$) (ANOVA: char size as a single factor, $F_{1,13}$= 7.08, $p$=0.020). Extractable C followed a similar trend, except the lowest concentrations were from the R+/powdered char and R-/1mm char treatments (ANOVA: char size as a single factor, $F_{1,13}$= 5.01, $p$=0.043).

The main char size effect was to increase water extractable concentrations of Ca, K, Mg, Mn, Ni, P and S in soil containing powdered char compared to soil alone, or soil amended with 1 mm char. The other main size effect was to reduce concentrations of Al, Co, Cr and Fe in soil containing 1 mm char compared to the other two soil treatments. With the exception of Mn and Ni, the response appears to be split between macro- and micronutrients (Table 2.5).

**Table 2.5:** Concentrations of water extractable elements from the potting medium at the end of the experiment. Data show the effects of char size as a single factor. Data are mg $kg^{-1}$ apart from Co and Cr which are µg $kg^{-1}$.

| **Macro-nutrient** | **No char** | **Powdered char** | **1mm char** | ***LSD** | **p** |
|---|---|---|---|---|---|
| **Ca** | 67.1 | 87.2 | 71.3 | 13.4/16.4 | 0.024 |
| **K** | 40.2 | 105.9 | 31.5 | 24.2/29.6 | <0.001 |
| **Mg** | 23.84 | 29.18 | 22.14 | 4.1/5.0 | 0.002 |
| **P** | 8.81 | 17.61 | 7.48 | 2.0/2.5 | <0.001 |
| **S** | 176.3 | 199.2 | 165.1 | 27.4/33.5 | 0.019 |
| | | | | | |
| **Micro-nutrients** | **No char** | **Powdered char** | **1mm char** | ***LSD** | **p** |
| **Al** | 21.61 | 20.05 | 8.13 | 3.7/4.5 | <0.001 |
| **Co (µg)** | 8.23 | 9.27 | 5.49 | 1.0/1.2 | <0.001 |
| **Cr (µg)** | 35.0 | 32.0 | 14.8 | 5.7/7.0 | <0.001 |
| **Fe** | 10.52 | 9.14 | 6.31 | 1.7/2.1 | 0.003 |
| **Mn** | 0.35 | 0.70 | 0.27 | 0.09/0.1 | <0.001 |
| **Ni** | 0.053 | 0.062 | 0.033 | 0.008/0.01 | <0.001 |

*Two LSD values are given because of the uneven replicate number. The lower value should be used when comparing between the two char treatments and the larger value should be used when comparing between the no char and plus char treatments.

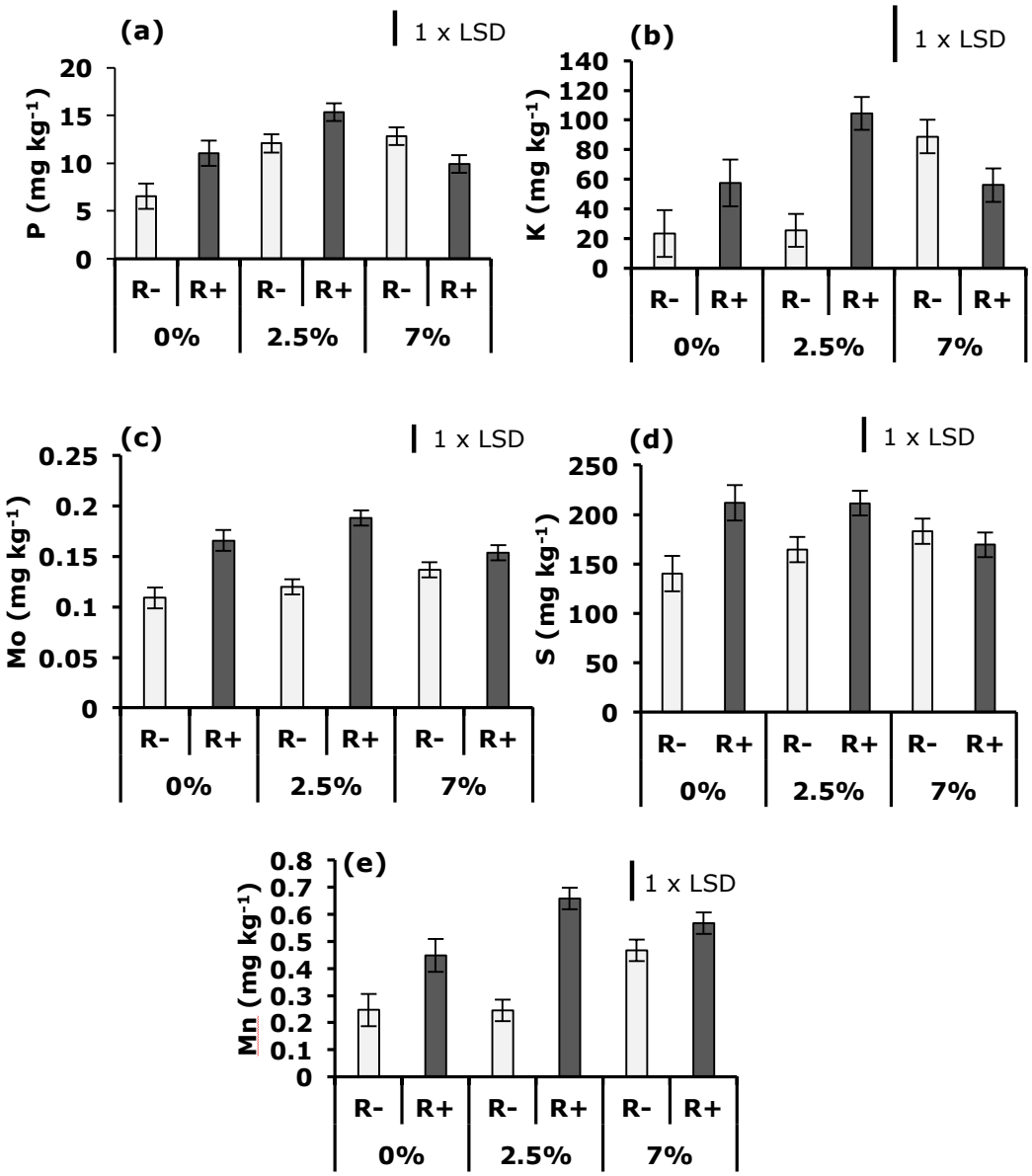



**Figure 2.19:** Concentrations of water soluble elements in soil treated with *Rhizobium* and char showing a char conc. x *Rhizobium* inoculation interaction for: **(a)** P ($F_{1,13}$=11.16, $p$=0.005); **(b)** K ($F_{1,13}$=25.02, $p$<0.001); **(c)** Mo ($F_{1,13}$=11.44, $p$=0.005, LSD=0.023); **(d)** S ($F_{1,13}$=5.75, $p$=0.032) and **(e)** Mn ($F_{1,13}$=12.89, $p$=0.003). Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%).

A significant increase in water soluble B (29%), K (50.5%), Mn (73.4%) and S (16.4%) was associated with *Rhizobium* inoculation (as a single factor) whilst *Rhizobium* inoculation decreased water extractable Ni (15.7%) and Zn

(25.8%). However, the effect of *Rhizobium* inoculation on water soluble K ($F_{1,13}$=25.02, $p$<0.001, LSD=34.2), sulfur ($F_{1,13}$=5.75, $p$=0.032, LSD=38.7), molybdenum ($F_{1,13}$=11.44, $p$=0.005, LSD=0.023), phosphorus ($F_{1,13}$=11.16, $p$=0.005, LSD=2.8) and manganese ($F_{1,13}$=12.89, $p$=0.003, LSD=0.16), was modified by char concentration. The overall trend common to these data is that *Rhizobium* supplementation increased water soluble concentrations in soil without char and with 2.5% char. This effect was negated in soil amended with 7% char (Figure 2.19).

### *2.3.6.2 Total elemental concentrations*

Both char and *Rhizobium* amendments affected total elemental concentrations of the experimental soils measured after hydrofluoric acid digestion. Sulfur data were discounted because of likely volatilisation during digestion. There was a consistent trend across all the elements of increased total concentration in soils amended with powdered char compared to the char-free soil, or soil with the 1 mm char fraction when char was considered as a single factor. The highest char concentration applied consistently led to a reduction in total concentrations of all elements apart from Ca when char concentration was considered as a single factor. Calcium was significantly higher in soils containing char than in soil without char (ANOVA: char as a single factor, $F_{1,13}$=21.46, $p$<0.001; data for soil only, 8.2 g $kg^{-1}$ and soil + char, 10.6 g $kg^{-1}$).

*Rhizobium* inoculation did not influence total elemental concentrations as a single factor, but all elements apart from B and Ca responded to a char x *Rhizobium* inoculation interaction (Table 2.6), or to a variation of that interaction (i.e. char concentration x *Rhizobium* inoculation (K) or char size x *Rhizobium* inoculation (K and Fe)). Total [K] was lower in soils amended with 7% char but without added *Rhizobium*, than in soils subjected to the other treatments. More interestingly, soil with 2.5% powdered char added had significantly higher total [K] than soil with the other char size x concentration treatments. Soil amended with 7% powdered char and the 1 mm fraction (at 2.5% and 7%) had similar total K concentrations and these were all lower than that of the no char (soil only) treatment (ANOVA: char size x char concentration interaction, $F_{1,13}$=39.83, $p$<0.001, LSD=0.35; Fig. 2.20a). Total [Fe] was

higher in soil amended with 2.5% powdered char than in any other soil/char combination. Iron concentration in the 1 mm char treatments was similar irrespective of concentration added and both had lower Fe concentrations than the soil without char (ANOVA: char size x char concentration interaction, $F_{1,13}$=13.07, $p$=0.003, LSD=0.59; Fig. 2.20b).

Total soil %C was higher in treatments with char than without.

**Table 2.6:** Total elemental concentrations in soil assayed at the end of the experiment showing a char x *Rhizobium* inoculation interaction.

| Element | No char | | + Char | | *LSD | *p* |
|---|---|---|---|---|---|---|
| | R- | R+ | R- | R+ | | |
| **Al (g)** | 22.9 | 27.8 | 24.8 | 22.6 | 2.7/2.1/1.4 | <0.001 |
| **Co** | 5.2 | 5.7 | 5.4 | 5.2 | 0.3/0.2/0.1 | <0.001 |
| **Cr** | 38.0 | 42.2 | 37.4 | 36.8 | 2.7/2.1/1.3 | 0.004 |
| **Cu** | 14.5 | 18.7 | 16.3 | 15.1 | 1.6/1.3/0.8 | <0.001 |
| **Fe (g)** | 15.6 | 17.1 | 15.9 | 15.6 | 0.8/0.7/0.4 | 0.001 |
| **Mg (g)** | 6.3 | 7.6 | 7.4 | 7.0 | 0.8/0.7/0.4 | 0.001 |
| **Ni** | 21.6 | 26.0 | 22.7 | 21.3 | 1.7/1.4/0.9 | <0.001 |
| **P** | 375.3 | 428.8 | 393.3 | 364.3 | 33.2/26.2/16.6 | <0.001 |
| **Zn** | 38.1 | 54.7 | 52.2 | 42.6 | 16.1/12.7/8.1 | 0.008 |

*Three LSD values are shown because of different numbers of replicates in each group (soil or char). First LSD value: for comparing within the soil (no char) group; second value: for comparing between soil and char groups; third value: for comparing within the char group.

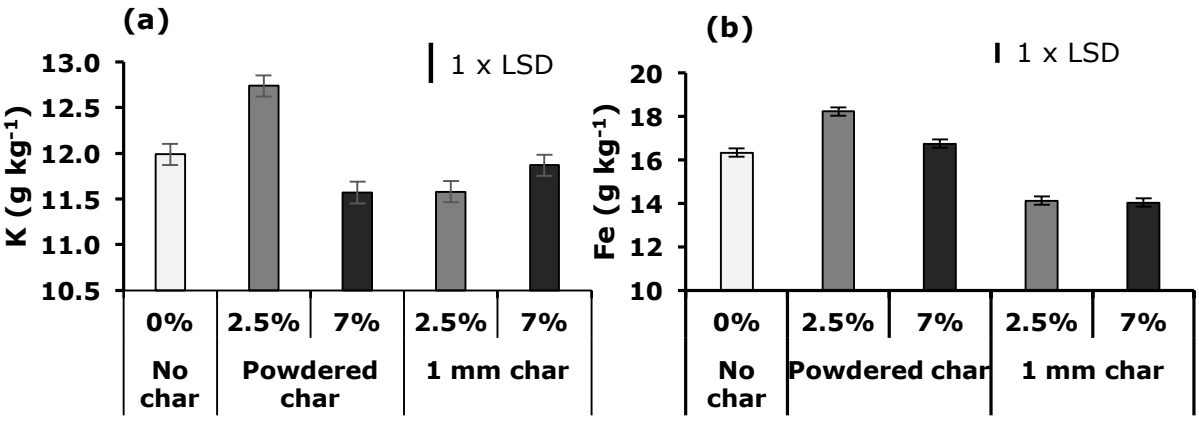



**Figure 2.20:** Total **(a)** K concentration ($P$<0.001) and **(b)** Fe concentration ($p$=0.003) in soil showing a char size x char concentration interaction for each. Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%).

### *2.3.6.3 Soil pH*

Presence of powdered char increased soil pH (char size as a single factor, $F_{1,13}$=13.46, $p$=0.003, LSD=0.22). The 7% amendment increased pH over that of the 2.5% amendment and soil only (char concentration as a single factor, $F_{1,13}$=11.93, $p$=0.004, LSD=0.22; Fig. 2.21a).

