EFFECT OF BIOCHAR AND RHIZOBIUM INOCULATION ON NODULATION, CHLOROPHYLL CONTENT, GROWTH AND YIELD OF CHICKPEA (*Cicer arietinum L.*)

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DECLARATION

I, Patricia Jozina Macil, student number:11618261, hereby declare that this Dissertation for Master of Science in Agriculture (Plant Production) submitted to the Department of Plant Production, School of Agriculture, University of Venda has not been submitted previously for any degree at this or any another University. It is original in design and execution, and all references have been duly acknowledged.

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DEDICATION

I dedicate this work to my late sister Reneilwe Ragosebo Macil who always inspired me to do my best and work hard at all times. May her soul rest in peace.

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ABSTRACT

Soil infertility, water scarcity, and availability of high yielding and drought tolerant crop genotypes remain major constraints for agricultural production in semi-arid regions. These constraints are major threats to sustainable crop production and food security. Management practices in such areas should always be geared towards improving productivity at a low cost while sustaining soil fertility. Preliminary studies showed the huge potential of chickpea in the dry environments of the North Eastern South Africa. However, lack of nodulation in chickpea has been reported in these regions probably due to low soil pH, insufficient rhizobial populations or total lack of infective native rhizobia. Therefore this study assessed the effect of biochar and rhizobium inoculation on soil pH, nodulation, growth, yield and chlorophyll content of chickpea in Mpumalanga (Nelspruit) and Limpopo (Thohoyandou) Provinces, South Africa.

Two field experiments were planted during winter 2015 and 2016. Treatments consisted of three levels of biochar (0, 10 and 20 t ha^{-1}), two Rhizobium inoculation levels (with and without Rhizobium inoculation) and three chickpea genotypes (ACC #4, ACC #5, and ACC #6) in a factorial combination arranged in randomized complete block design replicated three times. Crop phenology (days to 50% emergence, flowering, podding, and physiological maturity), crop growth (plant height, canopy cover, number of primary and secondary branches), nodulation (number of nodules per plant and nodule dry weight), yield and yield components (number of pods per plant, number of seeds per pod and 100 seed weight [100-SW]), and chlorophyll content were determined at various crop growth stages. Identification and isolation of native rhizobia from soils was done using standard protocols. Data obtained were subjected to analyses of variance using the general linear model of Genstat software version 17. Significant differences between the treatments means were compared using the standard error of difference (SED) of the means at 5% level. Correlation analyses were performed to assess the relationship between parameters. Molecular data was subjected to BLASTn in National Centre for Biotechnology Information (NCBI) searches for identification of isolated strains

Application of biochar at 10 and 20 t ha^{-1} increased soil pH by 0.7 pH units in Thohoyandou (clay soil) in 2015 and 2016, respectively. Soil pH increased by 0.77 pH units at 10 t ha^{-1} and 1.2 pH units at 20 t ha^{-1} in Nelspruit (loamy sand) in 2015 and 2016, respectively. Similarly, rhizobium inoculation increased soil pH by 0.2 (Thohoyandou) and 0.5 (Nelspruit) pH units in 2015 and 2016, respectively. There was a 100% increase in nodulation in inoculated compared to uninoculated treatments. There was no effect of biochar and rhizobium inoculation on number of days to 50% flowering, podding,

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physiological maturity and on plant height. However, plant height varied with genotypes. Biochar application increased above ground biomass by 17% (10 t ha^{-1}) and 12% (20 t ha^{-1}), and 100 seed weight by 9% (10 t ha^{-1}) and 7% (20 t ha^{-1}) in Thohoyandou in 2015. Rhizobium inoculation increased yield and yield components in Thohoyandou in both seasons; biomass was greater by (31 and 23%), grain yield (26 and 24%), number of pods per plant (18 and 31%), and 100-SW (10 and 13%) in 2015 and 2016, respectively. Similarly, rhizobium inoculation increased biomass (53.4%), grain yield (81%), number of pods per plant (54%) and number of seeds per pod (89%) in Nelspruit in 2015. Genotype did not affect yield and yield components in Nelspruit. In contrast, genotype affected above ground biomass, grain yield, harvest index, number of pods per plant, and number of seeds per pod in 2015 in Thohoyandou with ACC #6 producing greater yield compared to ACC #4 and 5. The analysis for native rhizobia showed that agricultural fields in Nelspruit and Thohoyandou lack effective strains of rhizobium. The identified strains according to 16s gene region were *Klebsiella variicola*, *Burkholderia cenocepacia*, *Bacillus subtilis* and *Ochrobactrum spp*. The effects of biochar and rhizobium inoculation were more pronounced in Thohoyandou compared to Nelspruit. Therefore biochar and rhizobium inoculation may improve chickpea productivity in Limpopo and Mpumalanga Provinces through improved soil pH, nodulation, growth, yield and yield components.

Key words: Biochar, rhizobium inoculation, genotype, chickpea

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
B	Biochar
BNF	Biological nitrogen fixation
C	Carbon
CH_4	Methane
CHL	Chlorophyll
CO_2	Carbon dioxide
CEC	Cation exchange capacity
DAE	Days after emergence
DAP	Days after planting
EC	Electrical conductivity
g	Gram
IR	Intercepted radiation
N	Nitrogen
P	Phosphorus
PH	Plant height
µL	Micro litre
nm	Nanometres
OC	Organic carbon

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CHAPTER 1: INTRODUCTION

1.1 Background information

There is an increasing demand for production of food crops to maintain steady food supply to the growing population. This increasing demand for food supply has led to a challenge of designing resource-efficient and low cost production strategies. The challenges of poor soil fertility, water supply, and availability of high yielding and drought tolerant crop genotypes remain major constraints for agricultural production especially in semi-arid regions and are major threats to sustainable crop production and food security. Management practices in such areas should always be geared towards improving productivity at a low cost while sustaining soil fertility. The use of soil amendments (such as biochar) and bio-fertilizers (such as rhizobium inoculants) could be crucial for improving productivity and ensuring food security for the growing population. Also, this could mitigate problems caused by chemical pesticides on beneficial soil microorganisms. Biochar application reduces the chemical toxicity and improves microbial activity (Nigussie *et al*., 2012)

Legume crops have gained increased importance especially in smallholder farming systems of Sub-Saharan Africa due to their contribution to household nutrition, health, and ability to grow in low fertility soils (Sennhenn *et al*., 2016). Most soils in semi-arid regions are characterized by poor fertility and hence incorporation of drought tolerant legumes such as chickpea in existing cropping systems may be crucial in ensuring improved productivity. Chickpea (*Cicer arietinum* L.) is a minor pulse crop in South Africa which is not cultivated commercially despite increased demand for domestic use. Globally, chickpea ranks second after common bean (*Phaseolus vulgaris*) (FAO, 2015). The crop is mainly grown for human consumption, animal feed and for medicinal purposes (Gaur *et al*., 2010). It is one of the most important food crops in sustaining food security due to its supply of high quality protein, carbohydrates, minerals and trace elements to the diet of human and animal population especially in developing countries (Basir *et al*., 2008). Chickpea is a significant contributor to agricultural sustainability through biological nitrogen fixation (BNF) and as a rotation crop allowing the diversification of agricultural production systems (FAO, 2004). Under favourable conditions, symbiotic nitrogen (N) fixation can produce greater than 100 kg N ha^{-1} (Beck, 1992), and provide up to 85% of the N required by a chickpea crop thus reducing the need and cost for external inputs (Walley *et al*., 2005; Chemning' wa & Vessey, 2006). Chickpea is adapted to environmental stresses such as drought, high temperatures and poor soils and therefore could be a suitable crop for semi-arid regions such as Limpopo and Mpumalanga provinces. Thangwana and Ogola (2012) reported that chickpea may be an important food security crop for smallholder farmers in the semi-

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arid regions of Limpopo and Mpumalanga Provinces. Farmers in these regions could practise double cropping where fields are currently fallowed during winter. The incorporation of chickpea in these systems during winter may not only improve soil fertility but also increase the land productivity of the cereal-based cropping systems and economic returns of farmers. However, for winter cultivation, supplementary irrigation should be available especially during critical plant growth stages. Despite the ability of chickpea to do well in poor soils, it also produces higher yield compared to other legumes in winter seasons and therefore makes it a suitable crop for the semi-arid regions such as the North Eastern part of South Africa (Thangwana and Ogola, 2012; Lusiba, 2015)

Crop growth and yield are generally reduced by poor soil fertility. Glaser *et al.* (2002) reported that to overcome the problems of poor soil fertility and low crop yield, it is important to adapt new technologies such as using biochar as a soil amendment, which improves soil fertility, crop yield and also sustains the environment for future use while mitigating climate change. Biochar is one of the most important soil amendments especially in soils with low organic carbon (OC). It is the product of biomass produced in the pyrolysis process where biomass is pyrolysed at high temperatures in the complete absence of, or little presence of oxygen (Glaser *et al*., 2002). The quality of biochar is affected by the type of biomass used, time of pyrolysis and handling after pyrolysis processes. Application of biochar in crop production has gained popularity and its beneficial effects have been well documented (Laird, 2008; Glaser *et al*., 2002; Major *et al*., 2010; Macdonald *et al*., 2014). It improves soil physical, chemical and biological properties and hence improves crop productivity. Its application to the soil increases soil quality, water retention, and plant nutrient availability (Glaser *et al.,* 2002 and Steiner *et al.,* 2007; Laird, 2008). Application of biochar to soils add basic cations and acts as liming agent to acid soils. It also helps in the mitigation of climate change by reducing the emission of greenhouse gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) into the atmosphere through carbon sequestration (Glaser, 2007; Steiner *et al*., 2007). These benefits are however, mostly determined by the pyrolysis conditions such as temperature, time, pressure, and biomass type (Demirbas, 2004). However, application of biochar alone may not be a good source for plant nutrient availability; its benefits may be enhanced through the application of other materials such as organic, inorganic and bio-fertilizers (Lehmann *et al.,* 2007).

The use of rhizobium inoculants is crucial in legumes especially where native rhizobium populations are insufficient and/or ineffective (Bhuiyan *et al*., 2008). The problem of legume nodulation is prevalent in semi-arid regions due to insufficient or total lack of rhizobium bacterium in the soils

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(Nishita and Joshi, 2010). Also, soil pH and plant nutrition are contributors to the lack of nodulation in legumes (Khan *et al*., 2014). Therefore, inoculants may play an important role in ensuring nodulation, improving biological nitrogen fixation and improving crop yield. Also, it reduces the need and cost of nitrogen fertilizers with concomitant environmental benefits (Solaiman *et al*., 2010). However, the use of industrial inoculants is only beneficial if incorporated with good management practices.

Chickpea research and production was initiated in two representative locations of dry environments in South Africa (Limpopo and Mpumalanga Provinces) by the University of Venda (Department of Plant Production) and Department of Rural Development and Land Reform (DARDL) (Nelspruit). However, lack of nodulation has been prevalent despite the crop showing huge potential in these regions. The lack of nodulation of chickpea could possibly be due to: lack of infective rhizobium strains in the soil; inadequate populations of infective native rhizobium strains; and low soil pH in these regions. The lack of nodulation in these regions implies lack of biological nitrogen fixation by the crop and hence increased need for external inputs. Ogola (2015) attributed the lack of nodulation in chickpea in north eastern South Africa partly to the total lack or insufficient populations of effective native rhizobia and low soil pH. Therefore, this study assessed the effect of biochar and rhizobium inoculation on nodulation, growth, yield, and some chlorophyll content of three chickpea genotypes in two locations of South Africa.

1.2 Objectives

The main objective of the study was to determine the effect of biochar and rhizobium inoculation on chickpea productivity.

The specific objectives of the study were to:

- Identify the available strains of native rhizobia in the soil.
- Assess the effect of biochar and rhizobium inoculation on:
    - Soil pH.
    - Crop phenology and growth of 3 chickpea genotypes.
    - Root nodulation of three chickpea genotypes.
    - Chlorophyll content, stomatal conductance, and intercepted radiation of three chickpea genotypes.

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- Yield and yield components of three chickpea genotypes.

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CHAPTER 2: LITERATURE REVIEW

2.1 Background information

2.1.1 Chickpea origin and utilization

There are two types of chickpea (*Cicer arietinum L.*), Kabuli and Desi which originated in South-Eastern Turkey and India, respectively (Ladizinsky and Adler, 1975). Desi has small dark thick seed coat and adapted to low rainfall areas. It produces purple or violet flowers, is relatively short in height and sometimes contains two seeds in one pod, which are small and swollen (Gaur *et al.,* 2010). Kabuli has larger seeds with a smooth white seed coat and grows relatively taller in areas of moderate rainfall. It produces white flowers and sometimes contains one seed per pod. The lateral roots of chickpea develop nodules with the symbiotic rhizobium bacteria, capable of fixing atmospheric nitrogen for plant use (Gaur *et al.,* 2010). Chickpea is an upright annual legume 30-70 cm tall and fairly drought tolerant due to its extensive tap root system (Gaur *et al.,* 2010). .

Chickpea is one of the most widely grown grain legumes in the world ranking second after dry bean (*Phaseolus vulgaris*) and constitutes about 20% of the world's pulses production (FAOSTAT, 2015). The largest producer is India with about 70% of chickpea produced annually followed by Australia, Pakistan, Turkey, Burma, Iran, Ethiopia, Canada and the United States (FAOSTAT, 2015; ICARDA, 2016). In Africa, Ethiopia is the leading producer of chickpea and South Africa contributes 0% to world chickpea production (FAOSTAT, 2015). Chickpeas are mostly grown for human consumption and animal feed. The crop is eaten as a vegetable or side dish with the staple food (Jukanti *et al*., 2012). It contributes to food security and nutrition especially in developing countries where majority of the population cannot afford animal protein for balanced nutrition (Basir *et al*., 2008). It is a nutrient-dense food crop providing more than 20% of daily value protein, dietary fibre and other minerals such as magnesium and vitamin B_6. In animal feed, the crop provides a source of energy and proteins and it has fewer digestive problems in non-ruminants (Bampidis e*t al*., 2011). The crop meets 80% of its nitrogen (N) requirement from symbiotic nitrogen fixation and can fix up to 100 kg N ha^{-1} from the atmosphere (Beck, 1992). Its ability to fix atmospheric nitrogen makes it suitable for crop rotations and intercropping.

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2.1.2 Biochar properties and benefits

Biochar is defined as charcoal obtained when organic materials (biomass and manures) are burned under low pressure and high temperature condition through pyrolysis process (under low or absence of oxygen). Biochar is considered as a tool for improving soil fertility and sequestering carbon (C) due to its stable nature in the soil (Lehmann, 2007; Laird, 2008). It has gained popularity due to its contribution to soil health, fertility and crop production. Biochar can remain in the soil for a number of years and its application is not continuous which can reduce both labour and input costs. Chemical and physical properties of biochar are affected by a number of factors during pyrolysis process such as temperature, residence time, biomass type and pressure (Antal and Gronli, 2003; Demirbas, 2004). These properties have a large impact on soil properties and processes when biochar is incorporated into the soil, thus influencing soil fertility and crop production (Demirbas, 2004). Biochar produced from crop residues and wood material have shown great potential in improving both chemical and physical properties of the soil. Also, the type of pyrolysis greatly affects biochar properties, for example an increase in pyrolysis temperature increased pH, surface area, ash content, and total Ca and P content and decreased CEC and total N (Wang *et al*., 2013). The quality of biochar may be assessed by measuring its pH, water holding capacity, ash content, volatile compound content, pore size, surface area and bulk density.

2.1.3 Rhizobium strains and their importance

Rhizobia are bacterial strains that are responsible for infecting roots of legumes resulting in the formation of nodules. It is a diverse genus with a wide variety of species which are host specific and occupy a wide range of geographic locations. The symbiotic relationship between roots of legumes and rhizobia is very crucial in legume production as it enables the plant to fix sufficient amount of nitrogen and meet its nitrogen nutrient requirements (Solaiman *et al*., 2010). The effectiveness and competitiveness of rhizobia differ with type of strains; some may be suitable for use as commercial inoculants and others not. Also, their survival in the soil, reproduction and effectiveness may be affected by a number of factors such as soil pH, moisture, and availability of minerals such as phosphorus (P) and molybdenum (Mo). Rhizobium inoculants improve crop yield of many legumes and also reduce the use of chemical nitrogenous fertilizers (Solaiman *et al*., 2010). Although chemical pesticides are important in controlling crop pests, they also harm useful microbes in the soil. Application of commercial inoculants may be useful in increasing the populations of these microbes such as rhizobia (Nishi and Joshi, 2010; Gul *et al*., 2014). Most soils in arid and semi-arid regions lack sufficient population of infective rhizobia, hence the use of commercial inoculants

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become crucial and beneficial in legume production in such areas (Romdhane *et al*., 2009). Ogola (2015) attributed lack of nodulation in chickpea in North Eastern South Africa partly to absence of compatible native rhizobia probably because chickpea is not native to the region.

2.2 Effect of biochar on soil pH

The addition of biochar to agricultural soils has recently received much attention due to the apparent benefits of biochar on soil quality. Biochar has positive effects on soil chemical properties such as pH and cation exchange capacity (Rondon *et al*., 2007). These benefits are enduring and hence it does not need to be added to soil each year as the case with many agricultural fertilizers. Therefore, biochar may be a suitable tool for sustainable use of soils especially in the semi-arid regions where soil pH imbalance is one of the constraints in crop production. Biochar from woody materials is typically a soil enhancer, improving the pH, soil water relations and CEC (Macdonald *et al*., 2014). The effect of biochar on soil pH is well documented (Nigussie *et al*., 2012; Rondon *et al*., 2007; Berek *et al*., 2011; Carter *et al*., 2013; Milla *et al*., 2013). The increase in soil pH due to biochar application has been attributed to high specific surface area and porous characteristics of biochar that increases cation exchange capacity (CEC) (Nigussie *et al*., 2012; Berek *et al*., 2013). Therefore, biochar could partially substitute lime in some instances due to its liming effects on soil. However, the effect of biochar on soil pH is dependent on the properties of biochar and the type of soils (Carter *et al*., 2013; Milla *et al*., 2013). In a recent study, Lusiba *et al*. (2017) found that biochar application increased, albeit non-significantly, soil pH at two sites with contrasting soil types. Therefore, there is still a need to investigate the effect of biochar on soil pH under field conditions in these representative areas of dry environments of South Africa (Limpopo and Mpumalanga Provinces).