*Rhizobium* addition as a single factor increased pH from 6.2 to 6.5 ($F_{1,13}$=12.11, $p$=0.004). There was no *Rhizobium* amendment x char interaction.

Since pH affects nutrient availability and in turn is influenced by several factors, General Linear Model multiple regressions were carried out using a number of factors as possible explanatory variables for pH; e.g. total nodule number, soil [Ca], [Mg], [N], [P], [C], char concentration and char size. Char concentration and size were included separately in the model because they were aliased. The factors that explained most of the variation in pH were total root nodule number and char size. None of the other explanatory variables were significant in any combination within the models.

Soil pH was significantly influenced by the total number of nodules when fitted as a single explanatory variable (regression: $F_{1,23}$=7.69, $p$=0.011). Total

nodule number only explained 21.8% of the variability in the pH response suggesting other explanatory variables should be included. However, the only ones that helped explain the data were char size and char concentration.

When nodule number and char size were fitted as an accumulated GLM, 39.1% of the variation in the pH data was explained, although only nodule number was significant [nodule number ($F_{1,19}$=9.88, $p$=0.005) + char size ($F_{2,19}$=3.17, $p$=0.065)] and there was no interaction between the two variables. However, the $t$-statistic from the estimated coefficients, suggested that the coefficient for powdered char ($t$=2.49, $p$=0.002) was significant if other variables in the model were kept constant. In order to summarise the data, estimated means were predicted from the regression model for three nodule densities for soil, soil + powdered char and soil + the 1 mm fraction (Fig. 2.21b).

When nodule number was fitted with char concentration, 35.3% of the variance was accounted for in accumulated ANOVA generated from the GLM [nodule number ($F_{1,21}$=9.29, $p$=0.006) + char concentration ($F_{2,21}$=3.39, $p$=0.053)]. The coefficients for nodule number ($t$=-2.09, $p$=0.049) and 7% char concentration ($t$=2.51, $p$=0.020) were both significant if other variables in the model were held constant. The data are summarised as predictions from the regression model for three nodule densities for soil, soil + powdered char and soil + the 1 mm fraction (Fig. 2.21c).

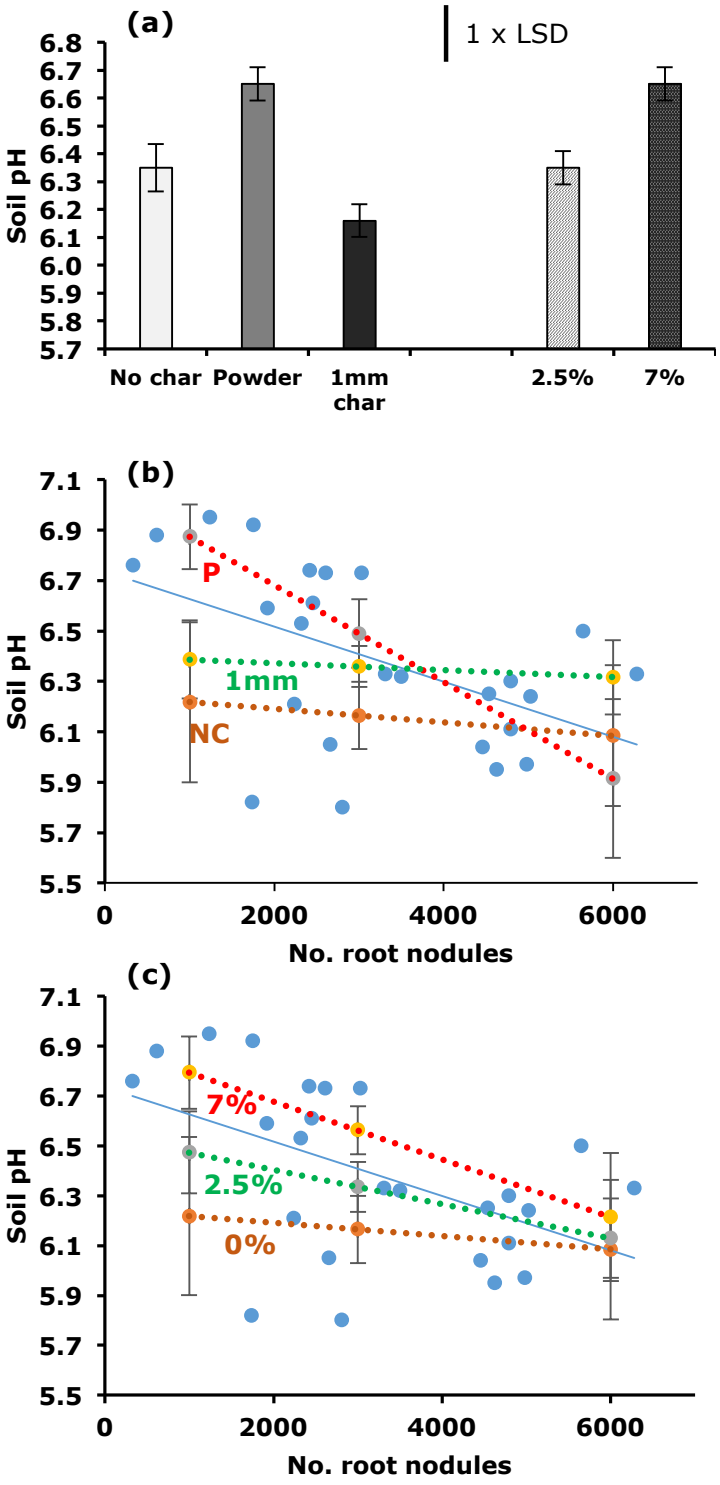



**Figure 2.21: (a)** Effect of char size as a single factor on soil pH ($p$=0.003; columns 1-3) and of char concentration as a single factor ($p$=0.004; columns 1, 4 & 5). Individual standard errors are based on the pooled variance estimate from the ANOVA with 13 degrees of freedom. Vertical bar represents the LSD (5%). **(b)** Soil pH as a function of total number of root nodules; blue trend line for observed data, $p$=0.011. Lines labelled P, 1mm and NC are predictions (± se) from regression model for powdered char (coefficient, $p$=0.002), 1mm char and no char treatments respectively. **(c)** As for (b) but predictions are for char concentration; 0% (no char), 2.5% and 7% char. (Coefficient for 7% char, $p$=0.020).

## 2.4 DISCUSSION

Although there are published papers which demonstrate a positive effect of char amendment on biological $N_2$ fixation in legumes, there is still a lack of data and limited understanding about the mechanisms behind any interactions. Furthermore, most papers vary soil char concentrations, but do not consider char particle size. This is of real importance, because this investigation has demonstrated that plant responses to powdered char are different from those to large particle sizes and these effects are in addition to those caused by delayed germination. Also, powdered char has fewer microhabitats for beneficial soil microbes, due to the small size and limited micropores. Previous literature reported that the structure of biochar provides a refuge for small beneficial soil organisms, such as symbiotic mycorrhizal fungi which can penetrate deeply into the pore space of biochar fungal hyphae where sporulation goes on with less competition from saprophytes (Saito and Marumoto, 2002).

Time from sowing to germination of beans growing in soil amended with 2.5% powdered char was extended compared to that of other treatments. The reason for this is unknown, but Buss and Masek (2011) demonstrated that re-condensation of volatile organic compounds during pyrolysis can lead to char with high concentrations of soluble phytotoxic compounds. Since the char came from the same batch, the reason for one set of plants exhibiting potential toxicity is unclear. In contrast, char increased wheat germination rates at application rates of <50 t $ha^{-1}$, but inhibited them at higher rates (Solaiman *et al*., 2012), so effects are char and plant species dependent. The cause of the inhibitory effect here is unknown, it is unlikely to be toxicity-related or other plants would have been affected. Delayed germination may have led to smaller plants, although plants were grown to the flowering stage before harvesting and had time to recover. This observation is important in general since char is used as a soil conditioner and fertilizer and has been proposed as a means of sequestering large amounts of carbon in soils; any unintended consequences of application should therefore be understood (Kuppusamy *et al.,* 2016).

Additional *Rhizobium* amendment (R+) resulted in reduced plant biomass relative to the uninoculated (R-) plants when grown in soil and in soil + 2.5%

char, as well as in soil containing powdered char. The negative growth effect due to *Rhizobium* addition was not observed in plants growing with the 1 mm char amendment. What is not known is whether the commercial (additional) rhizobia outcompeted the indigenous species for occupation of nodules, or whether they were more effective strains. Adding the commercial strain did not appear to be beneficial and it is possible that the bean plants could not regulate the relative resources required to maintain a mutualistic relationship (Lau *et al*., 2012). Nevertheless, there was a positive relationship between plant biomass and number of nodules per root system so the overall soil rhizobia had not shifted towards parasitism. The number of nodules formed was large, ranging from 1-2 per cm root length which translates to 1,200 $g^{-1}$ root dry weight and 2,935 nodules per root system (dry weight basis). Adding the commercial inoculum increased nodule number by 34%. Hansen *et al*. (1990) demonstrated that genetically modified hypernodulating soybean could not regulate allocation of resources to rhizobia in the same way that wild soybean varieties could, resulting in reduced growth. It is possible that the dwarf bean variety grown here could not regulate the commercially developed inoculum in tandem with the indigenous strains. Moawad and Bohlool (1984) reported that in the field, whilst cultured *Leucaena* rhizobia strains could compete with field strains the outcomes varied, with native strains forming 4-72% of the total nodules. The increase in biomass production after biochar application was also found in other legumes, including clover (Mia *et al*., 2014; Oram *et al*., 2014), common bean (Rondon *et al*., 2007), and alfalfa (Nishio and Okano, 1991). Steiner *et al.* (2007b) also observed that the application of biochar significantly increased nutrient uptake and plant growth.

Mia *et al*. (2014) found that when grown with mixtures of *Festuca rubra* and *Plantago lanceolata*, nitrogen fixation and biomass of red clover (*Trifolium pratense*) increased when grown with char at a rate of 10 t $ha^{-1}$, but not at 120 t $ha^{-1}$. These authors attributed the char-related increase in biological nitrogen fixation to increased soil K availability. In the current experiment, water extractable K concentration was enhanced by the highest char application (7%) when only native rhizobia were present (i.e. R- treatment), but by the 2.5% amendment when the commercial strain was also present. Water soluble soil K was reduced by the highest char amendment. It seems as if the same trend as

that reported by Mia *et al*. (2014) was observed here in the presence of the commercial rhizobia. However, in the presence of native rhizobia only, the higher char amendment led to a K enhancement; therefore, origin of the rhizobia affected the K response to char concentration. The char amendments in this study were approximately 18 t $ha^{-1}$ and 50 t $ha^{-1}$ on a dry weight basis; these are an approximation because they have not been adjusted for the bulk density of the soil/potting compost mixture (which was not measured) and were calculated based on a bulk density of 1.2g $cm^{-3}$. Furthermore, comparing tonnes of char $ha^{-1}$ across papers is difficult because not all state whether char is added on a field moist or a dry weight basis.

Interestingly P followed the same trends as K and P is well-known as an essential element for the growth of *Rhizobium* spp. (Leung and Bottomley, 1987). Previous literature also reported that a qualitative requirement for K has been demonstrated for some rhizobia (Sherwood, 1970). In addition, a bacterial $Ca^{2+}$-binding protein, designated as rhicadhesin, is involved in direct attachment of bacteria to the surface of the root hair cell (Smit *et al*., 1992). Sugawara *et al.* (2011) reported that strains of *Bradyrhizobium* spp. likely use organosulfur compounds for growth and survival in soils as well as for nodulation and nitrogen fixation.

The Mo concentration in leaves of plants from the R+/2.5% char treatment was higher than that in the corresponding leaves from the R-/2.5% treatment. This suggests that rhizobia present in these plants could have fixed $N_2$ more effectively than the R- plants, because Mo is essential for biological $N_2$ fixation (Rondon *et al*., 2007). The trend for increased Mo was particularly clear in the root tissues where the R+/2.5% char treatment significantly increased it compared to the R-/2.5% treatment. If root Mo concentrations could be considered as an indication for the potential, if not likelihood, that biological $N_2$ fixation occurred, it could be hypothesized that the 7% char amendment increased biological $N_2$ fixation when only indigenous rhizobia were present and the 2.5% char amendment increased biological $N_2$ fixation by the mix of native and commercial rhizobia. These findings corroborate those of Rondon *et al*. (2007) who concluded that the key reason for char-related increase in $N_2$ fixation was B and Mo availability.

Mo is required more for nitrogen fixation than for nitrate reductase or host plant growth (Parker and Harris, 1977), as it is an essential cofactor in the nitrogenase enzyme providing the site where nitrogen is reduced to $NH_3$ (Smith, 1977).

Most experiments considering the effect of char on biological $N_2$ fixation have been conducted in pots, but Quilliam *et al*. (2013) carried out long-term field trials in North Wales. These authors found that nodule numbers on roots of white clover (*T. repens*) were unaffected by 3-year old char amendment (reapplied 4-months before sampling) although a char-related increase in nitrogenase activity in individual nodules was recorded, but this did not translate into an increase across the whole root. Nevertheless, the results here support previous studies that biochar addition to soil can enhance soil fertility through increased biological nitrogen fixation when legumes are present (Nishio, 1996; Rondon *et al*., 2007).

*Rhizobium* amendment, total nodule number and char amendment increased soil pH over the duration of the experiment. Although there was no interaction between *Rhizobium* amendment and char addition, both factors explained much of the variation in the pH data, with powdered char and the 7% concentration having the greatest impact on pH. This is likely to be attributed to the higher concentrations of water soluble Ca and Mg in soil amended with powdered char. The char itself contains substantial amounts of total Ca (4.9 g $kg^{-1}$) and water extractable Ca (144 mg $kg^{-1}$). Powdered char is known to release higher concentrations of Ca into soil solution than larger particles due to the relatively greater surface area (Razzaq unpublished/in preparation).

Behaviour of soil nutrients is largely dependent on pH and consequent char-related changes. Water soluble P increased with char amendment and whilst the char contains P, the amount was not sufficient to explain the soil P changes. P in leaf tissue was increased by *Rhizobium* addition at low concentrations of char, but not at high concentrations. At high char concentrations phosphate may be less available to plants due to precipitation of soil P by Ca, resulting in insoluble calcium-phosphates. That may also explain the lower Ca concentration in plant leaves, although water soluble P concentrations do not appear to support this. However, when considering the regression predictions for the

relationship between root nodule number on soil pH, it is clear that the relationship between powdered char and pH differs depending on the degree of nodulation (and root growth). When nodule numbers are relatively low, the liming effect of the chars in relation to higher char concentration and particle size is clear, but this is lost with increasing number of nodules. In soil without char, the nodule-related pH decrease was small, but it increased in the highest char concentration and in the powdered char treated soils. The reasons for the decrease in pH are not known, but are likely to be related to root activity. For instance, in calcareous soils, Fe deficiency often occurs (Marschner, 1995). This may have happened in this experiment when char was added since water extractable soil Fe concentration was lower in soils with char amendment. Furthermore, powdered char reduced root Fe concentration, which translated to lower leaf Fe in plants grown with the highest rate of powdered char.

Fe is an essential micronutrient and is particularly important for beans since it is an important component of the nitrogenase complex (Ludden, 2001) and legumes require high amounts for nodule formation (Weisany *et al*., 2013). This may be one of the reasons for lower nodule numbers $cm^{-1}$ root length and total nodule numbers per root system when plants were grown with powdered char amendments. Char appeared to limit nodule development in roots where the additional inoculum had been added.

To overcome Fe deficiency in calcareous soils, plants produce organic acids (e.g. citrate and malate) to promote dissolution of ferric hydroxides and in so doing may excrete $H^+$ (see Jones, 1998). It is feasible that addition of high concentrations of powdered char resulted in plant production of organic acids which lowered the soil pH. This hypothesis is supported somewhat by the slope of the line with increasing nodule numbers (Fig. 2.19b).

Nitrification is known to lower soil pH, but it might be expected that roots would take up the resulting nitrate ions thereby limiting any acidification effect resulting from char-related increased nitrification. Char has been shown to increase nitrification and concomitant acidification in plant-free soil (Martin *et al*., 2015), but in this current experiment plants were present. Therefore, it is unlikely that nitrate leaching due to char-stimulated nitrification was a factor here. Nevertheless, Loss (1992; cited by Tang et al., 1999), determined that

40% of acidification under lupins was due to nitrate leaching. Since in this current experiment, nodule number was a factor in decreasing soil pH, this supports the view that acidification was related to root activity.

The reduced concentration of extractable N in soil with powdered char amendment coincided with an increase in %N in nodules grown in char-treated soils. The proportion of plant N obtained from fixation or from soil is unknown here. However, Tang *et al*. (1999) concluded from their study of legumes given a range of nitrate concentrations, that decreased acid production was related to lower $N_2$ fixation and increased uptake of nitrate. In their investigation, Tang *et al*. (1999) showed that the plant N derived from $N_2$ fixation was lessened by increasing soil nitrate. In this current experiment, no additional N was given, therefore N should have become limited as plant growth progressed, suggesting that $N_2$ fixation occurred since none of the plants showed signs of N-deficiency. On the other hand, there may have been sufficient organic matter in the potting medium to support ammonification and subsequent nitrification. Alternatively, surface oxidation of the char may have occurred in pots with more nodules, resulting in localized acidity around char particles (Cayuela *et al*., 2014).

## 2.5 CONCLUSION

Some of the results of this study support the previous literature stating that combined application of biochar and bio-inoculants may result in more prominent growth of plants compared with their separate use (Saranya *et al*., 2011; Saxena *et al*., 2013; Akhtar *et al*., 2015). However, it is clear that field strains of rhizobia and commercial inocula may respond differently to char amendment and to char particle size. Whilst char is chemically reasonably inert depending on the production mechanism and feedstock, it will be physically broken down in the soil to smaller particles over time and soil biota responses may alter. The hypotheses suggested at the start of the investigation were somewhat naïve given the complexity of the interactions between native and commercial rhizobia and between the char amendments; it is clear that longer term studies are required.

# 3 The effects of biochar and *Rhizobium* on the competitive outcome between wheat and broad bean

## 3.1 INTRODUCTION

Cereal-legume intercropping is practiced in many countries in order to enhance nitrogen-fertilisation and it is often used as a means of increasing wheat production in an economically sustainable manner (Pelzer *et al*., 2012). Wheat is an important crop across the world (Food and Agriculture Organisation of the United Nations, 2016) and enhancing yields whilst minimising chemical fertiliser inputs is desirable.

Within intercropping systems, two crops are grown simultaneously in the same field, often in strips. Increased yields of intercropped species have been observed, particularly in plants growing within adjacent rows (see Zhang and Li, 2003). These authors reported a 74% increase in wheat grain yield when intercropped with soybean. The crops complement each other in terms of nitrogen acquisition; legumes fix atmospheric nitrogen whilst wheat utilises soil mineral nitrogen (e.g. Mariotti *et al*., 2015). Intercropping provides a means of efficient nitrogen utilisation and is used for growing human food crops (Zhang & Li, 2003) or more commonly in temperate countries, for forage crops where grasses and legumes are grown together as a mixed crop (Frasier *et al*., 2017).

Beans may therefore be used for human consumption or grown for animal fodder. Whatever their purpose, biological nitrogen fixation (BNF) by legumes is a primary strategy used to improve soil fertility; ways of enhancing the potential of the rhizobia-legume symbiosis would be beneficial (Kahindi *et al*., 1997; Thies and Rillig, 2009). One possible means of increasing BNF is to apply biochar to soil (Rondon *et al*., 2007). Several studies have shown that biochar makes a positive contribution to improving soil fertility and plays a role in carbon sequestration when used as a soil amendment (Woolf *et al*., 2010; Park *et al*., 2013). Biochar can be formed from a range of feedstocks from hard-woods to bamboo to livestock bedding and manure (Marris, 2006; McHenry, 2009). It also has a favourable impact on the environment since it can absorb

various contaminants from soil and water (Uchimiya *et al*., 2011; Park *et al*., 2013).

In Chapter 2, inoculation with a commercial strain of *Rhizobium* resulted in an increased root:shoot ratio in plants growing in soil and in soil amended with powdered biochar. However, *Rhizobium* did not alter the root:shoot ratio of beans when grown with soil treated with larger sized particles of char. The powdered char resulted in less root nodulation whilst the 1 mm size char did not affect nodule numbers. Powdered char, irrespective of *Rhizobium*, resulted in lower foliar nitrogen concentrations, whilst plants grown with the larger char particles had higher leaf nitrogen concentrations than plants grown in unamended soil, or in soil with added powdered char. Powdered char amendment was generally detrimental to the growth of the broad bean plants, particularly when additional rhizobia was present. The **main aim** of the experiment described here in Chapter 3, was to determine if dual cropping broad bean with wheat would modify the negative effect of powdered char on growth of bean and to establish whether char and *Rhizobium* would alter the competitive relationship between beans and wheat. Understanding these relationships is important before char is used on a farm scale. The most promising use for char is likely to be for carbon sequestration (Dumbrell *et al*., 2016), in which case it is possible that it may be applied to fields used for forage crops, or on small farms which may practice intercropping.

The **specific objectives** of this experiment were:

(1) To quantify the effect of *Rhizobium* inoculation on competition between broad bean and wheat plant growth.

(2) To determine if char amendment modified the competitive outcome between bean and wheat.

The **main hypotheses** were:

(1) Presence of *Rhizobium* would shift the competitive balance in favour of beans.

(2) Enhanced competition for soil nitrogen would outweigh the detrimental effect of powdered char on bean plant growth (observed in Chapter 2), resulting in a positive competitive outcome for beans in the presence of *Rhizobium*.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Experimental approach

A pot experiment under controlled conditions was conducted to determine whether the outcomes of competition between a nitrogen fixing broad bean plant (*Vicia faba* var. Optica) and a plant that obtains all of its nitrogen from the soil (*Triticum aestivum* var. Willow) would be modified by addition of char.

### 3.2.2 Experimental set up

The char used for this investigation was purchased from the BioRegional Charcoal Co. Ltd., UK; full details are given in Chapter 2, Section 2.2.2.

The experimental design and treatment regime was the same as that described in Chapter 2 (Section 2.2.3), with the exception that here, in each pot, was one bean and one wheat plant growing together as a biculture.

Briefly, the treatment structure was:

(i) Soil/compost only
(ii) Soil/compost + 2.5% powdered char
(iii) Soil/compost + 7% powdered char
(iv) Soil/compost + 2.5% of 1 mm char
(v) Soil/compost + 7% of 1 mm char

Hereafter the soil/compost mix will be referred to as 'soil', or the 'no char' treatment.

A commercial *Rhizobium* inoculum (*Rhizobium leguminosarum* biovar *viciae*) provided by Legume Technology (Nottingham, UK) was added to half the pots. In total, there were 4 replicate pots per treatment.

### 3.2.3 Harvest and sample processing

Plants were grown to maturity and bean pods collected as they ripened. The experiment was terminated when the wheat grains were ready for harvest. At harvest, shoot material of each species was separated and shoot biomass (both species) and pod (beans) and grain (wheat) yield measured. For wheat plants, weight of wheat shoots (plus and minus ears), number and weight of ears and total number and weight of grains were determined. For bean plants, weight of bean shoots (plus and minus pods), number of bean pods and weight of bean pods were determined. Dry weight was determined by drying the samples in an oven (45°C) until constant weight.

### 3.2.4 Soil and plant analyses

Total elemental concentrations and total carbon and nitrogen concentrations of the experimental soils in each pot were carried out as described in Chapter 2, Sections 2.2.4.5 and 2.2.4.6.

Bean and wheat shoot elemental concentrations, wheat grain elemental content and total carbon and nitrogen concentrations of plant shoots and wheat grain were conducted as described in Chapter 2, Sections 2.2.5.1 and 2.2.5.2.

Bean yield was insufficient for full chemical analyses.

### 3.2.5 Statistics

Data were analysed by General Analysis of Variance using Genstat version 17 (Lawes Agricultural Trust/VSN International, Hemel Hempstead, UK). Normality was tested by plotting residuals against expected normal quantiles and post hoc comparisons between means were based on least significant differences at the 0.05 probability level where appropriate.

The factors used in analysis of the data were char size (soil, powdered or 1 mm), char concentration (0%, 2.5% or 7%) and *Rhizobium* inoculum (± commercial inoculum).

## 3.3 RESULTS

For logistical reasons, root data are not available for the plants in this study; however, unlike the bean plants described in Chapter 2, the uninoculated bean roots here were free of nodules.

### 3.3.1 Bean and wheat shoot biomass

*Rhizobium* inoculation significantly affected both bean and wheat shoot biomass as a single factor and wheat grain weight (Fig. 3.1a). Inoculation reduced bean biomass by 38% ($F_{1,27}$=30.68, $p$<0.001), but increased wheat biomass by 57% ($F_{1,27}$=11.62, $p$=0.002) and wheat grain yield by 81% ($F_{1,27}$=16.79, $p$<0.001; Fig. 3.1a).

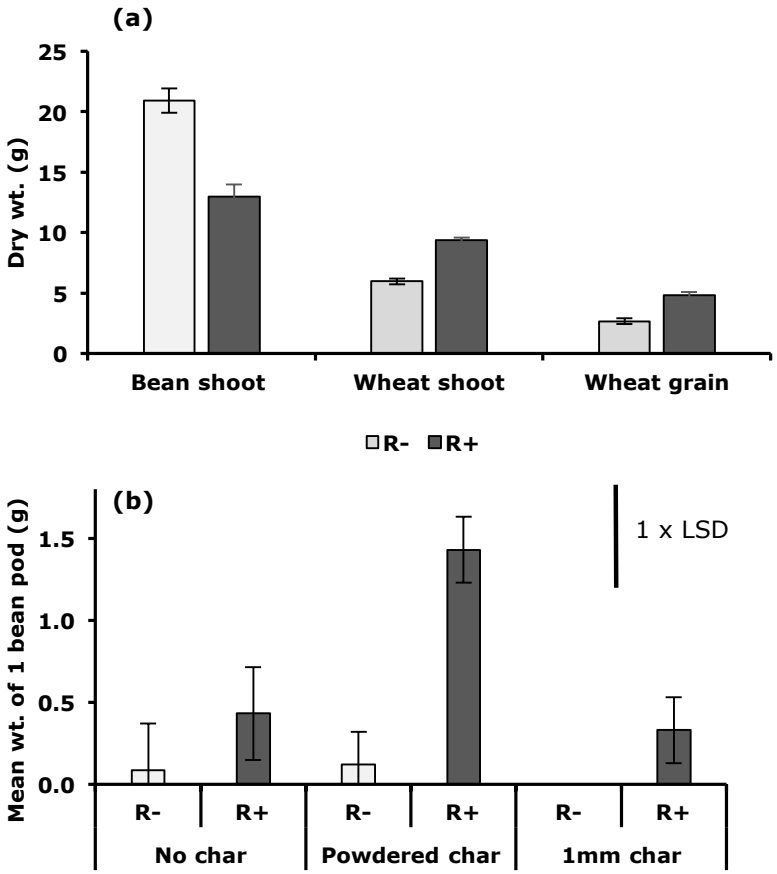



**Figure 3.1: (a)** Dry weights showing the effect of *Rhizobium* inoculation (as a single factor) on bean shoots ($p$<0.001), wheat shoots ($p$=0.002) and wheat grain

($p$<0.001). **(b)** Mean weight of 1 bean pod (*Rhizobium* x char size interaction, $p$=0.022). Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom. Vertical bars represent the LSD (5%).