2.3 Effect of biochar and rhizobium inoculation on growth of chickpea

The addition of biochar as a soil amendment has been identified as an important resource-efficient tool that does not only improve crop productivity but also has a positive impact on the environment (Glaser *et al.,* 2002). Biochar contains appreciable amounts of plant nutrients such as calcium, magnesium, carbon and potassium, and this could positively affect plant growth and development (Lusiba *et al*., 2017). Indeed, the effects of biochar on plant growth have been well documented in a number of crops such as wheat, peanuts, spinach and maize (Akhtar *et al*., 2015; Milla *et al.*, 2013; Major *et al.*, 2010b). Schulz and Glaser (2012) reported that the application of biochar, mineral fertilizer (N, P, and K), compost, and biochar plus compost in infertile sandy soil in the greenhouse increased plant growth of oats. In contrast, in a greenhouse study on alfisol, application of green

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waste biochar at 0, 10, 50, and 100 t ha $^{-1}$ did not affect crop growth of radish, but a significant increase in growth was observed when biochar and 100 N kg ha^{-1} fertilizer was applied (Chan *et al.,* 2008). Macil *et al*. (2017) reported an increase in the proportion of intercepted radiation with the application of biochar at 0, 5, 10 and 20 t ha^{-1} and 90 kg P ha^{-1} in the current study location. It is clear that the effect of biochar on crop growth varies with biochar type, application rate, soil type, crop type and other husbandry practices. Furthermore, biochar benefits may be greater if incorporated with both organic and inorganic fertilizers. Therefore, incorporation of biochar and rhizobium inoculation may not only improve growth of chickpea in dry environments but enhance root nodulation, nutrient availability and crop productivity through improved soil pH and increased availability of beneficial rhizobia.

Rhizobium inoculants are mostly used as bio-fertilizer and have been efficient in the production of legumes. The use of synthetic inoculants is mostly beneficial if the crop grown is not native to the area and also when there is insufficient population of effective native rhizobia in the soils. Soils in the semi-arid regions normally have low rhizobium populations due to low soil pH, insufficient soil moisture, and high average temperatures (Khattak *et al*., 2006). The effects of rhizobium inoculation on growth of chickpea and other legume crops have been reported in a number of studies (Solaiman *et al*., 2010; Mmbaga *et al.,* 2015; Khattak *et al*., 2006; Bhuiyan *et al*., 2006). Namvar *et al*. (2011) reported an increase in chickpea growth with rhizobium inoculation. Furthermore, Nishita and Joshi (2010) reported an increase in shoot length and shoot weight of chickpea with rhizobium inoculation. Similarly, Mmbaga *et al.* (2015) observed an increase in growth of common bean with rhizobium inoculation. An increase in number of branches, plant height and other growth parameters with rhizobium inoculation have been reported (Akhtar *et al*., 2015; Mmbaga *et al*., 2014; Namvar *et al*., 2011).

The increase in number of germinated seeds was observed in rhizobium inoculated seeds compared to the control (Vaishali *et al*., 2014). Increase in plant height with rhizobium inoculation have been reported at the site of the current study (Ogola, 2015) and in other areas (Solaiman *et al*., 2010). Clearly rhizobium inoculation improves the growth of chickpea and other legume crops and may be one of the useful strategies for sustainable enhancement of crop growth and yield of legumes. Yusif *et al.* (2016) reported an increase in number of leaves per plant and plant height of rhizobium inoculated groundnut grown in biochar amended soils compared to control which was associated with high nutrient availability, moisture retention and reduced leaching of mineral N (Yusif *et al*., 2016). Although the effects of biochar and rhizobium inoculation on the productivity of

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chickpea and other legumes are well documented in literature, there is scant information on the interactive effects of biochar and rhizobium inoculation on growth of chickpea especially in soils with low pH like the site of current study.

2.4 Effect of biochar and rhizobium inoculation on nodulation of chickpea

Biochar enhances biological nitrogen fixation (BNF) which is the process whereby atmospheric nitrogen is fixed in the root nodules that are formed as a result of a symbiotic relationship between rhizobium bacteria and roots of legumes. BNF is important in soils with low N availability where N fertilizer inputs are minimal, especially soils in arid regions (ABARES, 2011). Biochar addition in the soil does not only have an influence on microbial population and activity, but also the interaction between legumes and microbes through the effect of nutrient availability and adaptation of habitat (Lehmann and Rondon, 2006). In a greenhouse study, biochar additions (30 and 60 g kg^{-1}) significantly improved BNF by *Phaseolus vulgaris*; this was attributed mostly to increased availability of boron (B), molybdenum (Mo), potassium (K), calcium (Ca), phosphorus (P), and higher pH as well as lower aluminum saturation in the soil (Rondon *et al*., 2007). Application of biochar (10 and 20% by weight) increased soil nitrogen content of Anthrosol but not Ferralsol when cowpea and rice were used as test crops (Lehmann *et al*., 2003). The potential of increasing BNF with biochar application is poorly understood, with inconsistent reports on root nodulation (Quilliam *et al*., 2013). More recently at the site of the current study, biochar application did not affect nodulation of uninoculated chickpea (Lusiba, 2015).

Inoculating seeds with strains of rhizobia increases root nodulation of chickpea and other legumes (Bhuiyan *et al*., 2006; Khattak *et al*., 2006; Mmbaga *et al.,* 2015). Similarly, Sainia *et al*. (2003) showed that number of nodules per plant and nodule dry weight was significantly higher in the treatments receiving Rhizobium inoculation in both sorghum and chickpea. Biochar and rhizobium inoculation increased both the number of nodules per plant, nodule dry weight and root biomass of groundnut in Nigeria (Yusif *et al*., 2016). These results were associated with increased rhizobium populations due to inoculation and also alterations of soil pH and other soil properties which affect root growth and survival of rhizobia in the soil.

Also, Ogola (2015) reported an increase in the number of nodules and nodule weight in chickpea with rhizobium inoculation compared to control. However, nodulation was relatively poor even with inoculation. The poor nodulation in inoculated plots was partly attributed to low soil pH. Therefore,

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nodulation can be improved by combining inoculants (addition of rhizobia) and biochar (for soil pH balance and moisture retention). It is clear that inoculation alone may not enhance root nodulation but a balance of other factors affecting nodulation such as soil pH and moisture may lead to improved and effective nodulation (Ogola, 2015). The interactive effect of biochar and rhizobium inoculation improved nodulation of groundnuts in Nigeria (Yusif *et al*., 2017). However, the interactive effects of biochar and rhizobium inoculation on nodulation of chickpea and other legumes in South Africa are not well documented. Therefore, there is a need investigate the effect of biochar and rhizobium inoculation on nodulation of chickpea in different locations of dry environments in South Africa.

2.5 Effect of biochar and rhizobium inoculation on chlorophyll content and intercepted radiation of chickpea

The application of biochar may affect physiological parameters such as chlorophyll content, stomatal conductance and the proportion of intercepted radiation by the plant canopy. Pinewood biochar contains a number of important minerals such as magnesium, calcium, carbon and potassium (Lusiba, 2015), and also acts as an absorber, reducing nitrogen leaching and increasing N use efficiency thus increasing chlorophyll content (Lehmann *et al*., 2003). An increase in chlorophyll content with biochar application has been reported in chickpea (Macil *et al*., 2017) and other crops (Burke *et al*., 2014; Akhtar *et al*., 2014; Badar *et al*. 2015). The response of chlorophyll content to biochar application may be attributed to the effect of biochar on plant nutrient status. Moreover, chlorophyll content may be affected by the age of the crop, daily collection time, biochar application rate and properties (Milla *et al*., 2013). In our earlier study by Macil *et al*. (2017), rhizobium inoculation was not included. Therefore, it is not clear how leaf chlorophyll content would respond to the interactive effect of biochar and rhizobium inoculation. Therefore, there is a need for intensive investigation on the interactive effects of biochar and other organic fertilizers on chlorophyll content at different growth stages and different environments.

The use of bio-fertilizers such as rhizobium inoculants has been identified as a suitable substitute for nitrogen fertilizers especially in poor soils. The effect of rhizobium inoculants on chlorophyll content of crops is associated with improved BNF, which enables the legume crops to fix sufficient atmospheric nitrogen to meet their N nutrient requirement. Bambara and Ndakidemi (2009) showed that chlorophyll content of common bean increased significantly with rhizobium inoculation. In a greenhouse and field study, an increase in leaf chlorophyll content in rhizobium inoculated plots (by

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123 % and 178, respectively) compared to the control was observed (Mmbaga *et al*., 2015). The response of chlorophyll content to rhizobium inoculation may be attributed partly to the supply of N to the crop, which influences the amount of proteins, amino acids, protoplasm, and chlorophyll formed (Namvar *et al*., 2011). Although the individual effect of biochar and rhizobium inoculation on chlorophyll content are well documented, there is still a gap that exists in understanding the interactive effects of biochar and rhizobium inoculants on chlorophyll content of chickpea and other legumes.

The effect of biochar on the proportion of intercepted radiation (IR) by plant canopy has been reported in chickpea (Macil *et al*., 2017; Nishita and Joshi, 2010; Namvar *et al*., 2011) and other crops (Milla *et al.,* 2013). Biochar additions to soils result in increased leaf area, availability of essential nutrients such as N and prevents early senescence (Macil *et al*., 2017; Lusiba *et al*., 2017; Milla *et al*. 2013). These effects on plant growth will therefore lead to higher proportion of radiation intercepted by the plant canopy and hence more biomass accumulation.

The effect of rhizobium inoculation on IR is well documented in chickpea (Namvar et *al*., 2011; Solaiman *et al*., 2010; Ogola, 2015). Rhizobium inoculation improves the proportion of intercepted radiation through improved crop growth parameters such as leaf area index, canopy size and number of leaves. The increase of crop growth due to rhizobium inoculation was associated with adequate supply of N to crops and hence enhanced plant processes such as cell division, photosynthesis and growth rate (Namvar *et al*., 2011; Solaiman *et al*., 2010). Yusif *et al*. (2016) reported that application of both biochar and rhizobium inoculation increased the number of leaves per plant of groundnuts. Despite the foregoing, there is scant evidence in literature on the interactive effect of biochar and rhizobium inoculation on canopy development and hence the proportion of intercepted radiation by chickpea crop.

2.6 Effect of biochar and rhizobium inoculation on yield and yield components of chickpea

Field and greenhouse studies have shown that biochar application increased yields of chickpea and other crops such as cowpea, peanuts, cotton and rice (Lusiba, 2015; Namvar *et al*., 2011; Nishita and Joshi, 2010; Burke *et al*., 2014; Milla *et al*., 2013; Solaiman *et al*., 2010; Partey *et al*., 2016). The available evidence suggests that significant reductions in N fertilizer application may be achieved while maintaining similar yields, with the addition of biochar to soils (Chan *et al*., 2008). However, contradicting observations have been reported on the response of crop yields to biochar

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application; these conflicting results have been attributed to variations in environmental conditions and biochar properties (Filiberto and Gaunt, 2013).

Application of biochar increased biomass and crop yield of soybean compared with control due to increased available soil phosphorus (P), total soil nitrogen (N) and total soil carbon (C) (Mete *et al*., 2015). William and Qureshi (2015) reported an increase in yield of okra, coriander and beans due to addition of biochar to soils. It is clear that biochar additions to soils lead to increased crop yields, but the effects may not be immediate but observable in subsequent years (Major *et al*., 2010b). Biochar additions to acidic and nutrient poor soils, combined with organic and inorganic fertilizers are likely to produce yields greater than when fertilizer or biochar is applied alone (ABARES, 2011). Application of biochar and phosphorus fertilizer increased above ground biomass and grain yield of chickpea in the current study location (Lusiba, 2015). Therefore, there is need to assess the effect of biochar on yield and yield components of chickpea in different locations of South Africa since the responses may vary with locations and season.

The increase in crop yields with rhizobium inoculation has been reported widely (Nishita and Joshi, 2010; Romdhane *et al*., 2009; Khattak *et al*., 2006; Bhuiyan *et al*., 2008; Bejandi *et al*., 2012; Yusif *et al*., 2016; Khaitov *et al*., 2016). For example, increase in number of pods per plant, number of grains per pod, 100 seed weight, and average grain yield of chickpea with rhizobium inoculation was reported by Namvar *et al*. (2011) while Solaiman *et al*. (2010) observed an increase in dry biomass of chickpea inoculated with 4 different rhizobium strains. In contrast, the effect of rhizobium inoculation on crop biomass, grain yield, harvest index, and radiation use efficiency (RUE) was not significant at the site of the current study (Ogola, 2015). The response of crop yields to rhizobium inoculation depends on the strain characteristics and environmental conditions (soil pH, soil fertility status, temperatures, and soil moisture content). Although the effects of biochar and rhizobium inoculation on yield and yield components of chickpea are well documented, however, there is no study that has covered the interactive effect of both biochar and rhizobium inoculation on yield and yield components of chickpea in dry environments. Therefore, incorporating rhizobium inoculation with biochar application may be crucial for improving chickpea productivity in these regions.

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CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental site

The experiments were conducted at the University of Venda's Experimental Farm, Thohoyandou (22º58.08´S and 30º26.4´E, and 595 m above sea level), Limpopo Province, and University of Mpumalanga's Experimental Farm, Nelspruit (25°45′S and 30°97E, and 660 m above sea level) Mpumalanga Province, South Africa. Thohoyandou receives an annual rainfall of ± 500 mm that falls predominantly in summer. The average minimum and maximum temperatures are between 18ºC and 31ºC. Nelspruit receives an annual rainfall of ± 620 mm per annum that also falls predominately in summer. The average minimum and maximum temperatures are between 6.5°C and 28°C (Tadross *et al*., 2006). The sites are characterized by deep, well-drained clay and loamy sandy soils, respectively (Soil Classification Working Group, 1991).

3.2 Experimental design

The study was conducted over two seasons which, with the first experiment conducted in winter 2015 and the second experiment conducted in winter 2016. Treatments consisted of three levels of biochar (0, 10 and 20 t ha^{-1}), two Rhizobium inoculation levels (with and without Rhizobium inoculation) and three chickpea genotypes (ACC #4, ACC #5 and ACC #6) in a 2 x 3 x 3 factorial combination arranged in randomized complete block design with three replications. The choice of biochar rates used was guided by the previous findings at the current site of study which showed that biochar rates at 10 and 20 t ha^{-1} are suitable rates for soils in Thohoyandou and Nelspruit (Lusiba, 2015). The size of individual plots was 2m × 2m, with 18 plots per block giving a total of 54 plots in 3 blocks (Figure 3.1).

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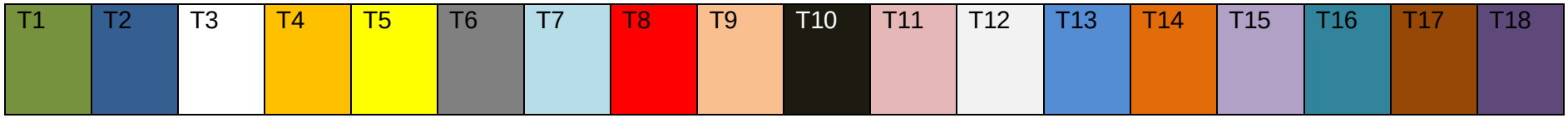
Block 1



Block 2

Block 3

Figure 3.1: Experimental design G=genotype B= biochar R= rhizobium inoculation (R1 without, R2 with)

T1- G4B0R1	T4- G4B0R2	T7- G5B0R1	T10- G5B0R2	T13- G6B0R1	T16- G3B0R2
T2- G4B10R1	T5- G4B10R2	T8- G5B10R1	T11- G5B10R2	T14- G6B10R1	T17- G3B10R2
T3- G4B20R1	T6- G4B20R2	T9- G5B20R1	T12- G5B20R2	T15- G6B20R1	T18- G3B20R2

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3.3 Soil sample collection and analysis

Soil samples were collected from the experimental plots at the two sites prior to biochar application (before sowing) and after harvesting. Two samples were randomly collected from each plot at a depth of 0 - 20 cm on each occasion. A composite sample for samples collected before sowing was obtained by mixing all the soil samples from the first set while the second set of samples were not mixed for determination of biochar effects on soil properties. Soil pH was measured using a pH meter with the ration 2:1 for water and soil.

3.4 Isolation and characterization of rhizobium bacteria

3.4.1 Soils

Serial dilutions of fresh soil for isolation of root associated bacteria were done using the soil samples collected before planting and after harvesting. 10 g of soil and rhizobium inoculant was suspended in separate conical flask containing 1 litre of distilled water. 10 ml of each mixture was transferred to another flask containing 1 litre of distilled water. Diluted solutions were transferred on the PCA (Plate Count Agar) media in petri dishes and incubated for 4 days at 28 ºC until the colonies appeared. The cultures were streaked on another PCA media in the second step. The nitrogen fixing bacteria was identified by streaking the pure cultures obtained from the PCA media on petri-dishes containing yeast extract-mannitol agar (YEMA) media (composition for 1L media: mannitol 10.0 g; k_2HP0_4 0.5 g; $MgSO_4$ -7H^2O 0.2 g; NaCl 0.1g; yeast extract 1.0 g; agar 20 g) (Figure 3.2). Petri dishes were incubated at 28 ºC until colonies of rhizobia appeared (Vincent, 1970).

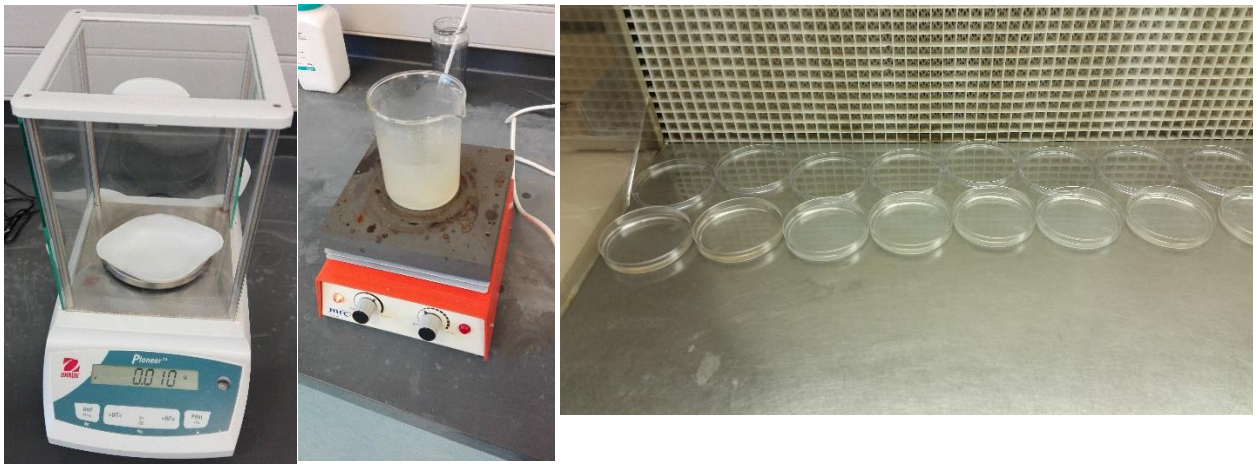

Figure 3.2: Preparing YEMA (media) for culturing

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3.4.2 Nodules

Root nodules were collected at 50% flowering by destructively harvesting four plants from inner rows. The nodules were separated from the roots, washed with tap water, sterilized in 70% ethanol and rinsed with sterile distilled water 5 times. The sterile nodules were crushed in 20 µL sterile solution and the nodule contents were streaked on plates containing yeast extract mannitol agar media (YEMA composition for 1L media: mannitol 10.0 g; k_2HP0_4 0.5 g; $MgSO_4$ -7H^2O 0.2 g; NaCl 0.1 g; yeast extract 1.0 g; agar 20 g) (Vincent, 1970) and incubated at 28 ºC until the bacterial colonies appeared. All colonies were purified by repeated streaking on YEMA plates. The isolates were stored at 4 ºC until molecular characterization.

3.4.3 Molecular characterization

3.4.3.1 DNA extraction

DNA was extracted using the CTAB (cetyl trimethylammonium bromide) method as described by Moller *et al*. (2001). A small amount of freshly cultured rhizobia was added into a tube containing 600 µL of CTAB buffer and mixed thoroughly. The mixture was incubated at 65 ºC for 1 hour, vortexing every 15-20 minutes. 700 µL of 24:1 chloroform: isoamyl mixture was added, vortexed and centrifuged at 12 000 rpm for 10 minutes. The mixture consisted of two layers (top and bottom layers). The top layer was transferred into a new tube and an equal volume of ice-cold isopropanol was added and vortexed. The samples were kept at -20ºC refrigerator overnight to precipitate the DNA. The samples were centrifuged at maximum speed for 20 minutes and the supernatant was discarded. 75% ethanol was added to wash the pellets, vortexed briefly and centrifuged for 5 minutes at maximum speed. The supernatant was discarded using yellow tips and the pellets were air dried. The pellets were then dissolved in 80 µL of Millipore water.

3.4.3.2 PCR preparations

Polymerase chain reaction (PCR) samples were prepared by adding 380 µL of Millipore water, 38 µL of buffer, 7.6 µL dNTP, 15.2 µL of both forward and reverse primers (F27 and R1485) and 1.52 µL Taq (enzyme) into a 15ml tube. The reactants were mixed together by vortexing and 24 µL of the mixture was transferred into PCR tubes. 1 µL of the DNA samples extracted was transferred into the PCR tube. Each PCR tube contained specific DNA and were marked according to the DNA sample name. The volume of the PCR mixtures was 25 µL per PCR tube. The tubes were placed in the PCR machine for 3 hours which consisted of 35 cycles following denaturation at 94 ˚C for 3

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minutes, annealing at 57 ˚C for 1 minute and extension at 72 ˚C for 1 minute. An elongation step of 72˚C for 5 minutes was followed by a final holding temperature of 20 ˚C (Steenkamp *et al*., 2008). The PCR products were viewed on agarose gel electrophoresis (Hassan *et al*., 2011). The un-purified PCR product were sent to Stellenbosch University Central DNA Sequencing Facility for post PCR clean up and sequencing reaction. The PCR amplifications was done in a GeneAmp® PCR system 2700 (Applied Biosystems, Foster City, Califonia USA) with the universal primers: forward primer (16 27F 5´-AGAGTTTGATCCTGGCTCAG–3´) and the reverse primer (16 1485R 5´-TACCTTGTTACGACTTCACCCCA-3´) amplifying nearly 1200 base pairs of the 16S rRNA gene (Estrella et al. 2009).

BLASTn algorithm (www.ncbi.nlm.nih.gov/BLAST/blast.cgi) software was used for comparing the rhizobial isolates. Sequences showing 98-100% similarity and 98-100% query were considered to be the closest matches to the already available sequences.

3.4.3.3 Agarose gel preparation and loading samples

One gram of agarose was suspended into 0.1% TBE (Tris-borate-EDTA) solution and placed into a microwave for 3 minutes. The agarose solution was left for 5 minutes to cool down and 5.2 µL of ethidium bromide was added into the solution. The agarose was placed into the gel tray with well comb in place and allowed to solidify for 30 minutes. The agarose was placed into a gel box (electrophoresis unit). The gel box was filled with TBE solution to cover it. The DNA samples were mixed with a blue dye before loading to ensure that it was successfully loaded. The samples were carefully loaded into the wells and the electrophoresis was set at 100V for 30 minutes. The gel was then placed under UV light for visualization of the DNA fragments and the visuals were captured.

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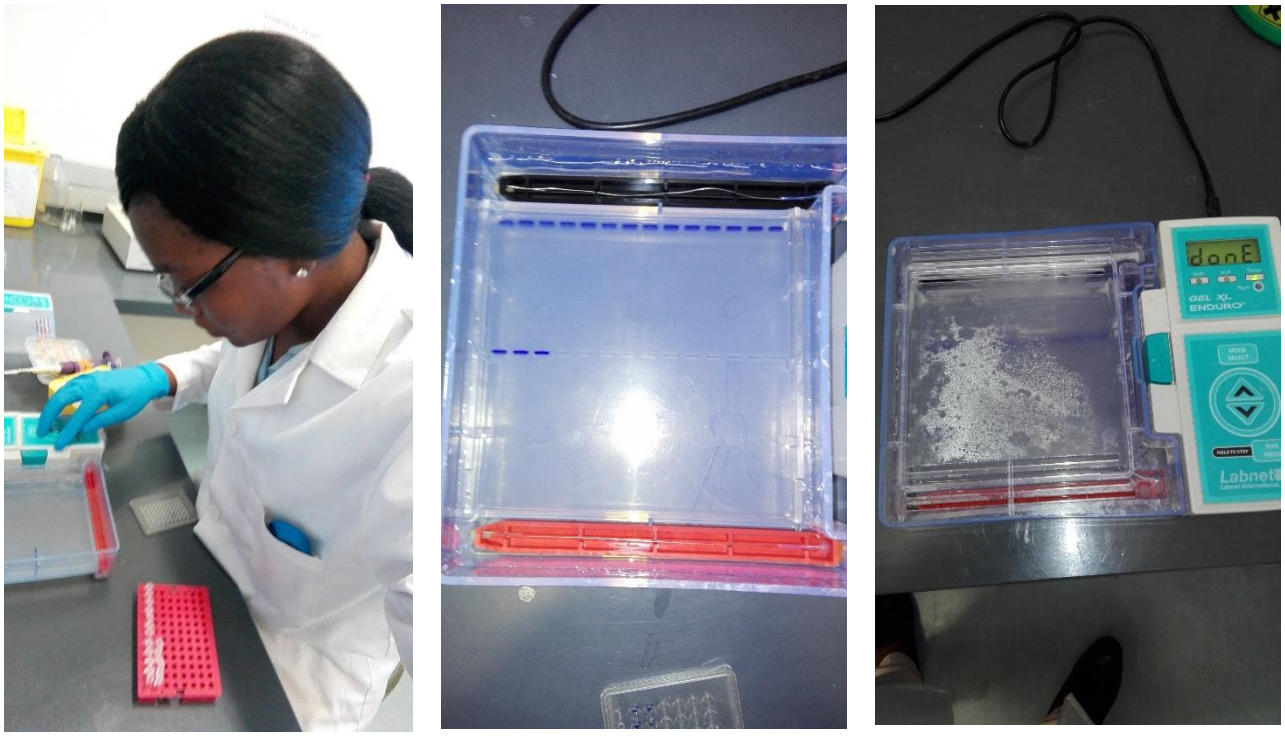

Figure 3.3: Loading and running DNA samples on agarose gel

3.5 Cultural practices

Biochar was applied a week before planting according to the treatments (0, 10, and 20 t ha^{-1}) and incorporated into the soil manually to a depth of 20 cm. Inoculated seeds (Figure 3.4) were soaked in a mixture of Rhizobium powder (Figure 3.5) and water at the rate recommended by the manufacturer and were planted immediately after inoculation. Uninoculated seeds were planted first to prevent contamination. First season's experiments were planted on the 24th and 30th May 2015 and the second experiments on the 13th and 14th April 2016 in Thohoyandou and Nelspruit, respectively. The seeds were sown manually at a spacing of 30 x 10 cm to give a plant population density of 33 plants per m^2 which was guided by the previous finding on the effect of planting density on growth of chickpea (Thangwana and Ogola, 2012). Phosphorus (P) and nitrogen (N) fertilizers were uniformly applied as single super phosphate at a rate of 90 kg P ha^{-1} and limestone ammonium nitrate (LAN) to all plots at the rate of 40 kg N ha^{-1}.

The plots were uniformly watered (close to field capacity) using micro jet and sprinkler irrigation system (Figure 3.6a & b) immediately after sowing to promote germination, emergence and crop establishment and supplementary irrigation was also provided during the growing season when necessary. The experimental plots were kept free from weeds by manual weeding throughout the

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growing seasons. Scouting for pests and diseases was done and Cyperin (Cypermethrin with pyrethroids as the active ingredient) was used as an insecticide to control pod borer at a rate recommended by the manufacturer (5 ml/ 100L). The insecticide was sprayed once in two weeks from post flowering until the crop has reached physiological maturity.

Figure 3.4 Inoculated chickpea seeds

Figure 3.5 Chickpea inoculant

Figure 3.6: Micro jet **(a)** and sprinkler **(b)** irrigation systems.

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Table 3.1, Summary of planting, crop emergence and harvesting dates for winter 2015 and 2016

Activities	**Thohoyandou**		**Mpumalanga**	
	Winter 2015 (Experiment I)	Winter 2016 (Experiment II)	Winter 2015 (Experiment I)	Winter 2016 (Experiment II)
Planting	24 May 2015	13 April 2016	30 May 2015	14 April 2016
Crop emergence	31 May 2015	20 April 2016	08 June 2015	22 April 2016
Harvesting	28 September 2015	31 August 2016	01 October 2015	14 August 2016

3.6 Measurements

Data was collected at different growth stages during the growing season and parameters measured included crop phenology (days to 50% emergence, flowering, podding, and physiological maturity), crop growth (plant height, canopy cover, number of primary and secondary branches), nodulation (no of nodules per plant, nodule fresh and dry weight), yield and yield components (number of pods per plant, number of seeds per pod and 100 seed weight (100-SW), and chlorophyll content.

3.6.1 Crop phenology

Crop phenology was assessed only in Thohoyandou by determining the number of days to crop emergence, number of days to 50% flowering, podding and physiological maturity. Days to crop emergence was determined by counting the number of days from the day of planting up to the day where 50% crop emergence was reached in all plots. The number of days to 50% flowering was determined from the day after 50% crop emergence up to the day in which 50% of the plants in each plot were flowering. The number of days to 50% podding was determined from the day after 50% crop emergence up to a day where 50% of the plants per plot were podding. The number of days to crop physiological maturity was determined from the day after 50% crop emergence up to a day where 50% of the plants in all plots have attained physiological maturity.

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3.6.2 Crop growth

Crop growth was only assessed in Thohoyandou by measuring plant height, number of primary and secondary branches, and canopy cover. Plant height was measured using a 30cm ruler during the early growth stage and then 5m measuring tape was used when the plant height increased to more than 30cm. Height was measured from the base of the plant to the apical bud of the plant and expressed in centimeters. The number of branches was determined by counting both primary and secondary plant branches from the middle row plants and the mean branches from middle row plants was recorded as the number of branches per plant. The measurements were taken weekly from 14 days after emergence until physiological maturity in each season. Canopy cover was determined by measuring the proportion of intercepted radiation using the AccuPAR, model LP-80 ceptometer (Decagon Devices Ltd., Pullman, USA). The ceptometer was placed perpendicular to the rows which measured photosynthetically active radiation (PAR) above and below the canopy (Figure 3.7). The PAR readings were then used to calculate the proportion of intercepted radiation. The measurements were taken at different plant growth stages during the growing season. The proportion of intercepted radiation was calculated using equation 1:

$$\alpha = 1 - (P_a / P_b) \qquad (1)$$

Where:

P_a is the photosynthetically active radiation (PAR) above the canopy

P_b is the photosynthetically active radiation (PAR) below the canopy

α is the proportion of the intercepted radiation

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Figure 3.7: Measuring intercepted radiation using ceptometer

3.6.3 Nodulation

Nodulation was determined at 50% flowering by randomly selecting 5 plants from the inner rows of each plot. The plants were carefully uprooted by excavating with a spade to a depth of 40cm. The roots were washed with tap water and the nodules attached to each root were removed (Figure 3.8). The number of nodules per root and nodule fresh weight were determined The root nodules were oven dried at a temperature of 65^{o}C for 48 hours and the nodule dry weight was determined.

Figure 3.8: Chickpea roots with nodules and isolated nodules in a petri-dish.

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3.6.4 Chlorophyll content

Chlorophyll content was only measured in Thohoyandou using two different methods; the extraction method and chlorophyll content meter at different plant growth stages (vegetative, flowering and podding) during 2015 and 2016 growing seasons. During winter 2015, only the extraction method was used but in 2016 both methods were used. Chlorophyll content meter (CCM-200 PLUS, Opti-Sciences, Tyngsboro, Massachusetts) was used to measure chlorophyll content in the field. The chlorophyll meter was calibrated before taking measurements and the leaves were inserted inside the chamber. The extraction method involved the use of leaf Samples that were collected from 5 plants in the middle rows from each plot. 0.5g of fresh leaf samples were taken and placed into 20ml vials containing 10ml of 95% ethanol. The samples were homogenized with tissue homogenizer and centrifuged for 10 minutes. The supernatant was separated and 0.5ml of it was mixed with 4.5ml of ethanol. The solution mixture was analyzed for chlorophyll a, b and total carotenoids content using a spectrophotometer at 470, 649, and 669 nm wavelengths (Lichtenthaler, 1987). Equation 2, 3 and 4 were used to calculate chlorophyll a, b and carotenoids content, respectively:

Chlorophyll a (C_a) = $12.25A_{664} - 5.19A_{649}$ (2)

Chlorophyll b (C_b) = $27.43A_{649} – 8.12A_{664}$ (3)

Total carotenoids = $1000A_{470} – 2.13C_a - 97.63C_b / 209$ (4)

Where A is the absorbance

3.6.5 Yield and yield components

Yield and yield components were determined after harvesting using all plants that were harvested from two inner rows in a sample area of 0.6m^2. The pods were separated from all the harvested plants from the sample area and the number of pods per plant were counted and then pod weight was also measured. Number of seeds per pod was determined by shelling the pods and counting the number of seeds found in each pod. The 100-SW was obtained by calculating the average of the 100 seed samples that were measured. The plants harvested from the sample area were oven dried in 65^oC for 48 hours to determine total above ground dry biomass. The above ground dry biomass and the grain yield were used to calculate harvest index.

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3.7 Weather data during winter 2015 and 2016 growing season

Daily weather data for winter 2015 and 2016 experiments in Thohoyandou was obtained from an automatic weather station located approximately 100 m from the experimental site. There was no weather station at the experimental site in Nelspruit hence weather data for the site is not included. Rainfall (mm), average daily temperatures (°C), solar radiation (MJ m^{-2} d^{-1}), and evapotranspiration (mm), relative humidity (%), were recorded each day during the experiments (Table 3.2). Summary of rainfall and temperature (maximum, minimum and average) during 2015 (Figure 3.9a) and 2016 (Figure 3.9b) growing season relative to the crop growth stages was also recorded.