The wheat ear:vegetative shoot ratio was 1.97 and 1.53 for wheat plants growing in potting medium with and without rhizobia respectively, thereby showing a proportionally higher allocation of resources into grain production. Bean pod production was sparse, ranging from 0-6 pods on any one plant, but yields across treatment groups were reasonable, for example, ranging from 0-16 pods for plants pooled into the char size x *Rhizobium* group. More pods were produced from plants inoculated with rhizobia than from uninoculated plants and this rhizobia effect was modified by char concentration (*Rhizobium* x char conc. interaction, $F_{1,27}$=8.14, $p$=0.008) where more pods were produced by plants growing in the R+/7% powdered char treatment.

The average dry weight of individual bean pods was greatest in *Rhizobium* inoculated plants grown in soil amended with powdered char (*Rhizobium* x char size interaction, $F_{1,27}$=5.94, $p$=0.022; Fig. 3.1b). The total cumulative yield across all pots within the treatment group (i.e. the *Rhizobium* x char size group) followed the same pattern as the data for mean individual bean pod weight. However, a different sum total number of bean pods was produced within each treatment group; the R+/powder and R+/1 mm groups each produced 14 bean pods and the R+/soil group produced 16 pods (adjusted for the different number of replicate pots) whilst the others produced fewer than 3 pods. This suggests that the plants growing in the R+/powdered char treated soil were fitter than the R+ plants growing in the soil with 1 mm char or those in the char-free soil, because bean pod weight did not decrease with increasing numbers. Since *Rhizobium* inoculation did not increase overall shoot weight, this infers that the plants that produced beans were preferentially allocating their resources into reproductive output.

A char concentration effect was also observed for bean pod biomass where plants grown with 7% powdered char produced pods that were heavier than those from plants grown in soil without char. The 2.5% char amendment did not affect pod weight (char conc., $F_{1,27}$=5.53, $p$=0.026). An effect of char concentration was also observed for wheat grain weight but in contrast to the

bean, the least grain was produced with the 7% char amendment (char conc., $F_{1,27}$=4.61, $p$=0.041; Fig. 3.2).

There was a significant negative relationship between bean and wheat shoot weight where larger beans resulted in smaller wheat plants and *vice versa*. This relationship was modified by the presence of rhizobia, apparently in favour of wheat growth and to the detriment of the beans (Fig. 3.3).

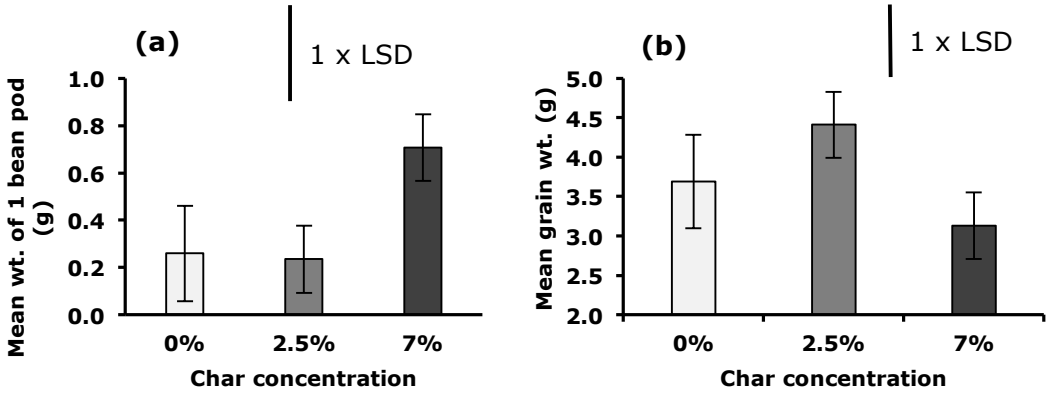



**Figure 3.2:** Effects of char concentration as a single factor on **(a)** average weight of 1 bean pod ($p$=0.026) and **(b)** wheat grain weight ($p$=0.041). Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom. Vertical bars represent the LSD (5%).

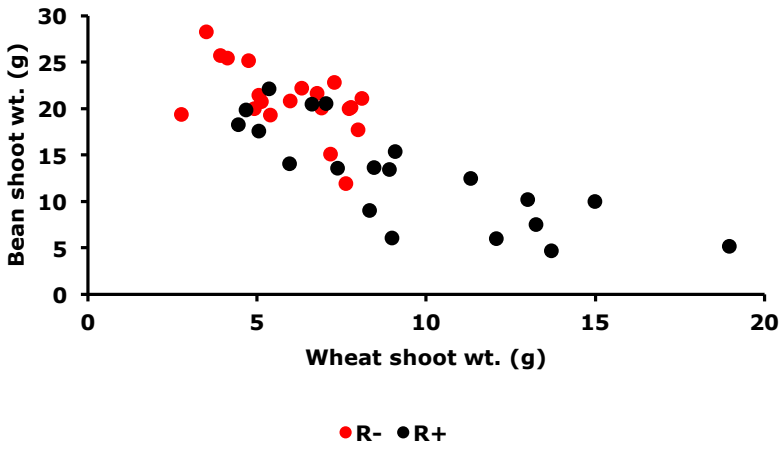



**Figure 3.3:** Relationship between bean and wheat shoot grown in the presence or absence of *Rhizobium* inoculum when one individual of each species shared the same pot.

### 3.3.2 Macro- and micro-nutrients in bean shoots

The same total macro- and micronutrients outlined in Chapter 2 were investigated in this experiment, but with the addition of selenium and sodium.

Once bean seeds were removed from their pods and ground in preparation for analysis, there was very little material to work with. Therefore, total CN analyses were performed but data cannot be analysed by ANOVA because there are too many zero values. Nevertheless, two observations were that: (i) the %C in the bean seed was higher in seeds from plants grown with powdered char (37% vs 31% for the 1 mm char and 32% for soil) and (ii) the %N in bean seeds was higher in seeds from plants grown in soil minus char (3.2%) than in soil amended with either size fraction of char (1.03%, 1 mm; 1.6%, powdered).

All other data for plant macro- and micronutrients were replicated and analysed by ANOVA.

Leaf nutrient content was primarily affected by *Rhizobium* addition as a single factor, with three macronutrients (K, P, and S) responding positively to the rhizobia inoculum. Four micronutrients also responded positively to the rhizobia treatment (B, Mo, Na and Se), whilst Mn did not (Table 3.1). The other key nutrients were unaffected by inoculum and therefore not mentioned, apart from Ni which was lower in shoots of plants grown in the R+/7% char treatment compared to the other treatments (*Rhizobium* x char conc. interaction, $F_{1,27}$=7.68, $p$=0.010) and Zn which was present at its highest concentration in shoots grown in the R+/2.5% treatment (*Rhizobium* x char conc. interaction, $F_{1,27}$=7.03, $p$=0.013). Shoot Zn was not significantly ($p$=0.054) affected by *Rhizobium* amendment, but it is included in Table 3.1 because it shows a trend towards a response.

**Table 3.1:** Elements in bean shoot that responded to *Rhizobium* inoculation as a single factor. Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom.

| **Element: g or mg kg$^{-1}$** | **R-** | **R+** | **s.e.** | $F_{1,27}$ | $p$ |
|---|---|---|---|---|---|
| **K** (g) | 12.8 | 15.8 | 0.64 | 10.66 | 0.003 |
| **P** (g) | 4.8 | 5.7 | 0.20 | 11.55 | 0.002 |
| **S** (g) | 1.6 | 2.1 | 0.75 | 17.12 | <0.001 |
| **B** (mg) | 12.2 | 15.9 | 0.62 | 17.27 | <0.001 |
| **Mn** (mg) | 130.6 | 106.5 | 0.62 | 6.65 | 0.016 |
| **Mo** (mg) | 15.1 | 23.3 | 1.42 | 16.79 | <0.001 |
| **Na** (mg) | 190.0 | 298.0 | 31.77 | 5.80 | 0.023 |
| **Se** (mg) | 0.004 | 0.006 | 0.0006 | 4.30 | 0.048 |
| **Zn** (mg) | 18.9 | 21.4 | 0.89 | 4.05 | 0.054 |

Nitrogen, K, Al and Ni shoot concentrations were affected by the size fraction of char added to the potting medium. Lower concentrations of all four elements were present in shoots of plants grown with powdered char compared to shoots of plants grown in either soil without char, or in soil with the 1 mm fraction (Table 3.2).

**Table 3.2:** Elements in bean shoot that responded to char size as a single factor.

| **Element: g or mg kg$^{-1}$** | **No char** | **Powdered char** | **1 mm char** | $F_{1,27}$ | $p$ | **LSD** |
|---|---|---|---|---|---|---|
| **N** (%) | 2.05 | 1.92 | 2.14 | 4.59 | 0.041 | 0.20 |
| **K** (g) | 15.00 | 13.02 | 15.20 | 4.61 | 0.041 | 2.10 |
| **Al** (mg) | 23.40 | 12.00 | 22.10 | 4.80 | 0.037 | 10.6 |
| **Ni** (mg) | 0.86 | 0.83 | 0.96 | 6.34 | 0.018 | 0.11 |

Shoot Cu was significantly lower in plants grown with 7% of the 1 mm size fraction of char than in plants subjected to any of the other treatments (char size x char conc. interaction, $F_{1,27}$=11.30, $p$=0.002). Apart from the various nuances and significances within the interactions for Ni, Zn and Cu, a broad observation is that shoot concentrations of these essential micronutrients were lower when plants were grown in soil supplemented with 7% char.

### 3.3.3 Macro- and micronutrients in wheat shoots and grain

#### *3.3.3.1 Nutrients in wheat grain*

Ten different elements measured in bean shoots were affected by *Rhizobium*, either as a single factor or as an interaction term. In contrast, wheat elemental concentrations did not appear as responsive to *Rhizobium*, but many were modified by the presence of char. Nevertheless, wheat grain chemistry was affected by *Rhizobium* amendment and other soil treatments.

Grain Cu and Mn concentrations both responded to *Rhizobium* within the potting medium; [Cu] increased (*Rhizobium* as a single factor, $F_{1,27}$=14.64, $p$<0.001; Fig. 3.4a) whilst Mn concentration decreased ($F_{1,27}$=20.04, $p$<0.001; Fig. 3.4b).

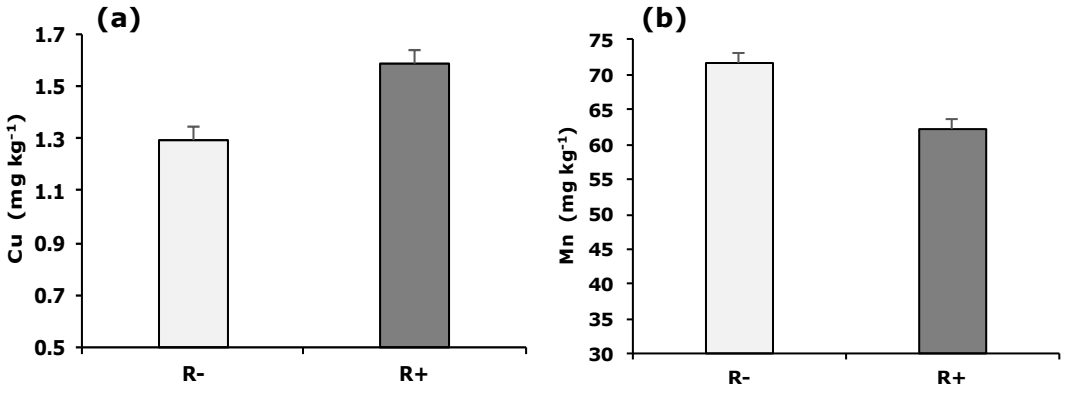



**Figure 3.4:** Effect of rhizobia inoculation on wheat grain concentrations of **(a)** Cu ($p$<0.001) and **(b)** Mn ($p$<0.001). Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom.

Aluminium concentrations in grain from wheat plants not subjected to rhizobia were similar irrespective of the presence of char. However, wheat plants grown with rhizobia, but in the absence of char, produced grain with higher concentrations of Al (*Rhizobium* x char interaction, $F_{1,27}$=4.57, $p$=0.042, LSD=0.45; Fig. 3.5). ('Char' as an explanatory term here refers to the presence of char in the potting medium, meaning different char concentrations and size fractions were in effect pooled in ANOVA and were compared with soil-only treatments.)

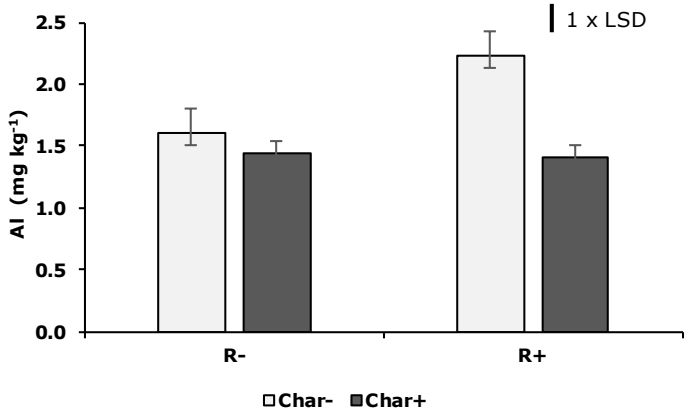



**Figure 3.5:** Concentrations of Al in wheat grain showing a *Rhizobium* x char interaction, $p$=0.042. Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom. Vertical bars represent the LSD (5%).

In addition to changes in grain Cu, Mn and Al concentrations, char-related decreases in carbon ($F_{1,27}$=55.49, $p$<0.001), phosphorus ($F_{1,27}$=10.05, $p$=0.004), molybdenum ($F_{1,27}$=6.13, $p$=0.020) and sulfur ($F_{1,27}$=26.43, $p$<0.001) were measured (Figs 3.6 a-d); all showed the same trend.

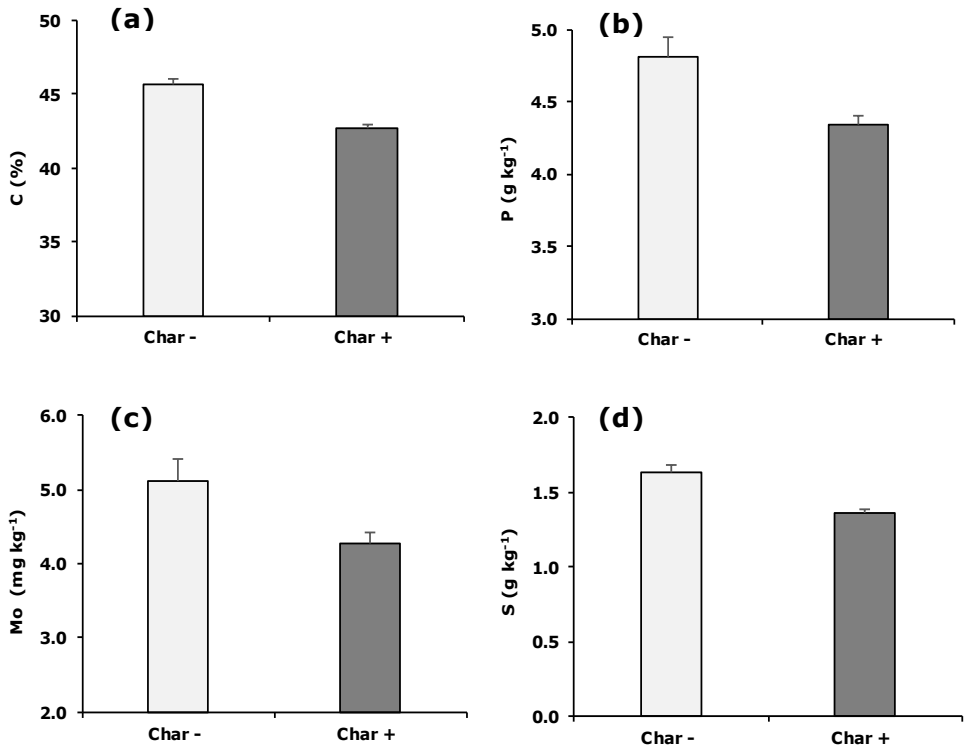



**Figure 3.6:** The effect of soil char amendment on wheat grain concentrations of **(a)** carbon ($p$<0.001), **(b)** phosphorus ($p$=0.004), **(c)** molybdenum ($p$=0.020) and **(d)** sulfur. Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom.

More complex interactions with char were observed with the micronutrients B, Zn and Mn (Fig. 3.7). Concentrations of B and Zn were significantly lower when parent plants had been grown in soil amended with the 1 mm char fraction. Both elements follow the same trend (Fig. 3.7 a & b). Manganese concentrations were also reduced by parental growth in soil with the 1 mm char fraction, but only when amendment was at the 7% level. Interestingly, the 7% application of char resulted in significantly higher grain Mn concentration when present as a powder. Therefore, the highest char amendment altered Mn bioavailability and /or uptake depending on the char particle size (Fig. 3.7c).

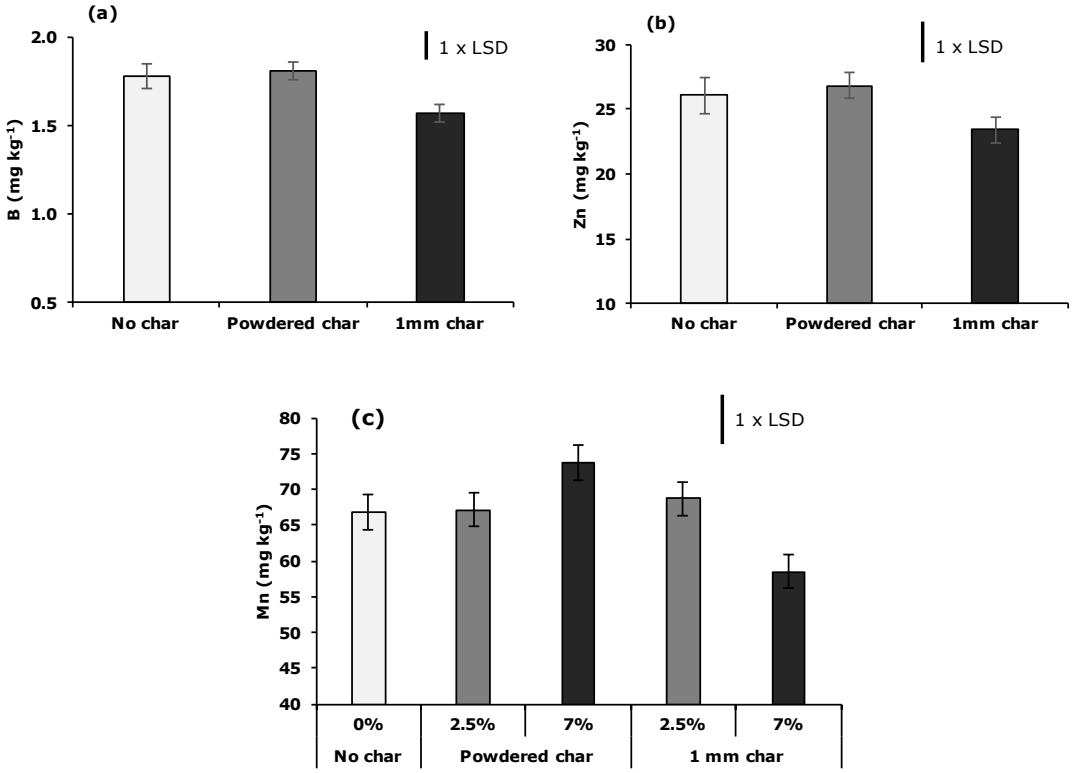



**Figure 3.7:** The effect of char particle size as a single factor on concentrations of **(a)** B ($F_{1,27}$=10.25, $p$=0.003) and **(b)** Zn ($F_{1,27}$=5.77, $p$=0.023) in wheat grain. **(c)** Interaction between char particle size x char concentration on grain Mn ($F_{1,27}$=12.45, $p$=0.002). Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom. Vertical bars represent the LSD (5%).

### *3.3.3.2 Nutrients in wheat shoots*

Most of the treatment-related effects on shoot elemental content were due to presence of char, although concentrations of B, Co, K, Mn and Zn were affected by *Rhizobium* amendment. Only shoot Co and Zn concentrations appeared to be influenced by a *Rhizobium* x char interaction. The char effect on the foliar nutrients was generally negative, with lower concentrations in the foliage of plants grown in char amended soil than in soil without char (Table 3.3). Only boron and selenium were enhanced due to char amendment. However, any subtle effects of particle size or amount applied may have been lost over time. Calcium concentration was not significant (or was weakly significant) when the presence of char was considered as a single factor. However, foliar Ca concentrations followed a gentle (but significant) stepwise decline with

increased char amount (char conc. as a single factor, $F_{1,27}$=4.40, $p$=0.045) and Co concentrations were lower in foliage of plants grown with 1 mm char (char size as a single factor, $F_{1,27}$=5.81, $p$=0.023).

**Table 3.3:** Elements in wheat shoot that responded to presence of char as a single factor in the potting medium.

| Element: mg kg$^{-1}$ | Soil ± char treatment | | $F_{1,27}$ | $p$ |
|---|---|---|---|---|
| | Char - | Char + | | |
| **B** | 3.84 | 4.60 | 9.99 | 0.004 |
| **C** | 56.39 | 50.62 | 5.78 | 0.023 |
| **Ca*** | 3.54 | 3.09 | 4.02 | 0.055 |
| **Fe** | 20.00 | 15.53 | 5.26 | 0.030 |
| **Na** | 41.50 | 13.50 | 5.97 | 0.021 |
| **Ni** | 0.19 | 0.14 | 5.22 | 0.030 |
| **P*** | 3.98 | 3.42 | 4.83 | 0.037 |
| **S** | 925 | 761 | 13.39 | 0.001 |
| **Se** | 0.004 | 0.007 | 56.69 | <0.001 |
| **Zn** | 14.86 | 8.27 | 8.04 | 0.009 |

*g kg$^{-1}$

Shoot boron and potassium concentrations were significantly enhanced by presence of rhizobia in the soil ($p$=0.015 and $p$=0.013 respectively), but Mn exhibited the same negative pattern as that observed for grain Mn ($p$=0.014).

Both foliar Co and Zn were highest in plants grown in char-free soil without rhizobia amendment. Concentrations of both elements were lowered when rhizobia inoculum was added to char-free soil. This effect did not occur in soils containing char (Fig. 3.8 a & b). An opposite interaction was observed for Al in grain char-free soil, but an equivalent lack of response to rhizobia when char had been added to the system (Fig. 3.5).

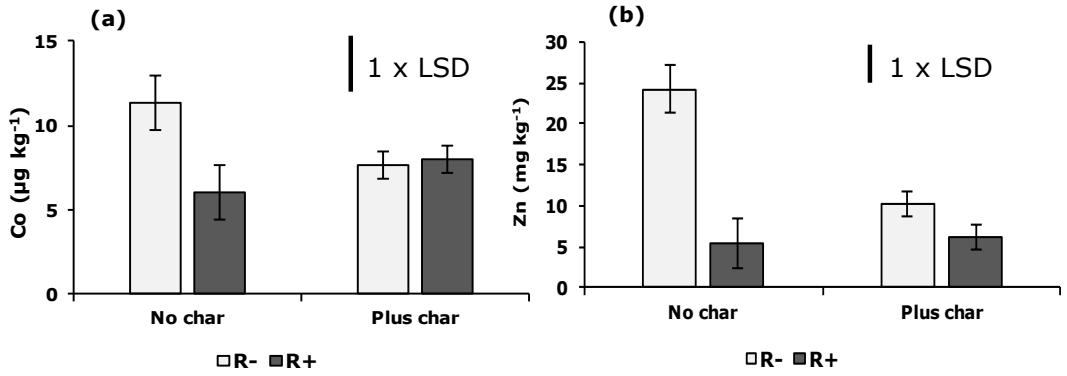



**Figure 3.8:** The effect of a char x *Rhizobium* interaction on shoot concentrations of **(a)** cobalt ($F_{1,27}$=4.79, $p$=0.037) and **(b)** Zn ($F_{1,27}$=10.07, $p$=0.004) in wheat shoots. Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom. Vertical bars represent the LSD (5%).

### 3.3.4 Total macro- and micronutrients in soil

There was a significant interaction between *Rhizobium* inoculation and char size on total soil P concentration ($F_{1,27}$=6.33, $p$=0.018) (Fig. 3.9a) where soils that received rhizobia inoculum and the 1 mm char size fraction had the highest total P concentration of all the soils (578.8 mg $kg^{-1}$). In addition, K concentration was significantly affected by an interaction between char size and char concentration ($F_{1,27}$ = 4.42, $p$=0.045) where 2.5% of the 1 mm char amendment resulted in the highest K concentration (11.7 g $kg^{-1}$) compared to other treatments. Soils that received a powdered char treatment had lower concentrations of Al (char size as a single factor, $F_{1,27}$=19.49, $p$<0.001), Fe ($F_{1,27}$=7.88, $p$=0.009) and Mg ($F_{1,27}$=10.18, $p$=0.004) (Fig. 3.9b).

No other treatment-related effects were observed.

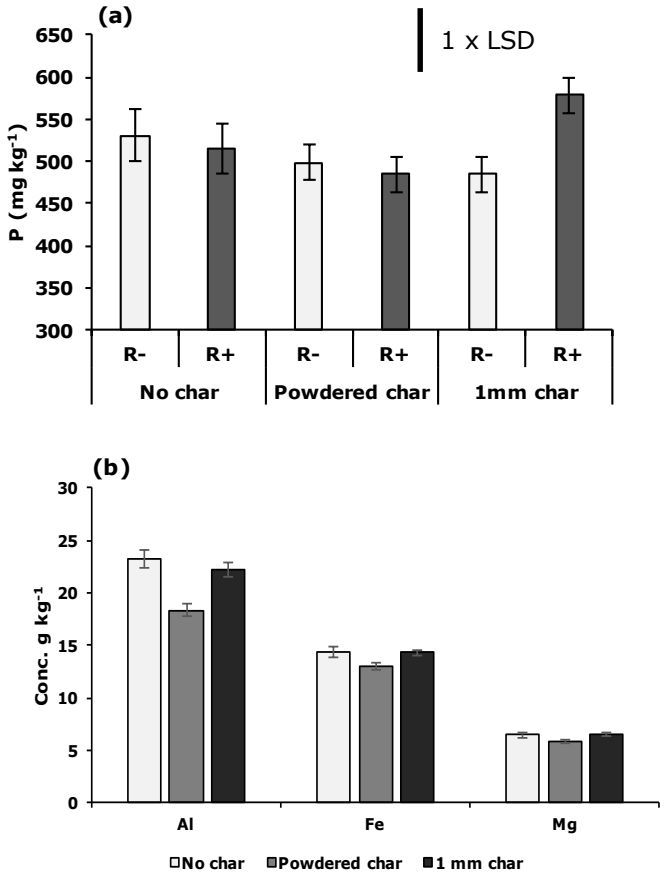



**Figure 3.9: (a)** The effect of a char x *Rhizobium* interaction on concentration of total soil phosphorus ($p$=0.018). The vertical bar represents the LSD (5%). **(b)** The effect of char particle size on total soil concentrations of three micronutrients; Al ($p$<0.001, LSD=1.8), Fe ($p$=0.009, LSD=0.9, Mg ($p$=0.004, LSD=0.5). Individual standard errors are based on the pooled variance estimate from the ANOVA with 27 degrees of freedom.