Table 3.2 , Mean solar radiation, relative humidity and evapotranspiration in Thohoyandou during winter 2015 and 2016

Month/ Season	Solar radiation (MJ m^{-2} d^{-1})	Relative humidity (%)	Rainfall (mm)	Evapotranspiration (mm)
Winter 2015				
May	14.84	62.43	0	2.97
June	12.48	61.06	1.01	2.31
July	13.05	58.74	0.76	2.62
August	15.22	5.74	1.52	3.05
September	15.55	64.78	102.86	3.18
Mean/Total	14.23	50.55	106.15	14.13
Winter 2016				
April	15.26	68.01	0.75	3.08
May	12.85	71.45	55.11	2.39
June	11.95	68.32	7.61	2.14
July	11.59	62.69	9.65	2.22
August	16.94	54.46	3.81	3.34
Mean/Total	13.72	64.99	76.93	13.17

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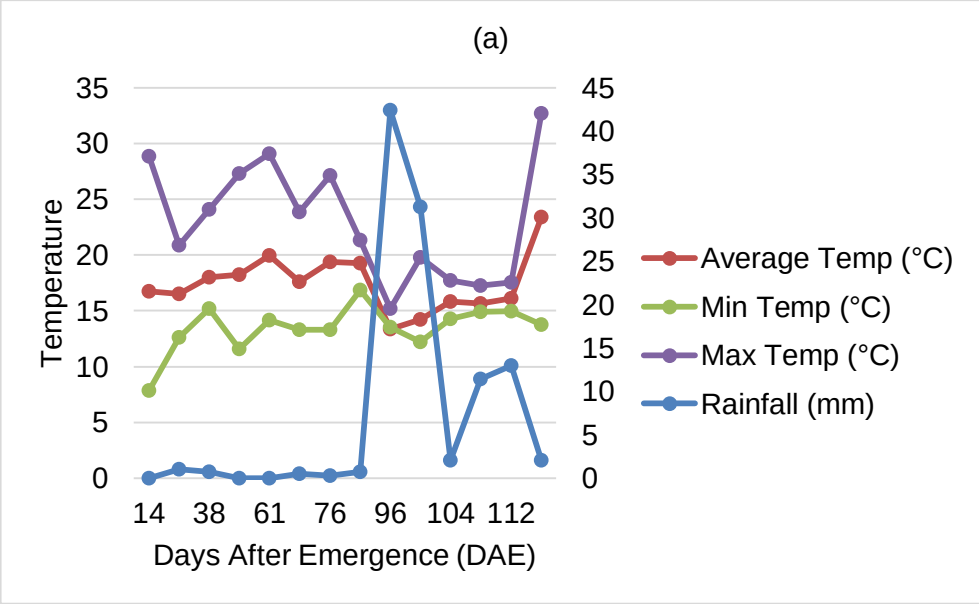

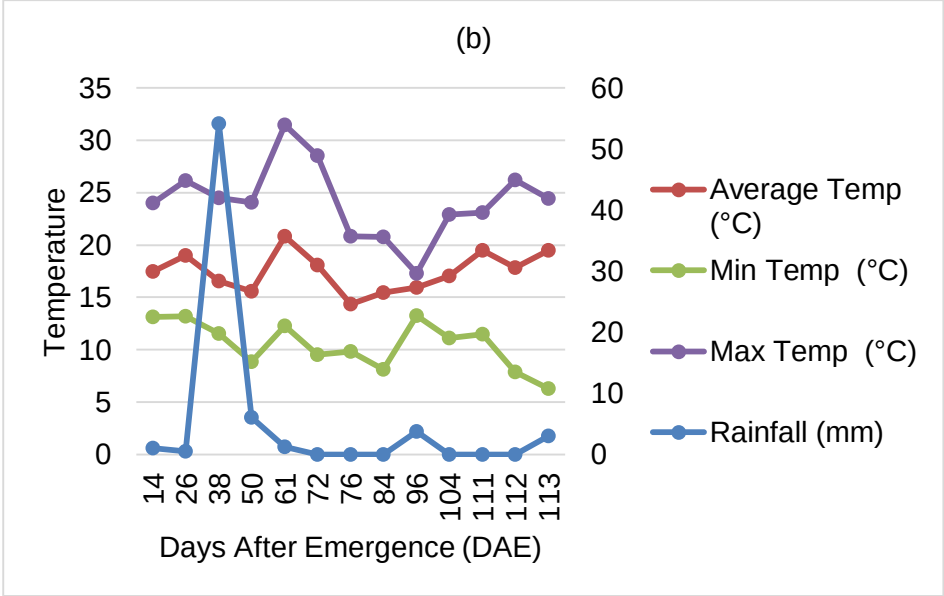

Figure 3.9: Rainfall and temperature during winter 2015 **(a)** and 2016 **(b)**

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3.9 Data analysis

Data obtained was subjected to analyses of variance (three factor ANOVA) using the general linear model (GLM) of Genstat software version 17. Significant difference between treatment means was compared using the standard error of difference (SED) of the means at 5% level. Correlation analyses were conducted to assess the relationship between the various parameters measured. Bio-edit and Staden version 4 were used to align the sequences. The aligned sequences were subjected to BLASTn from National Centre for Biotechnology Information (NCBI) and 98-100% similarities were considered a match.

Table 3.3, ANOVA Table

Source of variation	Degrees of freedom
Replication	r-1= 2
Genotype (G)	a-1= 2
Biochar (B)	b-1= 2
Rhizobium(R)	c-1= 1
G X B	(a-1)(b-1) = 4
G X R	(a-1)(c-1) = 2
B X R	(b-1)(c-1) = 2
G X B X R	(a-1)(b-1)(c-1) = 4
Error	(r-1)(abc-1) = 34
Total	rabc-1 = 53

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CHAPTER 4. EFFECT OF BIOCHAR AND RHIZOBIUM INOCULATION ON SOIL pH

4.1 Introduction

Soil pH is one of the factors affecting soil microbial population and their survival. Most soils in semi-arid areas generally have low population of rhizobia which may be able to form nodules on legumes and hence improve biological nitrogen fixation (Gul *et al*., 2014). Biochar has been used as a relatively affordable input for improving soil pH and has shown a great potential to substitute lime. Biochar additions provide both macro and micronutrients such as N, P, K, Ca, and Mg to the soil (Macdonald *et al*., 2014). Long term cultivation without the addition of external inputs, particularly by resource-poor smallholder farmers, contributes to alterations in soil pH, reduced soil fertility and low crop yields especially in arid and semi-arid areas (Jien and Wang, 2013). The use of lime to adjust soil pH is a common management practice for commercial farmers, however it requires regular application (Glaser *et al.,* 2002). The use of recalcitrant organic materials, such as biochar, as soil amendment may not only improve soil pH but also mitigate climate change through carbon sequestration and improve soil fertility and crop yield as well (Glaser *et al.,* 2002. Lehmann, 2007; Jeffery *et al*. 2011). These benefits makes biochar a suitable input for sustaining the environment while improving crop productivity.

Biochar is a carbonaceous, recalcitrant by-product of the pyrolysis process, produced through the thermal decomposition of organic materials in low or the absence of oxygen at a certain temperature and pressure (Verheijen *et al.,* 2010; Jeffery *et al.,* 2011). Biochar application is considered as a crucial tool for long-term carbon sequestration while simultaneously improving soil properties and functions (Islam *et al.,* 2014). Several incubation and greenhouse studies have shown remarkable results on the potential effect of biochar on soil chemical (Atkinson *et al.,* 2010; Lu *et al.*, 2014) and physical (Busscher *et al.,* 2011; Herath *et al.,* 2013) properties. However, these benefits in the soil depend on the feedstock type, pyrolysis condition (temperature and residence time) and also the type of soil the biochar applied to (Jeffery *et al*., 2011).

Inoculation of legumes seeds with synthetic rhizobium inoculants is one of the common practices which improves growth and yield of legumes especially in soils lacking native rhizobia (Khattak *et al*., 2006). Legumes have a special relationship with rhizobium bacteria which result in formation of nodules for biological nitrogen fixation process. Biological nitrogen fixation (BNF) provides appreciable amounts of free of cost nitrogen that can be used by the same crop or subsequent ones (Khattak *et al*., 2006). In low pH soils, the rhizobium populations are relatively low and

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rhizobium inoculation will aid in increasing the populations. Rhizobium inoculation also improves the microbial activity especially of rhizobia species. This enhances the competitiveness and effectiveness of rhizobia on the host plant relative to other competitors in the rhizosphere and ensures successful colonization (Laureen *et al*., 2016). Rhizobia secrets a variety of compounds in the soil which alters soil chemistry and provides nutrient source for other microbes in the rhizosphere. These compounds can either increase or decrease the soil pH depending on the soil properties and plant-microbe interactions (Laureen *et al*., 2016).

It is clear from the foregoing that biochar has more benefits on soil properties, and the effects may vary with soil type, feedstock type and the environment. However, most of previous studies have mainly been conducted in controlled environment on sandy textured soils (Atkinson *et al*., 2010; Lu *et al*., 2014; Herath *et al*., 2013). There are few studies in literature that shows the effect of biochar application on soil chemical properties under field conditions on both sandy and clay textured soils. More recently, Lusiba *et al*. (2017) at the site of the current study reported that biochar has a potential to increase soil pH of both clay and loamy sand soils in the North Eastern part of South Africa. However, there was no rhizobium inoculation used in the study. Therefore, more research is needed to assess the interactive effect of biochar and rhizobium inoculation on soil pH under field conditions in different soil types and environmental conditions. The main objective of this study was to assess the effect of biochar and rhizobium inoculation on soil pH of two contrasting soil types under field conditions.

4.2 Materials and methods

Full experimental details are given in chapter 3, but a brief summary is described below. Field experiments were conducted at two sites with contrasting soil types in winter 2015 (experiment I) and winter 2016 (experiment II). The experimental sites were the University of Venda's School of Agriculture, Experimental Farm, in Limpopo Province, and the University of Mpumalanga Experimental Farm, in Nelspruit, Mpumalanga Province, South Africa. The experiments consisted of three biochar rates (0, 10 and 20 t ha^{-1}), two rhizobium inoculation rates (inoculated and uninoculated) and three chickpea genotypes (ACC #4, 5 and 6) in a factorial combination arranged in a randomised complete block design with three replications. Soil samples were collected before sowing and after harvest between 15-20 cm soil depths to determine soil pH. Soil pH was measured with a pH meter with the ratio of 1:2 (soil to water). Biochar was applied according to the treatments a week before sowing. Data obtained was subjected to ANOVA using

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the general linear model of Genstat 17th Edition. Significant difference between treatments means were compared using standard error of difference of means at 5% level.

4.3 Results

4.3.1 Soil pH

The effect of biochar and rhizobium inoculation on soil pH was significant (P< 0.001) in both 2015 and 2016 in Thohoyandou (Table 4.1). Soil pH increased with biochar application rates and rhizobium inoculation. Application of biochar increased soil pH by 11% (0.70 pH units) at 10 and 20 t ha^{-1} compared to 0 t ha^{-1} in 2015 and 2016, respectively. Rhizobium inoculation increased soil pH by 3.3% (0.22 pH units) compared to uninoculated plots in 2015 and 2016. The interactive effect of biochar and rhizobium inoculation on soil pH was significant in both seasons (Figure 4.1a &b). Although inoculation increased the soil pH at all biochar rates, the increase was greater (6%) at 20 t ha^{-1} compared with 10 t ha^{-1} (1.4%) and 0 t ha^{-1} (2.4%).

Similarly, the effect of biochar and rhizobium inoculation on soil pH was significant (P< 0.001) in both 2015 and 2016 in Nelspruit (Table 4.1). Biochar application increased soil pH by 12% (10 t ha^{-1}) and 19% (20 t ha^{-1}) in 2015, and 13% (t ha^{-1}) and 21% (20 t ha^{-1}) in 2016. Rhizobium inoculation increased soil pH by 7.3 and 7.9% in 2015 and 2016, respectively. The interactive effect of biochar and rhizobium inoculation was significant on soil pH in both seasons. Although rhizobium inoculation increased soil pH at all biochar rates, the increase was greater (14%) at 0 t ha^{-1} compared with 10 t ha^{-1} (9%) and 20 t ha^{-1} (Figure 4.2a &b).

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Table 4.1: Effect of biochar and rhizobium inoculation on soil pH during winter 2015 and 2016 in Thohoyandou and Nelspruit.

Treatments	Location			
	Thohoyandou (clay soil)		Nelspruit (loamy sand soil)	
	Experiment I	Experiment II	Experiment I	Experiment II
Biochar (t ha^{-1})				
0	6.36^{a}	6.33^{a}	6.30^{a}	5.54^{a}
10	7.06^{b}	7.03^{b}	7.07^{b}	6.27^{b}
20	7.08^{b}	7.05^{b}	7.50^{c}	6.70^{c}
SED	0.03	0.031	0.035	0.039
Rhizobium				
Inoculated	6.94^{b}	6.91^{b}	7.20^{b}	6.40^{b}
Uninoculated	6.72^{a}	6.69^{a}	6.71^{a}	5.93^{a}
SED	0.02	0.026	0.029	0.032
F-Test probability				
Biochar (B)	***	***	***	***
Rhizobium (R)	***	***	***	***
B*R	***	***	***	***
CV (%)	1.4	1.6	1.5	1.9

Means followed by the same letter are not significantly different. *** (P<0.001), ** (P< 0.01), * (P<0.05), CV (coefficient of variation)

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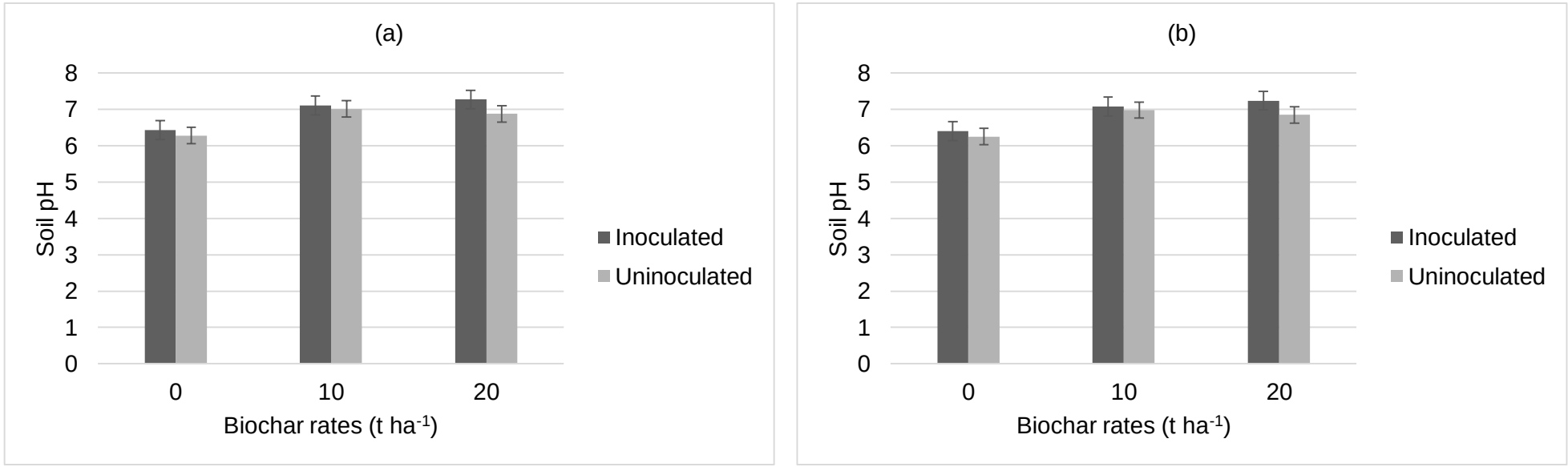

Figure 4.1: Effect of biochar and rhizobium inoculation on soil pH during winter 2015 **(a)** and 2016 **(b)** in Thohoyandou, Limpopo Province.

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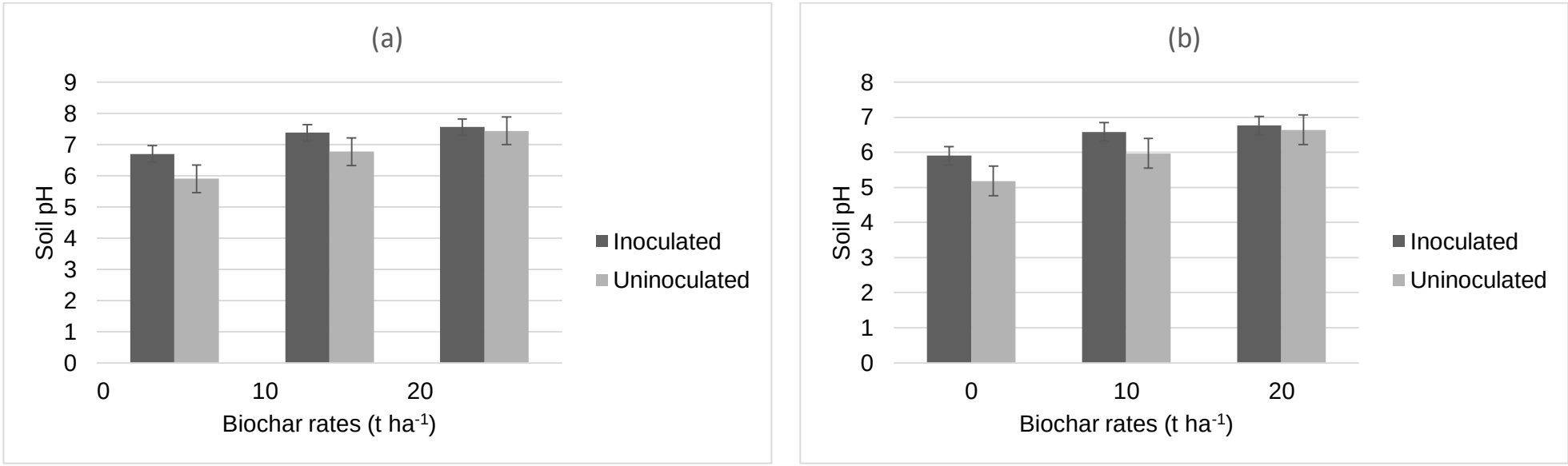

Figure 4.2: Effect of biochar and rhizobium inoculation on soil pH during winter 2015 **(a)** and 2016 **(b)** in Nelspruit, Mpumalanga Province

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4.4 Discussion

Biochar and rhizobium inoculation increased soil pH in clay and loamy sand soils in both seasons. Soil pH generally increased with biochar application rates. In loamy sand soils (Nelspruit), soil pH at 20 t ha^{-1} was significantly greater than at 10 and 0 t ha^{-1}. However, in clay soils (Thohoyandou) there was no difference in soil pH between 10 and 20 t ha^{-1} in both seasons. These results indicate that an increase in biochar rates above 10 t ha^{-1} in clay soils may not be effective in increasing soil pH but the trend may be different in loamy sand soils where soil pH was greater at 20 t ha^{-1} biochar rate. The results of the current study suggest that the liming potential of wood biochar does not only depend on the application rate but also soil type. The effect of biochar on soil pH could be attributed to the high ash content and pH of the biochar used in the current study (Appendix 1), and the high surface area and porous nature of the biochar that increases the cation exchange capacity of the soil thus allowing iron and aluminum to bind with the exchange site of the soil (Nigussie *et al*., 2012). Comparable results were reported by a number of researchers who indicated that biochar application reduced iron and aluminum toxicity (Nigussie *et al*., 2012, Saxena *et al*., 2013). Similarly, Lehmann *et al.* (2003) reported that Andisol (sandy soil) had higher pH value than the ferrosol (clay soil) after charcoal addition at 67.6 and 135 t ha^{-1} in Brazil. Most recently, Lusiba *et al*. (2017) reported an increase in soil pH, albeit non-significantly with application of biochar at the site of the current study.