## 3.4 DISCUSSION

The results of the investigation described here showed that the interactions between symbiotic nitrogen fixers (*Rhizobium*) and biochar, or each treatment as a single factor significantly affected growth and yield of the plants.

*Rhizobium* inoculation significantly improved wheat biomass and grain yield. Although it is accepted that *Rhizobium* is usually beneficial (e.g. Chen *et al*., 1999), the results of this experiment showed that *Rhizobium* inoculation decreased bean biomass, as the weight of bean shoots was significantly lower in plants inoculated with *Rhizobium* than in those without inoculum. The reason

for this may be that the inoculum was ineffective, or alternatively, the competition exerted by the wheat plants resulted in beans fixing atmospheric nitrogen, but being disadvantaged by the rhizobia-induced carbon drain. This is speculation and ideally, temporal and/or isotopic studies would need to be carried out. The findings are similar to those of Chapter 2. However, in this current experiment, *Rhizobium* benefited bean pod production when in the presence of char. Addition of *Rhizobium* as a single factor altered the competitive outcome between wheat and bean plants resulting in smaller beans and larger wheat plants. This is in line with the findings of Li *et al*. (2014) who demonstrated that when in competition with faba beans, wheat was a superior competitor and accumulated nitrogen more rapidly than the bean plants. Jensen (1996) earlier reported that cereals are stronger competitors than legumes for mineral nitrogen.

It is interesting that *Rhizobium* inoculation significantly improved bean performance when char was added to the growing medium. Char amendment significantly increased the benefits of *Rhizobium* additions by enhancing bean pods numbers and weight. It is likely that char stimulated biological nitrogen fixation (BNF) although BNF was not directly measured in this investigation. Nevertheless, char significantly improved biomass and yield in beans and this has been demonstrated by others (e.g. Rondon *et al*., 2007). Char-induced increased biological N fixation was previously shown for alfalfa (*Medicago sativa* L.), where BFN was measured by the N-difference and acetylene reduction assay methods (Nishio and Okano, 1991).

In the current experiment, the interactions between *Rhizobium* inoculation and char concentration, *Rhizobium* inoculation and char size, and char concentration and char size resulted in significantly improved biomass of beans. The combined use of bio-inoculants with organic amendments, would not only ensure high crop yields but also improve the physicochemical and biological properties of the soil (Miller and Miller, 2000). In this experiment, powdered char amendment produced an impressive effect on the weight and number of bean pods compared to the uninoculated plants. This may be because powdered char has fewer microhabitats for beneficial soil microbes, due to the small surface area and fewer micropores; therefore, there is less competition between *Rhizobium* and other soil microbes, but the effects of char on cation exchange capacity or

nitrogen immobilisation by the microbial community are more likely to have been a contributing factor. Beans grown with *Rhizobium* inoculation and char amendment (7% of the powdered char application), produced the highest biomass, whilst wheat performed least well. It is hypothesised that available nitrogen was limiting in those soils because of a char-induced altered C:N ratio and wheat therefore struggled to obtain sufficient mineral N, whereas beans could potentially fix atmospheric N. This suggestion is supported by the improved wheat performance at the lower soil char concentration, where the C:N ratio would be in favour of more efficiently securing mineral N. However, Oram *et al*. (2014) concluded from their study of competition between red fescue (*Festuca rubra*), red clover (*Trifolium pratense*) and plantain (*Plantago lanceolate*), that the beneficial impact of biochar amendment on the competitive ability of red clover was due to an increase in potassium availability when N was limiting. In the current experiment, bean plants growing in soil with rhizobia had higher shoot K concentrations than those grown in soil without inoculum, as did wheat shoots (but not grain). Here, K uptake was stimulated by rhizobia in both bean and wheat; perhaps the facilitated uptake of P by co-cropped plants as demonstrated by Li *et al.* (2016) occurred with K in this experiment.

Spencer *et al*. (1994) stated that *Rhizobium* has the potential to colonise roots of non-legumes and concentrate in tissues such as xylem where they act as plant growth-promoting bacteria for the non-legumes and improve their growth through multiple mechanisms other than nitrogen fixation. Their good survival ability makes them efficient competitors with other microbes in the root region (Kennedy, 2005). Rhizobial inoculation has been shown to promote growth of non-legumes like rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mays*) (Yanni *et al*., 1997, 2001; Gutiérrez-Zamora and Martínez-Romero, 2001; Mehboob *et al*., 2011). Improvement of growth and nutrient uptake in maize in response to rhizobial inoculation might be attributed to biosynthesis of phytohormones (Zahir *et al*., 2010), siderophore production (Hussain *et al*., 2014a), and increased availability of nutrients (Hafeez *et al*., 2004; Fatima *et al*., 2006; Pandey and Maheshwari, 2007). Therefore, the better growth and improved physiology of maize in response of rhizobial inoculation might be due to one or more of these mechanisms (Ahmad *et al*., 2015). Rhizobia have been reported to induce improved growth and yield of maize under normal as well as

stressed conditions (Qureshi *et al*., 2013; Hussain *et al*., 2014a) but the degree of rhizobial effect in non-legumes is variable (Hussain *et al* 2014a, 2014b).

In this experiment, *Rhizobium* inoculation resulted in increased concentrations of macro- and micro-nutrients in the bean shoots compared to bean shoots harvested from uninoculated soil. Phosphorus (P) is an essential nutrient which is not always readily available in soils, but was present in a higher concentration in *Rhizobium*-amended soil containing the 1mm size fraction of char than in soils from the other treatments. Bean shoots grown with rhizobia had higher P concentrations than those grown without inoculum and the P concentration was higher than that in the wheat shoot and grain. It has been demonstrated that intercropping legumes and cereals leads to rhizosphere acidification and production of phosphatases by legumes which solubilise the P, enabling uptake by both the legume and the intercropped cereal (Li *et al*., 2016 and references therein). Since the wheat shoot P concentration was lower in plants grown with char, it is possible that mobilised P was taken up locally by the bean roots and either insufficient was available for wheat uptake too, or it was adsorbed onto char particles and rendered unavailable again. It has been hypothesized that *Rhizobium* strains having low tolerance to phosphate-deficient environments may have difficulty in competing with other indigenous soil rhizobia for nodulation (Leung and Bottomley, 1987). The tolerance of the strain used here is unknown.

Sulfur concentration was influenced by *Rhizobium* inoculation. According to Sugawara *et al*. (2011) strains of *Bradyrhizobium* spp. likely use organosulfur compounds for growth and survival in soils as well as for legume nodulation and nitrogen fixation, and soil amendments that modify sulfur dynamics could be important for maintaining BNF.

Boron and molybdenum were both higher in shoots from inoculated bean plants. Boron plays a role in pre-infection events at the root surface (Redondo-Nieto *et al*., 2003) and the number of rhizobia and infection threads infecting host cells are reduced in boron-deficient plants. Bolaños *et al*. (1996) reported that B is an essential micronutrient for the development of nitrogen-fixing root nodules in pea (*Pisum sativum*). The involvement of Mo within the process of nitrogen fixation was given biochemical meaning by the isolation of the

molybdenum iron protein (MoFe protein) of nitrogenases from a variety of bacteria (Miller, 1990; Dilworth and Loneragan, 1991).

Manganese limitation plays a role in induction of infection threads and attachment of rhizobia to root hairs; in contrast, sufficient Mn results in cell growth (Kijne *et al*., 1988). However, these findings were based on laboratory cultures and interactions between Mn and other ions in the rhizosphere will be complex. More recently, Hood *et al*. (2017) demonstrated that published variability in the requirement for Mn uptake during symbiosis was due to whether the host plant involved belongs to a particular clade (galegoid or phaseoloid). The authors reported that $Mn^{2+}$ transporters are needed by rhizobia that infect plants belonging to the galegoid clade; the broad bean, *V. faba* belongs to this clade. Mn is therefore important in nodule development and for $N_2$ fixation. Data from the current experiment shows that shoot Mn concentrations were significantly lower in *Rhizobium* inoculated plants than in uninoculated ones. This might be a result of high affinity Mn transporters moving Mn to nodule development and function; nodule requirements would limit Mn transfer to the shoots, which would not be the case in plants without the symbiosis. It is interesting to note that of the shoot elements that were affected by the symbiosis, Mn was the only one to be lowered by inoculation. Wheat grain concentrations of Mn were lower in plants grown in inoculated pots, suggesting that beans preferentially sequestered Mn ions, possibly early in the growth cycle.

Zinc is needed by all plants and its importance in crop production has increased in recent years (Weisany *et al*., 2013), but it often has low bioavailability in more alkaline soils (Alloway, 2008). In the present study, wheat shoot Zn concentrations were significantly lower in plants grown in inoculated pots, whilst bean shoot concentrations were higher than in plants without rhizobia. This is indicative of superior uptake by the beans and in particular by beans growing in inoculated soil. Abreu *et al*. (2017) identified a model for zinc uptake in the *Medicago truncatula*-rhizobia symbiosis and the data here indicate that the symbiosis required and successfully obtained Zn present in the system.

Char amendment affected shoot concentrations of various nutrients; in particular, powdered char lowered bean shoot N and K and high char

concentration limited bean shoot Cu; similar trends were observed in wheat shoots and grain, but for different elements. The physical and chemical characteristics of char mean it is able to adsorb various organic compounds and exchange metal and other ions (Oliveira *et al*., 2017). It is therefore not surprising that char amendment will drive many of the interactions within the soil and rhizosphere, either directly by adsorption/desorption or indirectly through altered pH.

Observations in the literature vary depending on soil type, crop and char feedstock. Glaser *et al.* (2015) showed that biochar addition to mineral and organic fertilizers increased both K concentration and uptake in maize, whilst Lehmann *et al*. (2003) demonstrated positive effects of char on K uptake by cowpea grown in a Ferrosol, but not when grown in Terra Preta soils. Uzoma *et al*. (2011a) reported 2-6 kg $ha^{-1}$ higher P, Mg and N concentrations in maize grain after application of 10-20 tonnes $ha^{-1}$ biochar and 2 kg $ha^{-1}$ higher Mg uptake.

Clearly the relationship between char, plant and soil microorganisms is complicated, variable and dynamic. The aim of modifying nutrient-use-efficiency during growth of two intercropped species is beneficial for achieving sustainable intensification and soil char amendments may enable this. Ideally, consistent benefits should be obtained and the results highlight the need for further work in this area.

## 3.5 CONCLUSION

Whilst *Rhizobium* inoculation resulted in lowered bean shoot biomass, the positive interaction between inoculation and addition of powdered char led to the greatest production of bean pods in terms of number and weight. Interestingly wheat plants did not benefit from this treatment combination as evidence by reduced grain yield. Char is known to alter the C/N ratio in soils and beans may have coped better with this than wheat, because of their potential for fixing N. In the absence of the powdered char, wheat appears to be the stronger competitor and addition of *Rhizobium* pushed the competitive dynamic in favour of wheat. Nitrogen fixation was not measured and it is not known in this situation whether bean plants fixed N when *Rhizobium* was

present, or whether the rhizobia were being supported at a metabolic cost with minimum benefit. This is unlikely to be the case when beans were grown with powdered char. Wheat is known to be a superior competitor for soil N and the shifts in competitive balance with treatment are likely to be due to char-related differences in soil nutrient availability.

The key finding is that the interactions between *Rhizobium* and biochar were important in determining competitive outcomes between the plant species.

With regard to the original hypotheses at the start of the Chapter; *Rhizobium* did not shift the competitive balance in favour of beans, unless conditions were N-limited (i.e. which may have been the case in the presence of powdered char). The data suggest that competition for soil N outweighed the detrimental effect of interspecific competition.

# 4 Impact of biochar addition along with *Rhizobium* and arbuscular mycorrhizal fungi on growth and yield of broad bean

## 4.1 INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with approximately 80% of all vascular land plants (Wang and Qiu, 2006). They are important in helping plants acquire nutrients, particularly phosphorus and nitrogen (Smith and Read, 2008), protecting their host from invertebrate or pathogen attack and from abiotic stress (Yang *et al*., 2014; Spagnoletti *et al*., 2017). AMF are therefore important drivers of ecosystem services and there is much interest in encompassing them into sustainable agricultural methodologies. AMF diversity is affected by farming practices (Manoharan *et al*., 2017), although the fungi respond positively to soil char application (Warnock *et al*., 2007).

It has been known for some time that dual inoculation of legumes with AMF and rhizobia can enhance plant growth due to increased P uptake by the plant (Ibijbijen *et al*., 1996). However, Larimer *et al*. (2014) demonstrated synergistic interactions between AMF and rhizobia within the late-successional legume, *Amorpha canescens*, that were independent of nutrient levels, although P concentration modified the relative benefits of each partner. These authors observed that AMF colonisation increased the number and biomass of root nodules, but rhizobia lowered AMF colonisation of roots.

Despite the published papers demonstrating synergistic or additive benefits of the dual symbiosis, Larimer *et al*. (2010) conducted a meta-analysis and concluded that whilst dual colonisation was generally beneficial to the plant, the effects were not synergistic. They hypothesised that the reason for this may be because of unlimiting nutrients in pot trials. More recently, Omirou *et al*. (2016) inoculated cowpea with commercial AMF and a strain of *Bradhyrhizobium* and reported

positive results. However, what was interesting about their study was their finding that the indigenous AMF community shifted following *Bradhyrhizobium* inoculation.