Soil pH increased with rhizobium inoculation in both soils and seasons. The response of soil pH to rhizobium inoculation may be attributed partly to enzyme activity and release of biomolecules by rhizobium in the soil. Laureen *et al*. (2016) reported that microbes in the soil release a variety of compounds to the rhizosphere which alter soil chemistry. Also, rhizosphere as a competitive site for microbes, microbes tend to release secondary metabolites to outcompete competitors that occupy similar niches. However, the effect of these biomolecules on soil pH is not well understood and require further investigation.

The interactive effect of biochar and rhizobium inoculation on soil pH was significant in both sites and seasons. Rhizobium inoculation increased soil pH at all biochar rates but greater increase was observed at 10 t ha^{-1} in Thohoyandou and 20 t ha^{-1} in Nelspruit, both with inoculation. Biochar application creates suitable condition for microorganisms such as bacteria and thus improving their survival and reproduction (Macdonald *et al*., 2014). The effect of biochar and rhizobium inoculation on soil pH varied with location with more effects in Nelspruit (loamy sand) than

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Thohoyandou (clay). These effects may also be attributed to the variation in cropping history of the experimental sites. The experimental site in Nelspruit was previously occupied by grass species which may lower soil pH while there was cultivation of seasonal vegetables (cabbage and spinach) in the experimental site in Thohoyandou. Effects of biochar are more in acidic soils compared with neutral and alkaline soils (Nigussie *et al*., 2012; Macdonald *et al*., 2014).

4.5 Conclusions

The application of biochar and rhizobium inoculation increased soil pH of soils in Limpopo and Mpumalanga provinces. The increase in soil pH due to biochar application was probably due to the liming effect of the biochar used in the current study. Rhizobium inoculation increased soil pH and was attributed to the release of bacterial biomolecules to the soil. The results of the study showed that the effects of biochar and rhizobium inoculation were more pronounced in loamy sand than clay soils. However, more studies should be done to assess the effect of biochar and rhizobium inoculation on soil pH over time in different soil types.

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CHAPTER 5. IDENTIFICATION OF NATIVE RHIZOBIUM STRAINS IN THE SOIL

5.1 Introduction

Rhizobia is a very diverse group of bacteria found in the soil and have a number of strains which are beneficial. The ability of these species to adapt and survive in different environments varies and hence some of these species are location specific. The uniqueness of this genus is because of its ability to form a symbiotic relationship with legumes for biological nitrogen fixation. However, the effectiveness and competitiveness of these species to establish a mutual relationship with legumes may be affected by a number of factors including soil moisture, pH, and temperature (Khaitov *et al*., 2016). These factors mainly affect the survival and reproduction of the species and hence the population. Rhizobia may be introduced in agricultural through commercial inoculants. Other rhizobia species may be native to the soil, however, through continuous cultivation of crops with the use of both herbicides and pesticides to control weeds and pests, may decrease populations of rhizobia and hence result to poor productivity of legumes.

Chickpea is a newly introduced legume crop in South. The crop has shown a huge potential in the dry environments since its introduction over 10 years ago (Thangwana and Ogola, 2012). However, lack of nodulation without inoculation and poor inoculation has been reported in the region was and attributed to: (1) lack of native rhizobia, (2) Insufficient populations of effective strains and (3) low soil pH (Ogola, 2015). Similarly, Khaitov *et al*. (2016) reported that lack of cultivation of chickpea in a region may lead to lack of nodulation due to the absence of suitable and compatible native rhizobium strains. The availability of adequate populations of native rhizobia are important for the production of legumes especially where commercial inoculants are not used (Vessey and Chemining'wa, 2006). Soils in semi-arid regions may be deficient in specific and effective rhizobium strain nodulating chickpea due to stress conditions (Romdhane *et al*., 2009). Therefore, it is important to assess availability of native rhizobia in the North Eastern region of South Africa in order to improve the productivity of chickpea. The objective of the study was to assess the availability of native rhizobia in the soils collected from agricultural fields in Limpopo (Thohoyandou) and Mpumalanga (Nelspruit) Provinces.

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5.2 Materials and methods

Full experimental details are given in chapter 3, but a brief summary is described below. Soil samples were collected from the experimental sites at the University of Venda's School of Agriculture, Experimental Farm in Limpopo Province, and at the University of Mpumalanga Experimental Farm in Nelspruit, Mpumalanga Province, South Africa before planting. The soil samples were kept in the freezer until the analysis. 1g of soil was suspended into 10ml distilled water and 20µL of the mixture was in a PDA media and incubated at 28ºC until colonies appeared. The individual colonies were sub-cultured twice in YEMA and incubated until colonies appeared. The cultures were stored in a fridge until further analysis. Before DNA extraction, the cultures were sub-cultured. The DNA extraction was done using the CTAB method as described by Moller *et al*. (1992). The successful PCR reactions were sent for PCR clean up and sequencing to a sequencing facility in Stellenbosch. Data obtained from the sequences was edited using Staden and Bioedit software then subjected to blast searches from NCBI to identify the strains.

5.3 Results

The results from molecular identification of the strains isolated from soils collected from agricultural fields in Thohoyandou and Limpopo showed lack of native rhizobia (Table 5.1). The species identified were *Burkholderia cenocepacia, Klebsiella variicola, Bacillus subtilis* and *Ochrobactrum spp* with 98 to 100% similarities according to the BLASTn search from NCBI. The *B. subtilis and Klebsiella variicola* (98%) were isolated from soils in Thohoyandou and *B. cenocepacia* from nodules. Also, Ochrobactrum spp was isolated from soils in Nelspruit and *B. cenocepacia* (98%) from nodules. The cropping history on the site shows that there was no cultivation of chickpea before.

Table 5.1, List of identified strains from Thohoyandou and Nelspruit using 16s gene segment.

Site	Source	Identified strain	Cropping history
Thohoyandou	Soil	*Bacillus subtilis* and *Klebsiella variicola*	Cabbage, chickpea
	Nodules	*Burkholderia cenocepacia*	
Nelspruit	Soil	*Ochrobactrum spp*	Fallow, chickpea
	Nodules	*Burkholderia cenocepacia*	

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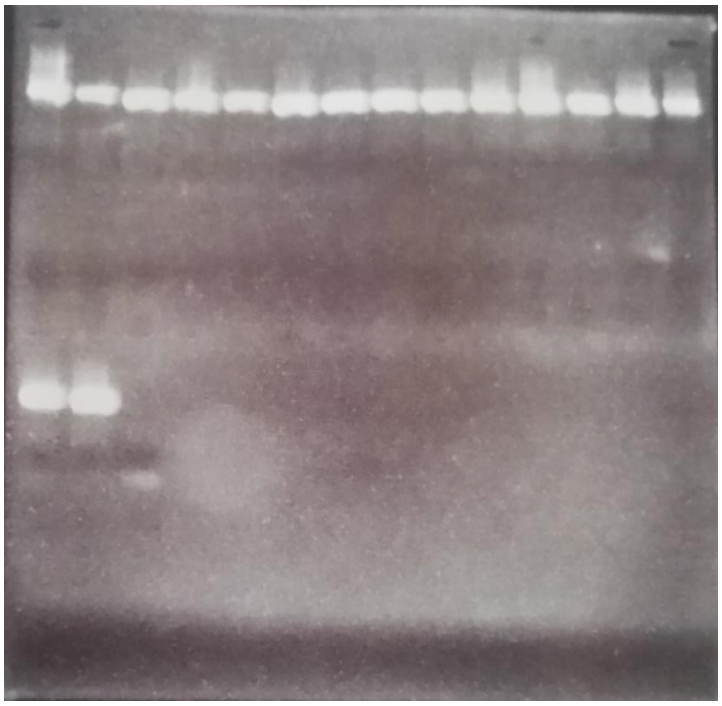

Figure 5.1. PCR image of the 16S rRNA gene bands for isolated strains.

5.4 Discussion

Burkholderia cenocepacia

Burkholderia are a group of bacteria which are known to be human and animal pathogens and occupy diverse ecological niches (Ngom *et al*., 2004). There are currently more than 50 identified *Burkholderia* species with only few species capable to nodulate legumes. *Burkholderia* species are mostly used in bioremediation, plant growth promotion and nutrient cycling. *B. cenocepacia* are known to solubilize organic and inorganic phosphate and make it available for uptake by plants (Zekic *et al*., 2017). However, there are limitations and restrictions in the use of *B. cenocepacia* in agriculture due to lack of molecular distinction between plant growth promoting rhizobacteria and human pathogenic strains (Zekic *et al*., 2017). The isolation of *B. cenocepacia* from chickpea nodules showed that the improved nodulation observed was partly attributed to the interactions between the resident bacteria and the commercial inoculant used. The results also suggest that the isolated *Burkholderia* strain may be an associate biological nitrogen fixer which may not be able to induce nodulation in chickpea but their ability and efficiency of nitrogen fixation

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may partly depend on interactions which rhizobia species. Although *B. cenocepacia* was isolated from chickpea nodules from the current study, there is scanty information in literature on the role of these strain in chickpea nodulation.

Bacillus subtilis

Bacillus are a group of bacteria which comprise a wide range of species found in different ecological ranges. These species are widely used in agriculture as plant growth promotion rhizobacteria and nutrient cycling. *B. subtilis* are known for their role as a potassium solubilizing bacteria. These species solubilize potassium and make it available for plant uptake and have been isolated from non-legumes such as citrus species (Lyer and Rajkumar, 2017). Some strains of *Bacillus* are known to be nitrogen fixing. The dual inoculation of chickpea with *B. subtilis* and *Rhizobium* increased nodulation and yield (Elkoca *et al*., 2007). These reports are in agreement with the results from the current study, that showed high nodulation which may have been attributed to the interactions of the resident bacteria (*Bacillus*) and the commercial inoculant.

Klebsiella variicola

Klebsiella species are a small group of endophytic bacteria which are distributed in different ecological origins. These species may be found within plants without causing any diseases. Their presence within the plant or rhizosphere do not harbor the role of nitrogen fixing bacteria. *K. variicola* was isolated from non-legume plants such as sweet potato, bananas, wheat and rice (Lyer and Rajkumar, 2017). Chickpea nodulating strains are often specific and *Klebsiella variicola* may be available in areas where chickpea is native in both natural and agricultural settings. However, the presence of this strain in the agricultural fields in Thohoyandou was probably due to the presence of banana plantations which are the host for these bacteria. Although, *K. variicola* was isolated in the soils, there is no evidence in literature that may be used to associate *K. variicola* with chickpea nodulation but may contribute in plant growth promotion.

Ochrobactrum spp

Ochrobactrum are a group of bacteria which were isolated from different ecological origins such as clinical and environmental sources (soil and water) (Ngom *et al*., 2004). Some of the species in this genus are known to nodulate legumes. The nitrogen fixing *Ochrobactrum* from acacia and lupine root nodules were isolated (Ngom *et al*., 2004; Trujillo *et al*., 2005). Also, some species were isolated from soils and nodules of a number of legumes including field peas, beans acacia trees and non-legumes such as sugarcane and bananas (Ngom *et al*., 2004). The ability of

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Ochrobactrum species to fix nitrogen is due to presence of nodulating genes such *nod* and *nif* (Balachandar *et al*., 2007). The isolation of *Ochrobactrum spp* from soils in Nelspruit may suggest that the high nodulation observed in Nelspruit was partly attributed to the interactions between the resident bacteria and commercial inoculant used. However, there are no reports which associates chickpea nodulation with *Ochrobactrum spp*.

Role of isolated strains in agriculture

The role of the isolated strains in agriculture is their involvement in plant growth promotion including: nitrogen fixation, nodulation, sequestration of iron, phosphate and potassium solubilizing and production of phytohormones. The isolated strains are known to be associate biological nitrogen fixers. Their ability and efficiency of nitrogen fixation may partly depend on interactions which rhizobia species. There are no reports associating chickpea nodulation with the isolated species. However, they have been found in other legumes such as lupine, peas and beans. The unavailability of chickpea nodulating strains and other rhizobia in these soils may also be due to the low soil pH since it affects both the survival and reproduction (Khattak *et al*., 2006; Gul *et al*., 2014). The availability of strains which are not symbiotic associates with chickpea may not induce nodulation hence the lack of nodulation in these regions. (Solaiman *et al*., 2010; Gul *et al*., 2014; Vandamme *et al*., 2002). The lack of native rhizobia in the soils may probably be the reason for lack of chickpea nodulation in these regions. Therefore, the use of commercial inoculants may be suitable to introduce the rhizobia in the soils and also improve crop productivity.

5.5 Conclusion

The results of the study revealed that there is lack of native rhizobia suitable for chickpea nodulation in the soils collected from agricultural fields in Thohoyandou and Nelspruit. Identified strains were *Burkholderia cenocepacia*, *Klebsiella variicola*, *Bacillus subtilis* and *Ochrobactrum spp* which are not directly associated with chickpea nodulation. Therefore, this explains the lack of chickpea nodulation in these regions.

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CHAPTER 6. BIOCHAR AND RHIZOBIUM INOCULATION IMPROVES ROOT NODULATION OF THREE CHICKPEA (Cicer arietinum L.) GENOTYPES.

6.1 Introduction

Chickpea is an important pulse crop ranking second after dry bean (*Phaseolus vulgaris* L.) and has a high nutritive value (FAO, 2015). It is an important source of protein for human diet. It can also be used for animal feed and medicinal purposes. Also, it plays a vital role in the sustainability of cropping systems (rotations and intercropping) due to its ability to fix atmospheric nitrogen. It has a specific symbiotic relationship with a distinct group of rhizobia needed for the formation of root nodules and successful biological nitrogen fixation (BNF). This contributes to reducing the cost for production inputs such as N fertilizers. However, availability of adequate populations of suitable rhizobium strains and their survival affect both nodulation and BNF (Khattak *et al*., 2006; Solaiman *et al*., 2010). Also soil type and crop genotype contribute to the competitiveness of rhizobia in formation of nodules. Therefore, commercial inoculants may be useful in ensuring root nodulation and BNF especially in semi-arid regions where the soils are poor with little or total absence of native rhizobia. Artificial seed inoculation with suitable rhizobia in soils lacking native rhizobia improves nodulation and reduce the uncertainty of root nodulation failure (Gul *et al*., 2014).

Biochar has both direct and indirect effects on soil properties and influences the productivity of soils. Improved biological properties of soils has been reported as one of the advantages of using biochar as a soil amendment (Macdonald *et al*., 2014). Increased microbial population, enzyme activity and survival has been reported in biochar amended soils (Khaitov *et al*., 2016 Yusif *et al.,* 2016; Carter *et al.,* 2013) probably due to the effect of biochar on soil physical and chemical properties. Biochar application improve nodulation probably due its ability to alter soil pH into a suitable range for favorable growth of microorganisms (Macdonald *et al*., 2014). Increased nodulation of legumes in biochar amended soils have been reported on soybean, chickpea and groundnut (Khaitov *et al*., 2016 Yusif *et al.,* 2016). Poor nodulation with rhizobium inoculation and lack of nodulation without inoculation was reported at the current site of study and was partly attributed to lack of infective native rhizobia and low soil pH in the soil (Ogola, 2015). Furthermore, lack of chickpea nodulation with biochar application was also reported at the current site of study (Lusiba, 2015). However, there is limited information in literature on the interactive effect of biochar and rhizobium inoculation on chickpea nodulation. Therefore, there is a need to assess the interactive effect of biochar and rhizobium inoculation on chickpea nodulation in different

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representative locations of dry environments in North Eastern part of South Africa. The study assessed the interactive effect of biochar and rhizobium inoculation on root nodulation of three different chickpea genotypes.

6.2 Materials and methods

Full experimental details are given in chapter 3, but a brief summary is described below. Two field experiments were conducted in winter 2015 (experiment I) and 2016 (experiment II) in Thohoyandou and Nelspruit. The experiments were conducted at the University of Venda's Experimental Farm, in Thohoyandou, Limpopo Province, and University of Mpumalanga's Experimental Farm, in Nelspruit, Mpumalanga Province, South Africa. The experiment consisted of three levels of biochar (0, 10, and 20 t ha^{-1}), two rhizobium rates (inoculated and uninoculated) and three chickpea genotypes (ACC #4, 5 and 6) in a factorial combination arranged in a randomised complete block design with three replications. Biochar was applied according to treatments a week before planting. Seeds were inoculated at planting at a rate recommended by the manufacturer. Uninoculated seeds were planted first to avoid contamination. 4 plants were destructively harvested at flowering from each plot for nodulation assessment. The plants were dug using spade and the roots washed with tap water. Root nodules were separated from the roots and counted to determine number of nodules per plant. The nodules were oven dried for 48 hours at 65ºC and nodule dry weight was measured. The data was subjected to ANOVA using the general linear model of Genstat 17th Edition. Significant differences between treatment means were compared using standard error of difference (SED) at 5% level.

6.3 Results

The effect of biochar and rhizobium inoculation on number of nodules and nodule dry weight per plant was significant during winter 2015 and 2016 seasons in Thohoyandou (Limpopo Province) (Table 6.1). The number of nodules and nodule dry weight increased with biochar application rates and rhizobium inoculation in both seasons. Number of nodules increased with biochar application rates by 89% (10 t ha^{-1}) and 163% (20 t ha^{-1}) in 2015, and 70% (10 t ha^{-1}) and 116% (20 t ha^{-1}) in 2016. Similarly, nodule dry weight increased with biochar application rates by 74% (10 t ha^{-1}) and 133% (20 t ha^{-1}) in 2015, and 121% (10 t ha^{-1}) and 293% (20 t ha^{-1}) in 2016. Rhizobium inoculation increased number of nodules and nodule dry weight by 100% in 2015 and 2016. The effect of genotype on the number of nodules and nodule dry weight was significant in 2015 only. Greater number of nodules and nodule dry weight were observed on ACC #6.

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Genotype ACC #6 had greater number of nodules (19 and 22%) compared to ACC #4 and #5, respectively. Similarly, nodule dry weight was greater on ACC #6 (27 and 25%) compared to ACC #4 and 5, respectively. The interactive effect of biochar and rhizobium inoculation on the number of nodules and nodule dry weight was significant in 2015 and 2016. Number of nodules and nodule dry weight increased with increase in biochar application rates and rhizobium inoculation. Although rhizobium inoculation increased the number of nodules at all biochar application rates, the increase was greater (88 and 70%) at 10 t ha^{-1} and 20 t ha^{-1} (163 and 116%) in 2015 and 2016, respectively **(**Figure 6.1a & b). Rhizobium inoculation also increased nodule dry weight at all biochar rates with greater increase at 20 t ha^{-1} (145 and 299%) and 10 t ha^{-1} (100 and 121%) in 2015 and 2016, respectively (Figure 6.2a & b). In general, higher number of nodules and nodule dry weight was obtained during winter 2016 compared to 2015 growing season.