Since biochar is known to affect soil pH, water holding capacity, soil structure, nutrient retention and adsorption of heavy metals (Cao *et al*. 2009; Spokas *et al*. 2009; Atkinson *et al*. 2010; Sohi *et al*. 2010) it is likely to alter the relationship between dual symbionts. In particular, if any synergistic interactions result from nutrient limitation in the rhizosphere then biochar amendment may alter the direction of the relationship. Since legumes form tripartite relationships with AMF and rhizobia (Barea *et al*. 2005; Meghvansi *et al*. 2008; Kaschuk *et al*. 2010), an experiment was conducted to test the effects of char.

The **main aim** of the experiment was to determine if the plant/AMF/rhizobia symbiosis is affected by biochar.

The **specific objectives** of this experiment were:

(1) To quantify the benefit (if any) of a dual inoculation with rhizobia and AMF.

(2) To determine if char amendment modified the outcome of any synergism between rhizobia and AMF.

The **main hypotheses** were:

(1) Dual inoculation with rhizobia and AMF will result in a positive outcome for the plants.

(2) Biochar amendment will increase the plant's requirement for rhizobia to the detriment of the AMF symbiont because of increased C:N ratio in soil and the requirement of the plant to support both species.

(3) Biochar amendment will reduce the synergistic effects but will not alter the level of root colonisation by each symbiont because both N and P could be limiting.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Experimental approach

A pot experiment was conducted to determine the interactions between biochar addition the plant growth promoting microorganisms (*Rhizobium leguminosarum* and a commercial AMF inoculum) on growth and yield of broad bean (*Vicia faba*).

### 4.2.2 Soil and char preparation

The char used for this investigation was purchased from the BioRegional Charcoal Co. Ltd., UK; full details are given in Chapter 2, Section 2.2.2.

To minimise available nutrients, only soil was used and not the soil/potting compost mixture as for the previous experiments (Chapters 2 and 3). Soil was potted and amended with char and *Rhizobium leguminosarum* biovar *viciae* as described in Section 2.2.2, except that only powdered char was used here.

AMF inoculation was carried out by adding a 5 mL volume of commercial inoculum (Empathy Root Grow, RHS) as a layer 1/3 of the way down the soil profile. The inoculum was purchased from a local Garden Centre.

The same pot size was used as in the previous two experiments.

### 4.2.3 Experimental set up

The experimental treatments were:

(i) Soil only
(ii) Soil + 7% powdered char
(iii) Soil + 7% powdered char + *Rhizobium*
(iv) Soil + 7% powdered char + AMF
(v) Soil + 7% powdered char + *Rhizobium* + AMF
(vi) Soil + *Rhizobium*
(vii) Soil + AMF

(viii) Soil + *Rhizobium* + AMF

Pots were placed in a growth room with a 12 hour light-dark cycle, a daytime temperature of 20°C and a night-time temperature of 18°C and grown as described in Section 2.2.3. There were 4 replicate pots for each treatment.

### 4.2.4 Harvest and sample processing

Plants were grown for 6 weeks from germination and were harvested at the vegetative stage of growth. Harvesting procedures are described in Section 2.2.4. The only difference was that additional sub-samples of fresh root were taken to stain in order to quantify AMF colonisation. Amounts removed were weighed fresh and dry weight equivalent values added back into the summative calculation of total root biomass.

### 4.2.5 Staining procedure for quantifying AMF root colonisation

At harvest a subsample of root was selected and divided into two portions. One portion was kept in the freezer (-20°C) for staining and estimation of the extent of root colonization by the AMF, and the other half was oven dried (45°C until constant weight) so that the biomass of both portions could be determined. The method used for staining is after Brundrette *et al*. (1984) and is as follows: Roots were cleared in 10% KOH (w:v) at 90°C for 30 minutes followed by rinsing with tap water. The roots were gently rinsed using a tea strainer to remove residual KOH. The roots were then immersed at 90°C for 1 hour in a staining solution (chlorazol black E: lactic acid: glycerol: deionized water). After an hour, root samples were removed from the stain and destained in a clean tube containing glycerol. After 24 h of destaining, the roots were examined using a compound microscope at x200 and colonisation quantified using the gridline intersect method (McGonigle *et al*., 1990).

### 4.2.6 Soil and plant analyses

Total elemental concentrations and total carbon and nitrogen concentrations of the experimental soils in each pot were carried out as described in Chapter 2, Sections 2.2.4.5 and 2.2.4.6.

Bean shoot and root elemental concentrations and total carbon and nitrogen concentrations of shoots and roots were conducted as described in Chapter 2, Sections 2.2.5.1 and 2.2.5.2.

### 4.2.7 Statistics

Data were analysed by General Analysis of Variance using Genstat version 17 (Lawes Agricultural Trust/VSN International, Hemel Hempstead, UK). Normality was tested by plotting residuals against expected normal quantiles and post hoc comparisons between means were based on least significant differences at the 0.05 probability level where appropriate.

The factors used in analysis of the data were char (±), *Rhizobium* inoculation (±) and AMF inoculation (±).

## 4.3 RESULTS

### 4.3.1 Arbuscular mycorrhizal fungal (AMF) colonisation

AMF colonisation was very low and variable, ranging from 0% - 14.6% root length colonised. The grand mean for hyphal colonization was just 2.7%. The overall grand mean for arbuscules was 2.6%. None of the treatments significantly affected AMF colonisation, although rhizobia inoculation increased % root length colonised with arbuscules from 2.3% to 2.9% and a similar observation was made for intraradical hyphae. Only plants that received inoculum were colonised.

### 4.3.2 Plant growth

In this experiment, only plants receiving rhizobia inoculum were nodulated. Nodules were not counted, but the dry weight of 30 randomly selected nodules was determined. Nodule biomass was unaffected by any of the treatments.

Neither char amendment nor AMF inoculation affected plant growth. However, *Rhizobium* inoculation significantly increased bean biomass including both shoot and root (Fig. 4.1).

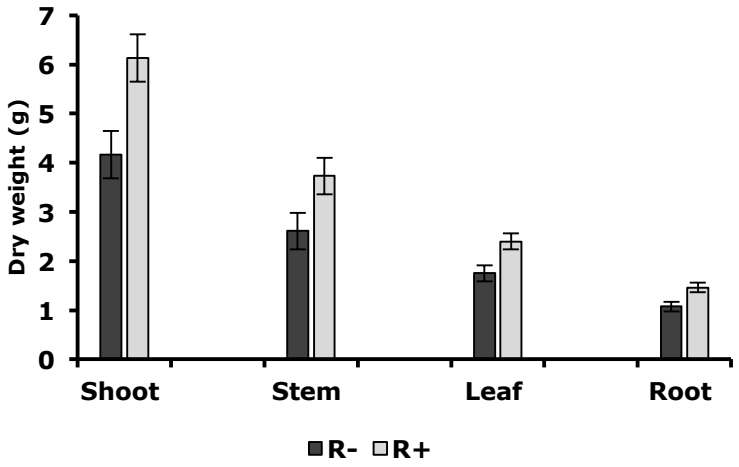



**Figure 4.1:** The effect of *Rhizobium* inoculum on plant biomass. Individual standard errors are based on the pooled variance estimate from the ANOVA with either 20 or 21 degrees of freedom. Shoot, $F_{1,21}=8.38$, $p=0.009$; stem, $F_{1,20}=4.51$, $p=0.046$; leaf, $F_{1,20}=7.65$, $p=0.012$; root, $F_{1,20}=7.92$, $p=0.011$.

### 4.3.3 Plant elemental concentrations

Char and *Rhizobium* amendments as single factors both affected leaf and root elemental concentrations. Not all elements measured were affected but the main trend for those that were, was for char amendment to reduce nutrient concentrations in both leaves and roots (Table 4.1). The exception to this was for root Ca which was increased in roots of plants grown with char.

**Table 4.1:** Effect of biochar amendment as a single factor on shoot and root concentrations of essential plant nutrients.

| g $kg^{-1}$ | - Char | +Char | *SE | *p* |
|---|---|---|---|---|
| **Leaves** | | | | |
| **Ca** | 33.25 | 30.25 | 1.20 | 0.014 |
| **Mg** | 7.14 | 6.29 | 0.338 | 0.052 |
| **S** | 6.70 | 5.57 | 0.317 | 0.020 |
| **Co‡** | 0.147 | 0.137 | 0.009 | <0.001 |
| **Roots** | | | | |
| **Ca** | 7.08 | 9.19 | 0.512 | 0.009 |
| **Mg** | 5.27 | 4.70 | 0.151 | 0.015 |
| **S** | 11.49 | 9.45 | 0.398 | 0.002 |
| **Fe** | 0.621 | 0.432 | 0.061 | 0.040 |

***** Standard errors are based on the pooled variance estimate from the ANOVA with 20 degrees of freedom for shoots and 19 d.f. for roots.

**‡** mg $kg^{-1}$

In the absence of *Rhizobium*, char amendment resulted in significantly lower leaf B concentrations than those in leaves from plants grown in the absence of char. In the presence of *Rhizobium*, this reduction was ameliorated, although in the char-free soil, rhizobia lowered leaf [B] relative to concentrations in plants from rhizobia-free char-amended soil. Root B was unaffected by any of the treatments (char x *Rhizobium* interaction, $F_{1,20}$=6.22, $p$=0.022; Fig. 4.2a). Leaf Zn concentrations followed a similar trend (char x *Rhizobium* interaction, $F_{1,20}$=6.92, $p$=0.016; Fig. 4.2b).

*Rhizobium* as a single factor had little effect on elemental concentrations within leaves or roots although leaf [S] was lower in leaves of plants grown with rhizobia, as was root K. Ni was increased slightly in root of plants growing with rhizobia, but this was only barely significant (Table 4.2).

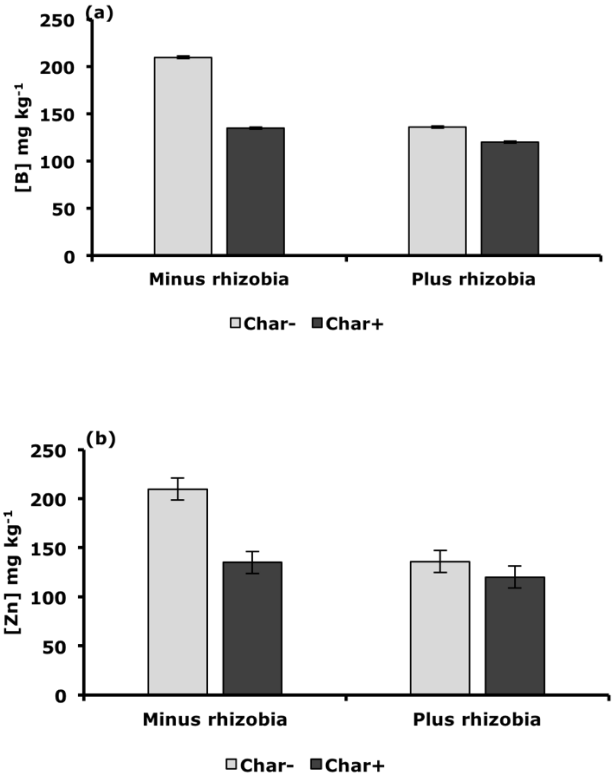



**Figure 4.2:** The effect of char on **(a)** leaf boron (char x *Rhizobium* interaction, $F_{1,20}$=6.22, *p*=0.022, LSD=3.78) and **(b)** leaf zinc concentrations (char x *Rhizobium* interaction, $F_{1,20}$=6.92, *p*=0.016, LSD=32.94). Individual standard errors are based on the pooled variance estimate from the ANOVA with 20 degrees of freedom.

**Table 4.2:** Effect of *Rhizobium* inoculation as a single factor on shoot and root concentrations of essential plant nutrients.

| g kg$^{-1}$ | - *Rhizobium* | +*Rhizobium* | *SE | *p* |
|---|---|---|---|---|
| **Leaves** | | | | |
| **S** | 7.19 | 5.08 | 0.317 | <0.001 |
| **Roots** | | | | |
| **K** | 23.81 | 16.71 | 2.309 | 0.043 |
| **Ni‡** | 2.77 | 3.13 | 0.122 | 0.054 |

***** Standard errors are based on the pooled variance estimate from the ANOVA with 20 degrees of freedom for shoots and 19 d.f. for roots.

**‡** mg kg$^{-1}$

### 4.3.4 Soil elemental concentrations

Exchangeable soil nutrients were measured following extraction in 1 mol $L^{-1}$ $NH_4NO_3$ solution. Not all of the elements measured were affected by any of the treatments, those that were affected are shown below (Table 4.3). The trend for the macro- and micro-nutrients below is one of being present in lower concentrations in soil which was inoculated with *Rhizobium*.

**Table 4.3:** Effect of *Rhizobium* inoculation as a single factor on $NH_4NO_3$ extractable soil elemental concentrations.

| mg $kg^{-1}$ | - *Rhizobium* | +*Rhizobium* | *SE | *p* |
|---|---|---|---|---|
| **P** | 95.2 | 69.6 | 5.46 | 0.003 |
| **Ca‡** | 8.33 | 7.84 | 0.148 | 0.031 |
| **Mg‡** | 0.465 | 0.410 | 0.112 | 0.002 |
| **Fe** | 2.00 | 1.42 | 0.155 | 0.014 |
| **Cu** | 0.256 | 0.180 | 0.0182 | 0.007 |
| **Mn** | 3.10 | 2.74 | 0.108 | 0.028 |
| **Zn** | 0.491 | 0.387 | 0.029 | 0.021 |
| **Co** | 0.040 | 0.035 | 0.0016 | 0.024 |

***** Standard errors are based on the pooled variance estimate from the ANOVA with 21 degrees of freedom

**‡** g $kg^{-1}$

Water extractions were also undertaken and these either gave similar results as those in Table 4.3, or they showed no treatment effect. However, char amendment as a single factor lowered Co concentrations from 0.012 to 0.010 mg $kg^{-1}$; a small but statistically significant reduction, $p$=0.021). Similarly, char amendment lowered water extractable Ni from 0.22 to 0.10 mg $kg^{-1}$ ($p$=0.022) and Fe from 5.0 to 3.5 mg $kg^{-1}$ ($p$=0.011).