In Nelspruit (Mpumalanga Province), the effect of biochar, genotype and rhizobium inoculation on number of nodules and nodule dry weight per plant was significant in 2015 and 2016 (Table 6.1). Biochar application rates increase number of nodules 45% (10 t ha^{-1}) and 91% (20 t ha^{-1}) in 2015, and 33% (10 t ha^{-1}) and 66% (20 t ha^{-1}) in 2016 compared with 0 t ha^{-1}. Similarly, nodule dry weight increased by 75% (10 t ha^{-1}) and 200% (20 t ha^{-1}) in 2015, and 6% (10 t ha^{-1}) and 17% (20 t ha^{-1}) in 2016. Rhizobium inoculation increased the number of nodules and nodule dry weight.

The number of nodules varied amongst the genotypes, with ACC #5 having greater number of nodules and nodule dry weight compared to ACC #4 and 6 in both seasons. The difference between genotype ACC #5 and 4 was 34%, 63% between ACC #5 and 6 and 43% between ACC #4 and 6 in winter 2015. During 2016 season, the difference in the number of nodules was 33% between ACC #5 and 5% between ACC #5 and 6, and 50% between ACC #4 and 6. Similar trend was also observed on nodule dry weight. The interactive effect of genotype and rhizobium inoculation on the number of nodules was significant in both seasons (Figure 6.3a & b). Rhizobium inoculation increased the number of nodules in all genotypes, however greater increase was on ACC #6 (91 and 32%) in 2015 and (95 and 28%) in 2016 compared with ACC #4 and 5, respectively. The interactive effect of biochar application rates and rhizobium inoculation on nodule dry weight was significant in both seasons (Figure 6.4a & b). Nodule dry weight increased with biochar and rhizobium inoculation rates with greater nodule dry weight observed at 20 t ha^{-1} compared to 0 and 10 t ha^{-1}. There was a slight increase in the number of nodules and nodule dry weight in 2016 compared to 2015.

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The effects of biochar, genotype and rhizobium inoculation on number of nodules and nodule dry weight per plant were greater in Thohoyandou compared to Nelspruit and also in 2016 compared to 2015 growing season.

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Table 6.1, Effect of biochar, genotype and rhizobium inoculation on chickpea nodulation during winter 2015 and 2016 in Mpumalanga (Nelspruit) and Limpopo (Thohoyandou) Provinces.

	LOCATION							
	THOHOYANDOU (clay)				NELSPRUIT (loamy sand)			
SEASON/YEAR	2015		2016		2015		2016	
Treatments	No of nodules per plant	Nodule dry weight (g)/plant	No of nodules per plant	Nodule dry weight (g)/plant	No of nodule per plant	Nodule dry weight (g)/plant	No of nodules per plant	Nodule dry weight (g)/plant
Biochar (t ha^{-1})								
0	3.00^{a}	0.0172^{a}	4.61^{a}	0.014^{a}	2.56^{a}	0.04^{a}	3.01^{a}	0.06^{a}
10	5.67^{b}	0.03^{b}	7.83^{b}	0.031^{b}	3.72^{b}	0.07^{b}	4.00^{a}	0.07^{b}
20	7.90^{c}	0.04^{c}	9.94^{c}	0.055^{c}	4.89^{c}	0.12^{c}	5.00^{b}	0.12^{c}
SED	0.63	0.005	0.4	0.005	1.05	0.02	1.00	0.03
Genotype								
ACC #4	5.22^{a}	0.0284^{a}	7.44	0.03	3.61^{b}	0.08^{b}	4.00^{b}	0.079^{b}
ACC #5	5.11^{a}	0.0289^{a}	7.22	0.031	5.50^{c}	0.10^{c}	6.00^{c}	0.11^{c}
ACC #6	6.22^{b}	0.0361^{b}	7.72	0.039	2.06^{a}	0.06^{a}	2.00^{a}	0.05^{a}
SED	0.77	0.006	0.4	0.005	1.05	0.02	1.00	0.025
Rhizobium								
Inoculated	11.04^{b}	0.062^{b}	14.93^{b}	0.067^{b}	0.74^{b}	0.15^{b}	8.02^{b}	0.17^{b}
Uninoculated	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
SED	0.77	0.006	0.4	0.004	0.86	0.02	0.8	0.02
F-Test probability								
Biochar (B)	**	**	***	***	*	*	*	*
Genotype (G)	**	**	ns	ns	**	**	**	**
Rhizobium (R)	***	***	***	***	***	***	***	***
B*G	ns	ns	ns	ns	ns	ns	ns	ns
B*R	**	**	***	***	ns	**	ns	*
G*R	ns	ns	ns	ns	**	ns	**	ns
B*G*R	ns	ns	ns	ns	ns	ns	ns	ns
CV (%)	4.8	6.1	4.2	10.9	28	7.2	18.1	4.6

Means followed by the same letter are not significantly different, *** (P<0.001),** (P<0.01) and *(P<0.05), CV (coefficient of variation)

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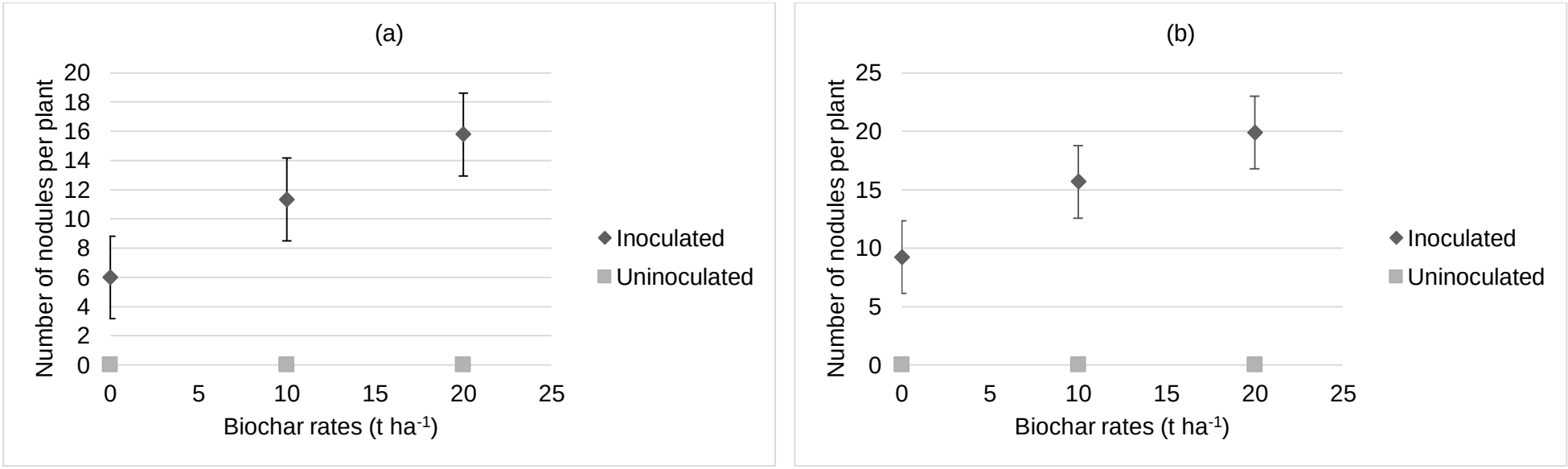

Figure 6.1: Effect of biochar and rhizobium inoculation on the number of nodules per plant in winter 2015 **(a)** and 2016 **(b)** in Thohoyandou (Limpopo Province).

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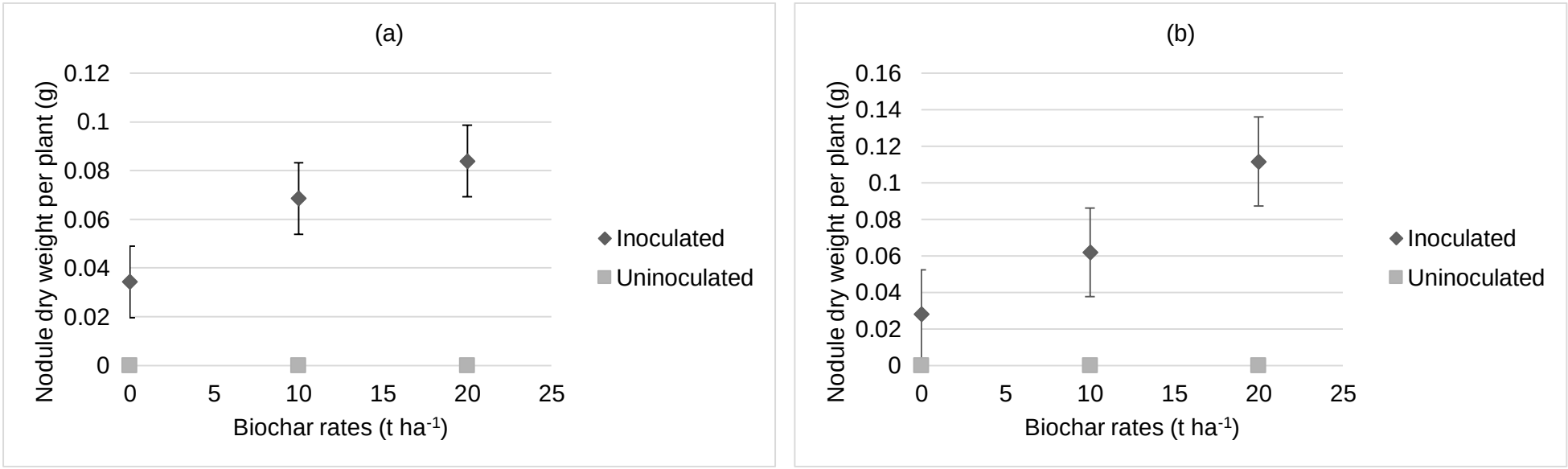

Figure 6.2: Effect of biochar and rhizobium inoculation on the nodule dry weight per plant in winter 2015 **(a)** and 2016 **(b)** in Thohoyandou (Limpopo Province).

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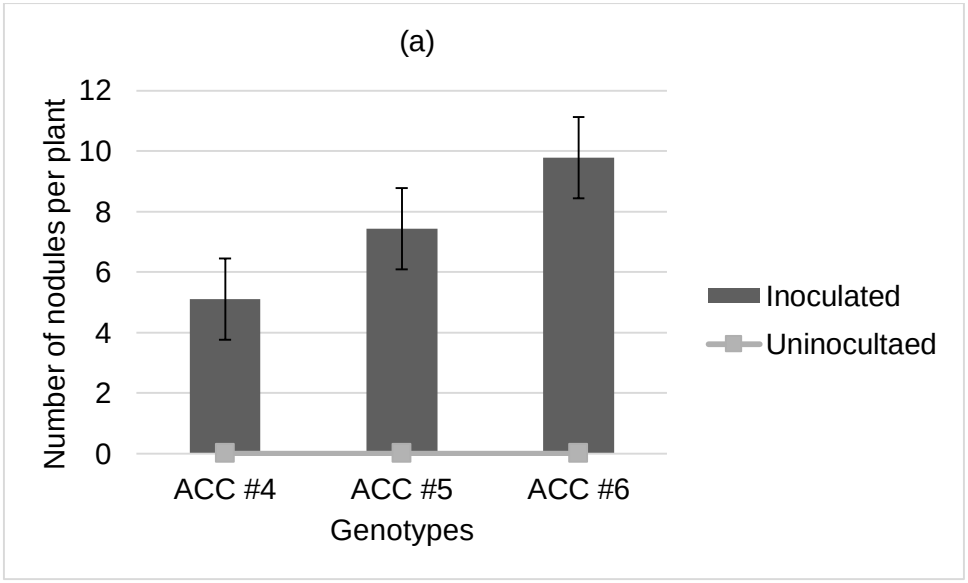

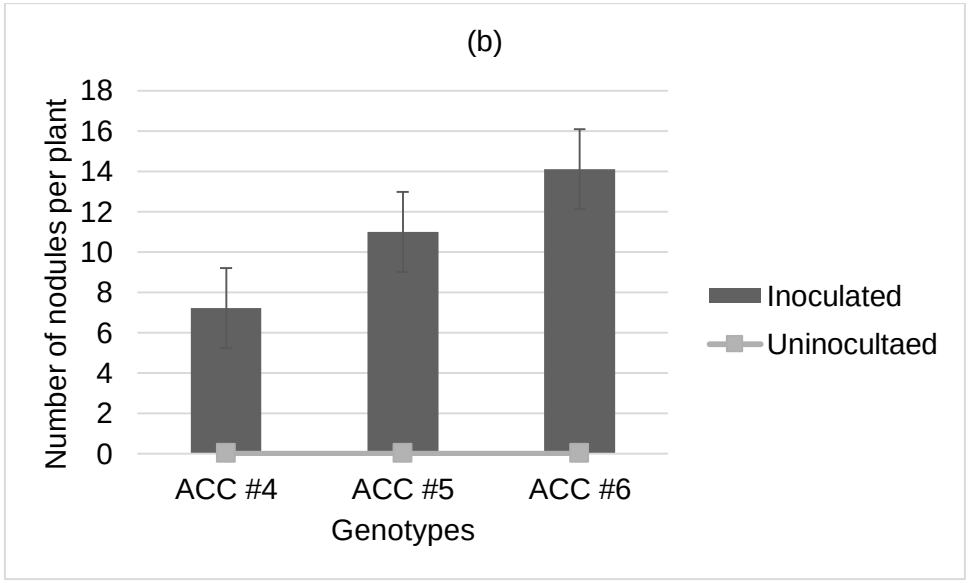

Figure 6.3: Effect of rhizobium inoculation and genotype on the number of nodules per plant during winter 2015 **(a)** and 2016 **(b)** in Nelspruit (Mpumalanga Province).

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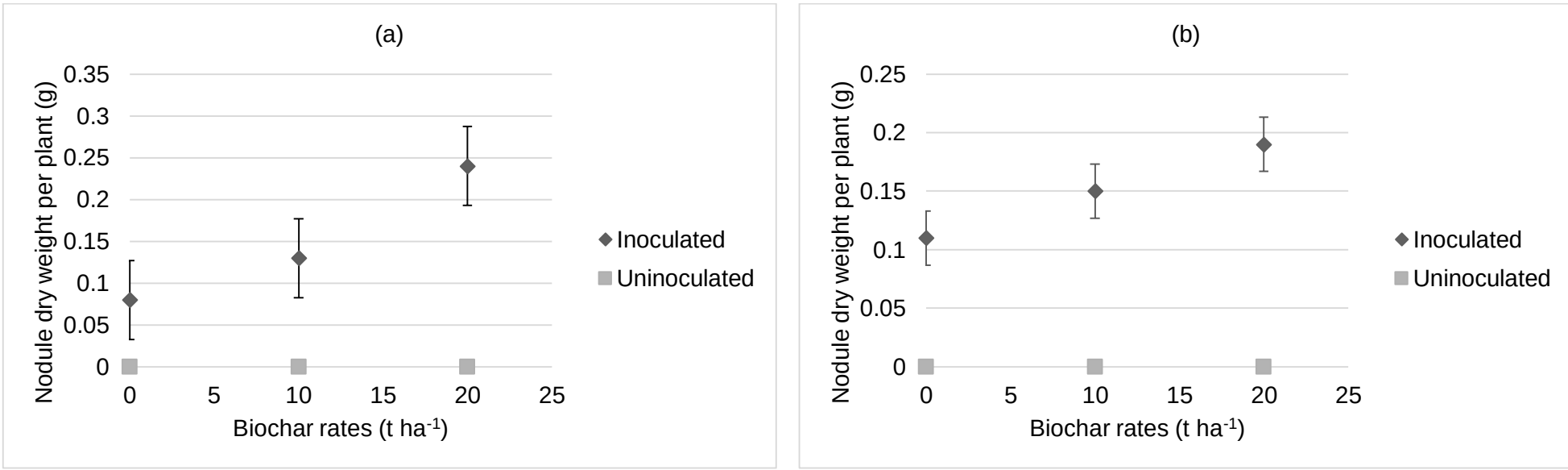

Figure 6.4: Effect of biochar and rhizobium inoculation on nodule dry weight per plant during winter 2015 **(a)** and 2016 **(b)** in Nelspruit (Mpumalanga Province)

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6.4 Discussion

The effect of biochar, genotype and rhizobium inoculation on root nodulation was significant in Thohoyandou and Nelspruit in 2015 and 2016 growing seasons. Number of nodules per plant and nodule dry weight increased with biochar application rates. The response of root nodulation (nodule number and nodule dry weight) to biochar application rates may be attributed to the effect of biochar on soil pH. Biochar application rates increased soil pH (by 11% in Thohoyandou and 21% in Nelspruit) to a range suitable for survival and proliferation of microorganisms such as rhizobia. Poor nodulation has been associated with poor survival of rhizobia especially in soils with extremely high or low soil pH (Khan *et al*., 2014). The high liming effect of the biochar used in the study may have contributed in improving the survival and competitiveness of the rhizobia ultimately improving both number of nodules and nodule dry weight. Earlier in a pot experiment, application of pinewood biochar increased root nodulation of soybean by 30% compared to other types of biochars and control (Khan *et al*., 2014). However, Khan *et al*. (2014) reported that extreme increase in soil pH due to biochar having high pH reduced the viable count of bradyrhizobia due to reduced growth and reproduction of bradyrhizobia, this could lead to reduced nodulation. Extreme increase in soil alkalinity adversely affects proliferation of bacteria which ultimately reduces nodulation and biological nitrogen fixation (Laureen *et al*., 2016).