It is interesting that plant/rhizobia utilisation of available nutrients is reflected in the total elemental concentrations (Table 4.4).

**Table 4.4:** Effect of char as a single factor on total soil elemental concentrations following HF acid digestion.

| g $kg^{-1}$ | *- Char* | *+Char* | *SE | *p* |
|---|---|---|---|---|
| **Mg** | 5.52 | 4.97 | 0.165 | 0.028 |
| **Fe** | 20.04 | 17.86 | 0.539 | 0.009 |
| **Ni‡** | 23.18 | 20.85 | 0.456 | 0.002 |
| **Co‡** | 5.76 | 4.98 | 0.222 | 0.023 |

***** Standard errors are based on the pooled variance estimate from the ANOVA with 21 degrees of freedom

**‡** mg $kg^{-1}$

## 4.4 DISCUSSION

The plants grown in this experiment did not become well colonized by AMF. The maximum percentage root colonisation recorded was 14% in one plant, but most values were around 0-2% with an average of 2.5%. The inoculum used was 'Rootgrow', manufactured by Empathy in the UK and consists of a carrier substance with propagules of more than one AMF species. The reasons for the lack of colonization are unknown. It is possible that the inoculum was ineffective, or the plants did not grow for sufficiently long to form the association. However, when viewing the stained root fragments, extraradical hyphae were observed suggesting some activity. Klironomos and Hart (2002) discussed the colonization strategies of different AMF species and concluded that some propagule types are better than others, but this varies with species. These authors harvested their experimental plants after 8 weeks and observed 10-50% colonization depending on the species. Therefore, the 6-weeks of growth in this current experiment should have been sufficient to have expected great colonization.

Klironomos (2003) stated that plants are able to limit AMF colonization if the species in an ineffective one. It is possible that this occurred here, particularly if the beans did not 'need' the association. Tavasolee *et al*. (2011) reported that

some strains of rhizobia can affect the ability of AMF to colonize roots. Since the *Rhizobium* strain used here formed nodules and was apparently an effective strain, it could be speculated that the *Rhizobium* prevented the AMF from 'taking hold'. However, since AMF root colonization was also low/absent in plants which had not been inoculated with rhizobia, this is an unlikely explanation here.

*Rhizobium* significantly increased both shoot and root biomass of the bean plants and the symbiosis resulted in some changes to shoot chemistry, although to a limited degree. Leaf S and root K were lower in plants grown with rhizobia. This might be a simple dilution effect because the plants were bigger, or might be because these elements are essential for protein synthesis and photosynthesis respectively (Nyoki and Ndakidemi, 2017). Perhaps of more interest are the rhizobia-induced decreased concentrations of leaf Zn and B when grown in the absence of char. Both these elements are essential for nodulation and $N_2$ fixation (Valenciano *et al*., 2011) and it is likely that during the first 6 weeks of plant growth demands due to nodulation would have been high. Interestingly, this pattern was not observed in plants grown in char-treated soil.

The demand for essential nutrients by the *Rhizobium* infected plants is also reflected by the reduced availability of many essential nutrients in the $NH_4NO_3$ soil extracts. Char as a single factor decreased leaf and root concentrations of a range of elements. Few interactions between char and rhizobia were observed, but where they occurred (with Zn and B), char appeared to change the direction of nutrient uptake by the plants infected with rhizobia.

The complementarity of between the two symbionts could not be explained here because of the failure of the AMF inoculum to colonize the plant roots. The reasons for this failure are worth exploring in case it was under the control of the beans rather than the inoculum itself. The experiment meant that the effects of char on early stages of bean growth and nodulation could be observed and is a good comparison with the data in Chapter 2 which considered plants at the early flowering stage, and in Chapter 3 in which beans had reached maturity.

# 5 GENERAL DISCUSSION

The main objective of this study was to determine the effects of amending soil with biochar on growth of broad beans (*Vicia faba* var. Optica). In each experiment, half the bean plants were inoculated with *Rhizobium leguminosarum* biovar *viciae* or left uninoculated and superimposed on that were soil char amendments where plants were grown in char-amended or in char-free soil. This was the basic approach of the first experimental chapter (Chapter 2). In a subsequent experiment (Chapter 3), beans were grown in a biculture with wheat (*Triticum aestivum* var. Willow) whilst simultaneously subjected to the same rhizobia and char treatments as in the first experiment. The final experimental chapter (Chapter 4), builds on the rhizobia and char treatments, but beans (grown individually) were also inoculated (or not) with arbuscular mycorrhizal fungi. A summary of the key findings of each chapter is at the end of this chapter.

One of the main findings across the whole investigation was that plants and rhizobia respond differently to different particle sizes of char. This response varied with rhizobia strain (commercial or indigenous) and with planting scheme (beans on their own, or co-cropped with wheat). This is potentially an important aspect of the work because to date, papers do not always state the particle size of the char used in agronomic studies. Most of the focus is on char production methods and feedstock type, both of which alter the response of soils and plants when char is applied, or on application rate (concentration). Once char is applied to the soil, because of its recalcitrant nature, it will remain there for thousands of years, so any erroneous application could have significant consequences for a small-scale farmer for example. To reduce the chance of possible contamination, European and International Biochar Standards are under development that will be used for agronomic purposes. To date, these focus on aromatic and heavy metal concentrations, stability and feedstock quality (European Biochar Certificate, 2012). Char certification is sensible, but the data obtained in this investigation show different responses to the same char, depending on particle size and growth situation. Therefore, even with application of certified standards, char amendment may still result in unintended consequences. Often, unintended consequences result from alterations in the soil microbiota. For example, Warnock *et al*. (2010)

found that biochar amendment decreased arbuscular mycorrhizal (AM) colonisation of *Plantago lanceolata* roots and of extrametrical hyphae in surrounding soil. The authors also recorded concomitant changes in soil P availability although the host plant increased in biomass; the mechanisms for the response were not elucidated. Others have reported increased microbial activity following biochar amendment. For example, Khadem and Raiesi (2017) measured higher soil enzyme activities following addition of low-temperature char than with high temperature char of the same feedstock. Zhou *et al*. (2017) recently highlighted the differential effects of biochar following a meta-analysis which demonstrated that soil respiration was increased by almost 21% at the global scale by biochar amendment; this was differentiated into no difference in sub-tropical forests and around 20% increase in temperate forests. Biological $N_2$ fixation by rhizobia in legumes can also be enhanced by biochar amendments, because of increased soil C:N ratio, higher pH and micronutrients such as B and Mo which biochar supplies (Rondon *et al*., 2007; Güereña *et al*., 2015; Van Zwieten *et al*., 2015).

Earlier studies showed the ability of *Rhizobium* spp. in improving root surface area, root volume and N uptake by rice seedlings (Biswas *et al*., 2000b; Naher *et al*., 2009). In addition, P solubilisation by *Rhizobium* has been reported (Chabot *et al*., 1993, 1996; Halder *et al*., 1991, 1992) and was shown to increase the growth of maize and lettuce (Chabot *et al*., 1996). Similarly, biochar application may stimulate $N_2$ fixation by beans in association with *Rhizobium* spp. (Lehmann and Rondon 2006; Rondon *et al*. 2007). The presence of biochar in the soil can provide a physical niche for growing hyphae and bacteria, devoid of fungal grazers (Saito 1990; Pietikäinen *et al*. 2000; Warnock *et al*. 2007).

Another key finding of this investigation was that commercial rhizobia behaved differently towards biochar amendment than did the indigenous rhizobia. Specifically, commercial inoculum and powdered char were detrimental to the growth of broad bean when grown singly. The reason for this effect is unknown, but was likely to be related to changes in soil C:N ratio, to pH or to electrostatic forces affecting the bacteria. That the powdered char amendment increased bean pod yield when beans were competing with wheat in the same pot demonstrates the complexity of these interactions and the possibility for niche separation when

faced with a competitor. Intercropping legumes with grains is gaining interest as a means of moving towards sustainable intensification whereby using plants with multifunctional traits will simultaneously benefit crop yields and the environment (Blesh, 2018). Growing legumes with forage crops is an increasing trend Baxevanos *et al*. (2017).

Li *et al*. (2009a, 2009b) proposed that a non-legume such as maize can absorb mineral N from the soil, stimulating nodule formation and biological nitrogen fixation in the legume partner. In addition, the exudates produced by maize may promote flavonoid synthesis (signalling compounds for rhizobia) in the intercropped legume, increase root hair deformation and nodulation, and stimulating nitrogen fixation (Li *et al*., 2016a). Moreover, biochar addition may also increase the performance of the non-legume (vegetative growth, nutrient uptake and root exudation), thereby indirectly stimulating nodule formation and biological nitrogen fixation in the legume partner (Liu *et al*., 2017). There are two pathways for N transfer from legumes to cereals in legume-based intercropping systems (Liu *et al*., 2017). Legume plants could release N to the rhizosphere through root exudates and turnover (Jalonen *et al*., 2009), and the mineralized inorganic N from these organic residues can then be absorbed by the intercropped cereals (He *et al*., 2003).

It is likely that given the evidence in the published literature and the findings here, that a combined management regime employing biochar and legumes, particularly within intercropping or bicropping situations has a role to play in sustainable agriculture, or in reclamation schemes although a greater understanding of localised interactions would be essential.

Biochar is known to improve water retention, specific surface area, cation exchange capacity, total N, organic C and extractable P, K, Mg and Ca (Laird *et al*. 2010; Sánchez *et al*., 2009; Thies and Rillig, 2009). Several studies also reported that the characteristics of biochar such as high porosity, water holding capacity, and elevated concentrations of organic carbon and nutrients, have had positive effects on soil quality and plant growth (Xu *et al*. 2012; Graber *et al*. 2010; Major *et al*. 2010b). Plant growth and yield improvements following biochar application

have been reported under field and greenhouse conditions for a variety of crops, including legumes such as soybean (*Glycine max*) (Tagoe *et al*., 2008) and common bean (*Phaseolus vulgaris*) (Rondon *et al*., 2007). Moreover, quail litter biochar application increased number of nodules, plant height, dry weight, yield and nutrient uptake in soybeans (Suppadit *et al*., 2012).

Despite the reported positive effects of char, a meta-analysis conducted by Jeffery *et al*. (2011) concluded that biochar effects globally range from negative (-28%) to positive (+39%) with the mean effect being 10%. These authors suggest the main mechanisms for benefits are a liming effect, improved water holding capacity and improved nutrient status.

A summary of the key findings in each chapter is as follows:

| **Experimental Chapters** | **Key Points** |
|---|---|
| Chapter 2 | • All roots were nodulated, either by an indigenous strain only, or by a commercial strain in addition to the native strain.<br>• Bean plant responses to the two char particle sizes (powder and 1mm) were different.<br>• Powdered char delayed germination.<br>• Additional commercial *Rhizobium* amendment led to lower plant biomass relative to plants infected by the indigenous strain, but only when growing with powdered char and not with the 1mm char size.<br>• Overall there was a positive relationship between nodule number and plant biomass.<br>• Adding the commercial strain increased nodule numbers by 34%.<br>• Commercial or indigenous rhizobia modified effect of char on water extractable soil K. [K] was highest with native rhizobia and 7% char but highest with commercial rhizobia with 2.5% char.<br>• Commercial rhizobia and 2.5% char had the highest degree of potential $N_2$ fixation (based on leaf Mo concentration as a proxy for BNF).<br>• The 7% char amendment had lower leaf [Mo] than the 2.5% char + commercial rhizobia, but higher |

| | |
|---|---|
| | than all other treatments. Char possibly therefore increased BNF but the commercial inoculum performed best when with the lower char concentration.<br>• pH was increased most by powdered char (concentrations pooled) and by the 7% concentration (size particles pooled). |
| Chapter 3 | • One bean and one wheat plant were co-grown in the same pot across all treatments.<br>• Nodules were generally present only on inoculated roots.<br>• *Rhizobium* inoculation resulted in improved wheat biomass and grain yield.<br>• *Rhizobium* altered competitive outcome between bean and wheat. Without *Rhizobium*, beans outcompeted wheat; with *Rhizobium*, wheat was the stronger competitor based on shoot weight.<br>• *Rhizobium* inoculation decreased bean shoot weight but increased pod production when grown with char.<br>• The highest char concentration (particle sizes pooled) increased bean pod weight and reduced wheat grain yield. High char concentration likely immobilised N; beans could presumably fix $N_2$ but wheat could not.<br>• Powdered char increased bean pod weight.<br>• Rhizobia inoculation enhanced shoot K and P concentrations in bean and wheat.<br>• Bean and wheat leaf Mn concentrations were lower in plants from inoculated pots. Mn is essential for $N_2$ fixation and *V. faba* has high affinity Mn transporters. Mn would likely be sequestered in the bean root. |
| Chapter 4 | • *Rhizobium* significantly enhanced shoot and root growth whilst char did not.<br>• Char amendment resulted in lower concentrations of a range of nutrients in plant leaves and roots.<br>• Leaf B and Zn were reduced in plants infected with Rhizobium when grown in char-free soil. The presence of char modified this so there was no longer a *Rhizobium* effect.<br>• Available/exchangeable elemental concentrations were lower in soil with *Rhizobium*. |

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