The effect of genotype on the number of nodules and nodule dry weight was significant in Thohoyandou (2015) and Nelspruit (2015 and 2016). The difference in nodulation amongst the three genotypes used in the study may be attributed to their genetic variability and response to rhizobium inoculation (Gul *et al*., 2014). The nodulation ability of legumes differs with crop type and genotype (Solaiman *et al*. 2010; Yusif *et al*., 2016). Also, environmental conditions greatly affect the performance and expression of different crop genotypes (Namvar and Sharifi, 2011). Due to the differences in both climatic and edaphic factors of the two locations used in the study, the response of genotypes varied with location. The different response of the three chickpea genotypes may partly be attributed to their compatibility with the rhizobium inoculant used and also their suitability in different environments. Different crop genotypes respond differently to rhizobium strains due to variation in compatibility and effectiveness. Similarly, Solaiman *et al*. (2010) reported variation in number of nodules and nodule dry weight with different rhizobium strains used for chickpea inoculation. The non-response of nodulation to genotype at time of nodulation assessment may be partly attributed to stage of growth and growth conditions. Genotypic differences amongst crop genotypes may be visible under stress conditions such as

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water and mineral deficiencies. Also, genotype did not affect chlorophyll content at flowering which was the period for nodule assessment. There was a high and significant correlation between nodulation and chlorophyll content and that may explain our findings.

The effect of rhizobium inoculation on number of nodules and nodule dry weight was significant in both seasons and locations. These results may suggest that either there is total lack of infective rhizobia for chickpea, or the populations of infective rhizobia are far too low to be effective. Ogola (2015) reported an increase in chickpea nodulation with rhizobium inoculation and suggested that lack of nodulation without inoculation was probably due to lack of native rhizobia, low soil pH and low populations of infective native rhizobia. Inoculating with suitable rhizobium strains may improve nodulation and BNF through increased rhizobium populations and improved competitiveness (Khaitov *et al*., 2016; Nishita and Joshi, 2010). Similarly, Khan *et al*. (2014) reported an increase in root nodulation due to rhizobium inoculation in chickpea. Improved chickpea nodulation may ultimately lead to increased crop yields. More recently, Muslikah *et al*. (2016) observed that the number of nodules per plant and nodule dry weight increased with rhizobium inoculation rates. The response of legumes to rhizobium inoculation has positive effects not only on nodulation and BNF but is also environmentally friendly and a cost efficient source of N fertilizer. The use of bio-fertilizer such as rhizobium inoculation has shown a great potential in sustaining and enhancing soil microbiota which plays an important role in recycling of nutrients (Yusif *et al*., 2016).

The interactive effect of biochar and rhizobium inoculation on number of nodules and nodule dry weight was significant in winter 2015 and 2016 seasons. This response could be due to the effects of both biochar application rates and rhizobium inoculation on soil microbial populations and pH. The increase in soil pH to 7. 08 at 20 t ha^{-1} biochar rate created a soil pH range suitable for enhancing microbial activity and with inoculation, the survival and proliferation of the introduced rhizobia was also improved. Rhizobium inoculation may improve nodulation provided that other factors affecting survival and reproduction of rhizobia such as soil pH and soil moisture are taken care off (Yusif *et al*., 2016). Recently, Lusiba (2015) reported lack of chickpea nodulation with application of biochar only at the site of the current study. These results may suggest that the lack of nodulation was partly due to the lack of infective native rhizobia. Similarly, Ogola (2015) reported poor nodulation with rhizobium inoculation which was attributed partly to low soil pH in the area. Rhizobium inoculation alone may not enhance nodulation, even if other factors such as soil pH should be optimal. Hence, it is likely that the interactive effects of biochar and rhizobium

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inoculation may improve nodulation through the addition of rhizobia in the soil with the inoculant and also adjusting soil pH with biochar. Therefore, the combination of rhizobium inoculation and biochar application could enhance root nodulation through improved soil pH and addition of rhizobia in the soil especially in soils with low soil pH and little populations of rhizobia.

Application of both biochar and rhizobium inoculation may be a great combination in improving root nodulation, growth and yield of legume crops especially in arid and semi-arid regions. Yusif *et al*. (2016) reported an increase in groundnut nodulation with biochar application rates and rhizobium inoculation. Even though the number of nodule and nodule dry weight increased with both biochar and rhizobium inoculation rates in both locations, the nodulation in Thohoyandou was higher compared to Nelspruit. The difference in nodulation in these areas may partly be due to the management practices such as irrigation and environmental factors. The soil type in Nelspruit is loamy sand and may require irrigation regularly compared to clay soils in Thohoyandou. Furthermore, the cropping history may also have contributed to the variation in nodulation between the locations. The site selected for cultivation in Nelspruit was in fallow for 2 years with grass species occupying the area. However, in Thohoyandou there were spinach and cabbage cultivation before cultivation of chickpea. These differences in cropping history influence the growth of the successive crops hence the difference in nodulation. Nodulation varied with seasons, Nelspruit had greater variation in nodulation compared to Thohoyandou. These differences were attributed partly to the effect of biochar and inoculation on soil pH. Biochar and rhizobium inoculation had more effects on pH of soils in Nelspruit and there correlation analyses showed a positive correlation between nodulation and soil pH. Other than seasonal variation, the increase in nodulation in 2016 could have been attributed to better management practices such as timeous irrigation and weeding. Even though chickpea is a drought tolerant crop, water stress and weed competition at critical growth stages may reduce chickpea nodulation.

6.5 Conclusions

The effect of biochar, genotype and rhizobium inoculation on number of nodules per plant and nodule dry weight was significant in 2015 and 2016 growing seasons. However, there were variations in nodulation with season and study location with the highest nodulation in Thohoyandou. Application of biochar improved soil pH which contributed to improved nodulation. It is clear that chickpea nodulation may be improved through rhizobium inoculation and biochar

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application. However, more research is required using a different strains of rhizobia for inoculation and biochar types.

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CHAPTER 7. EFFECT OF BIOCHAR AND RHIZOBIUM INOCULATION ON CROP PHENOLOGY AND GROWTH OF THREE CHICKPEA GENOTYPES.

7.1. Introduction

Chickpea is one of the minor pulse crops in South Africa. The commercial production of the crop has not yet been established despite the high demand (Ogola and Thangwana, 2012). Chickpea research has been successfully established in the North Eastern part of SA but the effect of different external inputs on phenological and morphological traits has hardly been studied. The knowledge and understanding of the pattern of plant growth and development as well as the effects of environment is very crucial especially for newly introduced crops such as chickpea (Namvar and Sharifi, 2011). Time available for crops such as chickpea to produce vegetative structures and yield may be affected by climatic conditions and management practices. The changes in crop growth and development may also be influenced by genotype, temperature, photoperiod, soil nutrient and moisture status (Namvar and Sharifi, 2011).

Rhizobium inoculation is an alternative way of providing legumes with mineral N by improving the availability of root colonizing bacteria which forms a mutual relationship with legumes and ultimately fixing atmospheric nitrogen. The use of rhizobium inoculation have been well investigated, and so far positive results have been reported on growth and yield of chickpea (Saxena *et al*., 2013; Nishita and Joshi, 2010; Gul *et al*., 2014; Namvar *et al*., 2011; Namvar and Sharifi, 2011), and other crops (Yusif *et al*., 2016). Therefore a better understanding of the effect of this management practice on both phenological and morphological changes may provide the basis to maximize crop yields in certain environmental conditions.

Biochar is a nutrient rich soil amendment which has gained popularity due to its benefits on soil health and fertility (Major *et al*., 2010). Incorporation of biochar in the dry environments has shown a huge potential in sustaining the environment while improving crop yields. However, the effects of biochar on plant growth and yield primarily depends on biochar and soil properties. The role of biochar as soil amendment in plant growth and development is well documented with contradicting reports due to variation in the type of crop and environmental conditions (Major *et al*., 2010; Namvar and Sharifi, 2011; Nigussie *et al*., 2012). Phenology directly affect biomass accumulation and yield and any management practices that may affect phenology will also influence crop productivity. Early emerging crops have an advantage to utilize resources such as

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soil nutrients, moisture and solar radiation which may lead to increased crop yields (Namvar and Sharifi, 2011).

The interactive effect of biochar and rhizobium inoculation on growth and phenological developments of chickpea is not well documented. Hence there is a need to investigate the effect of these external inputs on morphological and phenological developments of chickpea in dry environments. The objective of this study was to assess the effect of biochar and rhizobium inoculation on crop growth and phenology of three chickpea genotypes in two representative locations of dry environments in South Africa.

7.2 Materials and methods

Full experimental details are given in chapter 3, but a brief summary is described below. Field experiments were conducted in Thohoyandou during winter 2015 (experiment I) and 2016 (experiment II). The experiments were conducted at the University of Venda's Experimental Farm, in Thohoyandou, Limpopo Province, South Africa. The experiments consisted of three biochar rates (0, 10 and 20 t ha^{-1}), two rhizobium inoculation rates (inoculated and uninoculated) and three chickpea desi genotypes (ACC #4, 5 and 6) in a factorial combination arranged in a randomised complete block design and replicated three times. Biochar was applied according to treatments a week before planting. Seeds were inoculated at planting at a rate recommended by the manufacturer. Uninoculated seeds were planted first to avoid contamination. Number of days to 50% emergence was determined by counting the number of days from sowing until 50% of the plants have emerged in each plot. The number of days to flowering was determined by counting the number of days from emergence until the day 50% of the plants in a plot had flowered at least one flower. The number of days to podding was determined by counting the number of days from emergence until 50% of the plants in a plot had at least one pod. Plant height and number branches were measured using a 5 m measuring tape for plant height and manual counting for number of branches. The data obtained was subjected to ANOVA using the general linear model of Genstat 17th Edition. When differences between the treatment means were significant, the means were separated using SED at 5% level.

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7.3 Results

7.3.1 Crop phenology

Days to 50% crop emergence

The effect of biochar application on the number of days to 50% emergence was significant (P< 0.01) in winter 2016 but not in 2015 (Table 7.1). Application of biochar reduced number of days to 50% emergence by 3% (10 t ha^{-1}) and 10% at (20 t ha^{-1}). Genotype had a significant effect on the number of days to 50% emergence in both seasons (Table 7.1). ACC #4 emerged late by 9% (2015) and 11% (2016) compared to ACC #5 and 6. Rhizobium inoculation did not affect the number of days to 50% emergence in both seasons. However, the interactive effect of biochar, genotype and rhizobium inoculation on 50% emergence was significant in 2016 (Table 7.1). There were no significant difference in the number of days to emergence on inoculated ACC #6 at 20 t ha^{-1} and inoculated ACC #5 at 10 t ha^{-1} (Table 7.2). Also, there were differences in number of days to emergence on uninoculated ACC #5 at 10 t ha^{-1} and inoculated ACC# 6 at 20 t ha^{-1}. In general, uninoculated plots at 0, 10 and 20 t ha^{-1} biochar application rates recorded delayed emergence compared to inoculated plots.

Days to 50% flowering

The main effect of biochar and rhizobium inoculation on the number of days to 50% flowering was not significant in both seasons (Table 7.1). The effect of genotype on number of days to 50% flowering was significant (P< 0.05) in 2016 and not in 2015. ACC #4 had a 5% and 3% delay in flowering compared with compared to ACC #5 and ACC #6, respectively (Table 7.1). The interactive effect of biochar and rhizobium inoculation was significant (P< 0.01 and P< 0.05, respectively) in 2015 and 2016 (Table 7.1). Rhizobium inoculation decreased the number of days to 50% flowering at all biochar rates but the decrease was greater at 10 t ha^{-1} (9 and 6%) compared with 0 t ha^{-1} (3 and 2%) and 20 t ha^{-1}(1 and 4%) in 2015 and 2016, respectively (Figure 7.1a & b).

Days to 50% podding

The application of biochar, genotype and rhizobium inoculation did not affect the number of days to 50% podding in 2015 and 2016 (Table 7.1). However, the interactive effect of biochar, genotype and rhizobium inoculation was significant (P< 0.05) in 2015. Inoculation did not affect

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the number of days to 50% podding in ACC #6 at 10 t ha^{-1} and 20 t ha^{-1} but decreased the number of days to podding in ACC #6 at 0 t ha^{-1} (by 5%). Rhizobium inoculation did not affect the number of days to podding in ACC #5 at 0 t ha^{-1} but decreased the number of days to podding in ACC #5 at 10 t ha^{-1} (by 8 %) and 20 t ha^{-1} (by 9 %). Also, inoculation did not affect number of days to podding in ACC #4 at 10 t ha^{-1} by reduced the number of days to podding in ACC #4 at 0 t ha^{-1} (by 6 %) and 20 t ha^{-1} (by 6 %) (Table 7.2).

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Table 7.1, Effect of biochar, genotype and rhizobium inoculation o crop phenology during winter 2015 and 2016 growing seasons in Thohoyandou (Limpopo Province).

SEASON/ YEAR	2015			2016		
Treatments	**Days to 50% emergence**	**Days to 50% flowering**	**Days to 50% podding**	**Days to 50% emergence**	**Days to 50% flowering**	**Days to 50% podding**
Biochar (t ha^{-1})						
0	6.11	39.11	69.39	6.44^{c}	38.56	65.39
10	6.22	38.89	68.78	6.28^{b}	38.28	65.00
20	5.83	40.06	70.06	5.83^{a}	39.21	66.06
SED	0.33	0.77	1.13	0.2	0.73	0.8
Genotypes						
ACC #4	6.44^{b}	40.28	70.89	6.67^{b}	39.67^{b}	66.44
ACC #5	5.89^{a}	38.67	68.72	5.88^{a}	37.87^{a}	65.11
ACC #6	5.83^{a}	39.11	68.61	6.00^{a}	38.44^{a}	64.89
SED	0.33	0.77	1.13	0.24	0.73	0.8
Rhizobium						
Inoculated	6.00	39.41	69.44	6.29	38.63	65.44
Uninoculated	6.11	39.30	69.37	6.07	38.68	65.22
SED	0.19	0.63	0.92	0.67	0.59	0.65
F-Test probability						
Biochar (B)	ns	ns	ns	**	ns	ns
Genotype (G)	*	ns	ns	***	*	ns
Rhizobium (R)	ns	ns	ns	ns	ns	ns
B*G	ns	ns	ns	ns	ns	ns
B*R	ns	**	ns	ns	*	ns
G*R	ns	ns	ns	ns	ns	ns
B*G*R	ns	ns	*	*	ns	ns
CV (%)	1.8	1.2	1	1.9	1.6	0.9

Means followed by the same letter are not significantly different, * P< 0.05, ** P <0.01 and *** P<0.001, CV (coefficient of variation)

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Table 7.2, Interactive effect of biochar, genotype and rhizobium inoculation on the number of days to podding (2015) and emergence (2016) in Thohoyandou, Limpopo Province.

TREATMENTS			YEAR/SEASON	
			2015	2016
Biochar rates (t ha^{-1})	Genotypes	Rhizobium	Days to 50% podding	Days to 50% emergence
0	ACC #4	Uninoculated	70.67^{ab}	7.67^{b}
0	ACC #4	Inoculated	67.00^{a}	6.33^{ab}
0	ACC #5	Uninoculated	68.67^{a}	6.33^{ab}
0	ACC #5	Inoculated	67.33^{a}	5.67^{ab}
0	ACC #6	Uninoculated	73.00^{bc}	7.00^{ab}
0	ACC #6	Inoculated	69.67^{a}	6.00^{ab}
10	ACC #4	Uninoculated	71.33^{ab}	7.33^{ab}
10	ACC #4	Inoculated	70.67^{ab}	6.67^{ab}
10	ACC #5	Uninoculated	71.33^{ab}	7.33^{ab}
10	ACC #5	Inoculated	66.33^{a}	5.33^{a}
10	ACC #6	Uninoculated	66.67^{a}	6.00^{ab}
10	ACC #6	Inoculated	66.33^{a}	6.00^{ab}
20	ACC #4	Uninoculated	75.00^{c}	6.00^{ab}
20	ACC #4	Inoculated	70.67^{ab}	6.00^{ab}
20	ACC #5	Uninoculated	72.33^{ab}	6.33^{ab}
20	ACC #5	Inoculated	66.33^{a}	5.67^{ab}
20	ACC #6	Uninoculated	68.67^{a}	5.67^{ab}
20	ACC #6	Inoculated	67.33^{a}	5.33^{a}
SED			2.77	0.5
F-Test probability				
Biochar (B)			ns	**
Genotype (G)			ns	***
Rhizobium (R)			ns	ns
B*G			ns	ns
B*R			ns	ns
G*R			ns	ns
B*G*R			*	*
CV (%)			4.9	9.9

Means followed by the same letter are not significantly different, *** (P<0.001),** (P<0.01) and *(P<0.05), CV (coefficient of variation)

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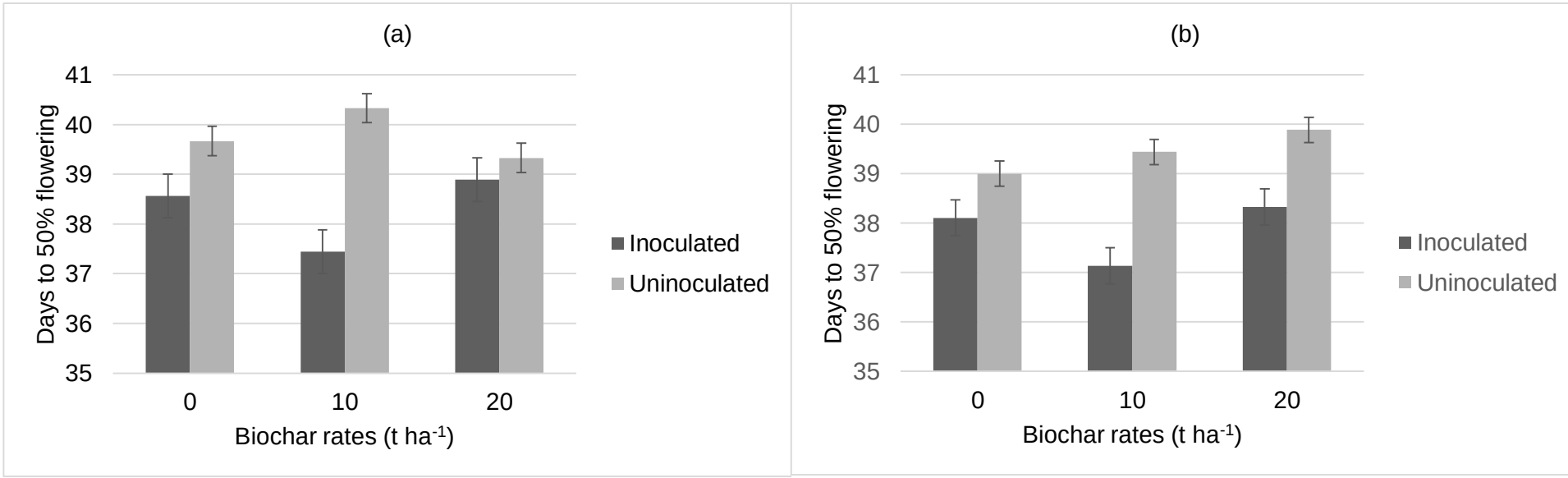

Figure 7.1: Effect of biochar and rhizobium inoculation on number of days to 50% flowering dung winter 2015 **(a)** and 2016 **(b)** in Thohoyandou (Limpopo Province).

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7.3.2 Crop growth

Plant height

The main effect of biochar on plant height was not significant at all measurement dates in both seasons (Table 7.3). In contrast, effect of genotype on plant height was highly significant (P< 0.001) in 2015 with ACC # 4 and 6 showing greater height compared to ACC #5 (Figure 7.2a&b). The effect of rhizobium inoculation on plant height was not significant in 2015 but affected plant height at 19 and 26 DAE in 2016 (Table 7.3). Rhizobium inoculation increased plant height (13 %) at 19 DAE and (7%) at 26 DAE, respectively in 2016. The interactive effect of biochar, genotype and rhizobium inoculation was significant (P< 0.05) at 73 and 80 DAE in 2015 (Table 7.3). Rhizobium inoculation increased plant height at all biochar rates with ACC #6 having greater height compared with ACC #4 and 5 (Table 7.4). Rhizobium inoculation increased plant height in ACC #6 at 0 t ha^{-1} (by 10 and 12 %), 10 t ha^{-1} (by 11 and 10 %), and 20 t ha^{-1} (by 11 and 7 %) at 73 and 80 DAE, respectively. Inoculation did not affect plant height in ACC #5 at all biochar application rates. Rhizobium inoculation did not affect plant height in ACC #4 at 20 t ha^{-1} but increased plant height in ACC #4 at 0 t ha^{-1} (11 and 8%) and 10 t ha^{-1} (8 and 7 %) at 73 and 80 DAE, respectively (Table 7.4). Generally, plant height increase with days after emergence in both seasons.

Number of branches

The effect of biochar, genotype and rhizobium inoculation on the number of primary branches was not significant in both seasons (Table 7.5). In contrast, biochar application affected the number of secondary branches in both seasons (Table 7.6). Number of secondary branches was greater by 21% (23 DAE), 17% (44 DAE), 13% (59 DAE) and 11% (68 and 79 DAE) in 2015, and 15% (26 and 47 DAE), 20% (61 DAE) and 21% (83 DAE) in 2016 at 20 t ha^{-1}. Similarly, number of secondary branches was greater by 18% (23 DAE), 7% (44 DAE), 9% (59 DAE), 11% (68 DAE) and 9% (79 DAE) in 2015, and 29% (26 DAE), 16% (47 DAE), 19% (61 DAE), 18% (75 DAE), and 14% (83 DAE) in 2016 at 10 t ha^{-1}. Genotype affected the number of secondary branches at 23 DAE in 2015 and 47 DAE in 2016 with ACC #5 having greater number of secondary branches compared with ACC #4 and 6. Rhizobium inoculation increased number of secondary branches at all measurements dates. Rhizobium inoculation increased the number of branches by 53% (23 DAE), 41% (44 DAE), 36% (59 DAE), 27% (68 DAE), and 29% (79 DAE) in 2015, and 4% (26 DAE), 39% (47 DAE), 35% (61 DAE), 33% (75 DAE) and 30% (83 DAE) in 2016. The interactive

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effect of biochar and genotype on the number of secondary branches was significant at all measurement dates in 2015 (Table 7.6).

Biochar application rates increased number of branches in all genotypes with greater number of branches at 20 t ha^{-1} on ACC #6, 10 t ha^{-1} on ACC #4 and 0 t ha^{-1} on ACC #5 (Figure 7.3c-e). Number of secondary branches was greater in ACC #5 at 0 and 10 t ha^{-1}, in contrast, at 20 t ha^{-1} secondary branches was greater in ACC #6 (Figure 7.3a&b). The interactive effect of biochar, genotype and rhizobium inoculation on the number of secondary branches was significant at 44 and 59 DAE (reproductive stage) in 2015 and at 26 DAE (vegetative stage) in 2016 (Table 7.7). Rhizobium inoculation increased number of branches of all genotypes at all biochar application rates. Rhizobium inoculation increased number of secondary branches in ACC #4 at 0 t ha^{-1} (41% , 54% and 22 %), 10 t ha-1 (21, 20 and 33%), and 20 t ha-1 (18, 9, and 50%) at 44, 59 and 26 DAE, respectively. Inoculation did not affect number of secondary branches in ACC #5 at 0 t ha-1 at 26 DAE but increased number of secondary branches in ACC #5 at 0 t ha-1 (22 and 25% at 44 and 59 DAE), 10 t ha-1 (74, 61, and 40%), and 20 t ha-1 (35, 35, and 40%) at 44, 59 and 26 DAE, respectively. Also, rhizobium inoculation increased the number of secondary branches in ACC #6 at 0 t ha-1 (50 and 35 % at 44 and 59 DAE, respectively), 10 t ha-1 (27 % at 44 and 59 DAE), and 20 t ha-1(80, 75, and 43% at 44, 59 and 26 DAE, respectively).

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Table 7.3, Effect of biochar, genotype and rhizobium inoculation on plant height during winter 2015 and 2016 in Thohoyandou.

Season/ Year	**2015**									
Treatment	**17 DAE**	**23 DAE**	**30 DAE**	**37 DAE**	**44 DAE**	**51 DAE**	**59 DAE**	**66 DAE**	**73 DAE**	**80 DAE**
F-Test probability										
Biochar (B)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Genotype (G)	***	***	***	***	***	***	***	***	***	***
Rhizobium (R)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*G	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
G*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*G*R	ns	ns	ns	ns	ns	ns	ns	ns	*	*
CV (%)	12	13.1	17.2	15.9	14.1	12.2	11.6	9.9	8.8	8.5

Season/ Year	**2016**									
Treatment	**19 DAE**	**26 DAE**	**31 DAE**	**38 DAE**	**47 DAE**	**54 DAE**	**61 DAE**	**68 DAE**	**75 DAE**	**83 DAE**
F-Test probability										
Biochar (B)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Genotype (G)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Rhizobium (R)	**	*	ns	ns	ns	ns	ns	ns	ns	ns
B*G	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
G*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*G*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CV (%)	5.8	6	4	8.6	10	10.7	9	10.1	7.5	11.3

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01) and *(P<0.05) and CV (coefficient of variation).

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Table 7.4, Interactive effect of biochar, genotype and rhizobium inoculation on plant height during winter 2015 at 73 and 80 days after emergence (DAE).

TREATMENTS			2015	
Biochar (t ha^{-1})	**Genotype**	**Rhizobium**	**73 DAE**	**80 DAE**
0	ACC #4	uninoculated	50.10^{c}	54.27^{b}
0	ACC #4	inoculated	55.50^{cd}	58.40^{bc}
0	ACC #5	uninoculated	36.73^{a}	39.50^{a}
0	ACC #5	inoculated	38.37^{a}	40.70^{a}
0	ACC #6	uninoculated	50.13^{c}	53.33^{b}
0	ACC #6	inoculated	55.17^{cd}	59.47^{cd}
10	ACC #4	uninoculated	51.97^{c}	55.80^{b}
10	ACC #4	inoculated	56.23^{de}	59.83^{cd}
10	ACC #5	uninoculated	39.27^{ab}	42.57^{a}
10	ACC #5	inoculated	41.50^{ab}	43.13^{a}
10	ACC #6	uninoculated	53.13^{cd}	56.47^{b}
10	ACC #6	inoculated	58.97^{de}	62.17^{d}
20	ACC #4	uninoculated	53.33^{cd}	57.50^{bc}
20	ACC #4	inoculated	54.13^{cd}	58.63^{bc}
20	ACC #5	uninoculated	38.00^{a}	40.73^{a}
20	ACC #5	inoculated	38.70^{a}	41.33^{a}
20	ACC #6	uninoculated	51.37^{c}	56.20^{b}
20	ACC #6	inoculated	57.17^{de}	59.87^{bc}
	SED		2.59	2.67
F-test probability				
Biochar (B)			ns	ns
Genotypes (G)			***	***
Rhizobium (R)			ns	ns
B*G			ns	ns
B*R			ns	ns
G*R			ns	ns
B*G*R			**	**
CV (%)			2.9	2.1

Means followed by the same letter are not significantly different, *** (P<0.001),** (P<0.01) and *(P<0.05) and CV (coefficient of variation).

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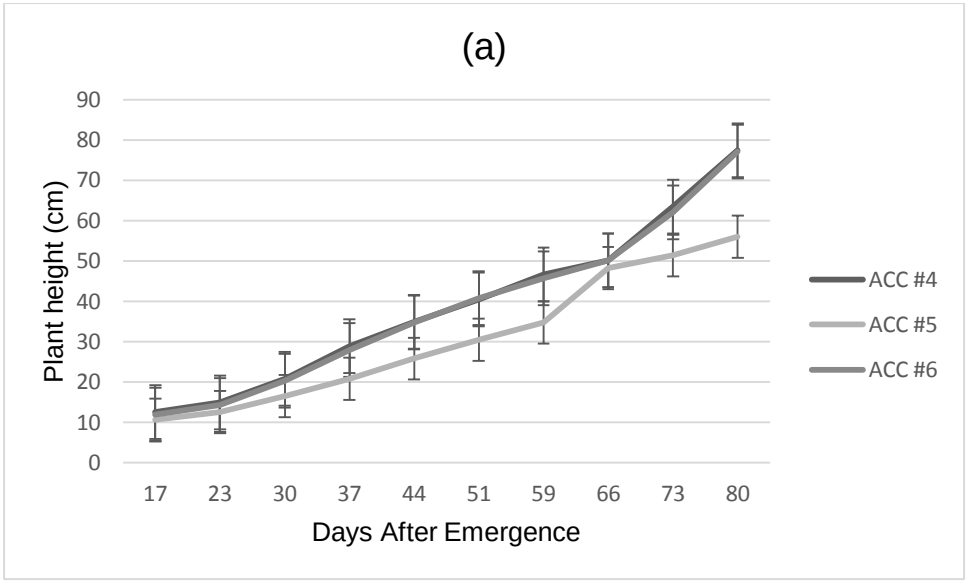

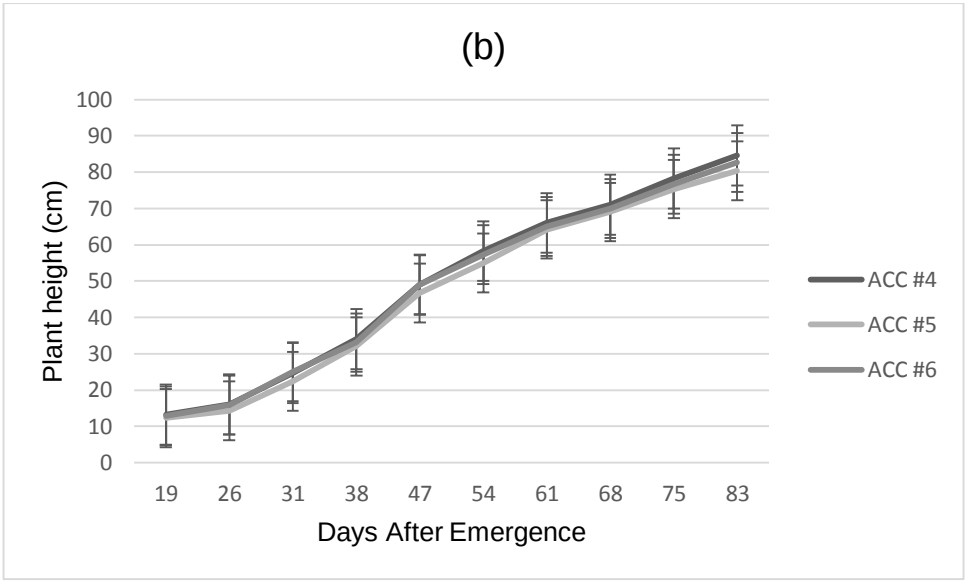

Figure 7.2.: Effect of genotype on plant height during winter 2015 **(a)** and 2016 **(b)** growing seasons in Thohoyandou

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Table 7.5, Effect of biochar, genotype and rhizobium inoculation on the number of primary branches during winter 2015 and 2016 in Thohoyandou.

	2015					2016				
Treatments	**23 DAE**	**44 DAE**	**59 DAE**	**68 DAE**	**79 DAE**	**26 DAE**	**47 DAE**	**61 DAE**	**75 DAE**	**83 DAE**
Biochar (B)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Genotype (G)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Rhizobium (R)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*G	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
G*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*G*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CV (%)	6.4	9.1	5.6	6.8	8.7	9.9	5.9	4.2	2.4	8.8

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Table 7.6, Effect of biochar, genotype and rhizobium inoculation on the number of secondary branches per plant during winter 2015 and 2016 growing seasons in Thohoyandou (Limpopo Province).

Season /Year	2015					2016				
Treatments	**23 DAE**	**44 DAE**	**59 DAE**	**68 DAE**	**79 DAE**	**26 DAE**	**47 DAE**	**61 DAE**	**75 DAE**	**83 DAE**
Biochar (t ha^{-1})										
0	2.14^{a}	3.81^{a}	5.39^{a}	6.83^{a}	8.50^{a}	1.47^{a}	4.11^{a}	4.64^{a}	5.58^{a}	6.94^{a}
10	2.53^{b}	4.06^{b}	5.86^{b}	7.56^{b}	9.28^{b}	1.89^{b}	4.78^{b}	5.52^{b}	6.58b	7.94^{b}
20	2.58^{b}	4.47^{c}	6.08^{c}	7.61^{b}	9.47^{b}	1.69^{b}	4.72^{b}	5.58^{b}	6.71^{b}	8.36^{c}
SED	0.180	0.20	0.26	0.312	0.347	0.154	0.215	0.243	0.334	0.397
Genotypes										
ACC #4	2.14^{a}	3.94	5.81	7.31	8.94	1.72	4.11^{a}	4.99	5.94	7.25
ACC #5	2.69^{c}	4.36	6.06	7.56	9.39	1.75	4.83^{b}	5.39	6.39	8.06
ACC #6	2.42^{b}	4.03	5.47	7.14	8.92	1.58	4.67^{b}	5.36	6.54	7.94
SED	0.180	0.2	0.275	0.312	0.347	0.154	0.215	0.243	0.334	0.397
Rhizobium										
Inoculated	2.93^{b}	4.79^{b}	6.67^{b}	8.2^{b}	10.22^{b}	1.72	5.28^{b}	6.03^{b}	7.18^{b}	8.76^{b}
Uninoculated	1.91^{a}	3.4^{a}	4.89^{a}	6.46^{a}	7.94^{a}	1.65	3.79^{a}	4.46^{a}	5.41^{a}	6.74^{a}
SED	0.320	0.160	0.225	0.255	0.283	0.126	0.175	0.199	0.272	0.324
F-Test probability										
Biochar (B)	*	**	*	*	*	*	**	***	**	**
Genotype (G)	**	ns	ns	ns	ns	ns	**	ns	ns	ns
Rhizobium (R)	***	***	***	***	***	ns	***	***	***	***
B*G	*	**	**	*	*	ns	ns	ns	ns	ns
B*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
G*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B*G*R	ns	*	*	ns	ns	*	ns	ns	ns	ns
CV (%)	4	1.2	4.3	5.5	4.3	7.4	5.3	1.5	2.6	2.9

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01), *(P<0.05) and CV (coefficient of variation).

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Table 7.7, Interactive Effect of biochar, genotype and rhizobium inoculation on the number of secondary branches per plant (44 and 59 DAE) during winter 2015 and (26 DAE) 2016 growing seasons in Thohoyandou (Limpopo Province).

TREATMENTS			YEAR/SEASON		
			2015		2016
Biochar rates (t ha^{-1})	Genotypes	Rhizobium	44 DAE	59 DAE	26 DAE
0	ACC #4	Uninoculated	2.83^{a}	4.00^{a}	1.50^{a}
0	ACC #4	Inoculated	4.00^{bc}	6.17^{ab}	2.00^{b}
0	ACC #5	Uninoculated	3.83^{bc}	5.33^{b}	1.33^{a}
0	ACC #5	Inoculated	4.67^{de}	6.67^{bc}	1.17^{a}
0	ACC #6	Uninoculated	3.00^{b}	4.33^{a}	1.33^{a}
0	ACC #6	Inoculated	4.50^{de}	5.83^{b}	1.50^{a}
10	ACC #4	Uninoculated	4.00^{d}	6.00^{bc}	1.50^{a}
10	ACC #4	Inoculated	4.83^{de}	7.17^{c}	2.00^{b}
10	ACC #5	Uninoculated	3.16^{b}	4.67^{a}	1.67^{a}
10	ACC #5	Inoculated	5.50^{e}	7.50^{c}	2.33^{b}
10	ACC #6	Uninoculated	3.00^{b}	4.33^{a}	1.83^{a}
10	ACC #6	Inoculated	3.80^{b}	5.50^{b}	2.00^{a}
20	ACC #4	Uninoculated	3.67^{bc}	5.50^{b}	1.33^{a}
20	ACC #4	Inoculated	4.33^{de}	6.00^{bc}	2.00^{b}
20	ACC #5	Uninoculated	3.83^{bc}	5.17^{ab}	1.67^{a}
20	ACC #5	Inoculated	5.16^{e}	7.00^{bc}	2.33^{b}
20	ACC #6	Uninoculated	3.50^{bc}	4.67^{a}	1.17^{a}
20	ACC #6	Inoculated	6.30^{f}	8.17^{d}	1.67^{a}
	SED		0.49	0.67	0.38
	F-test probability				
	Biochar (B)		**	*	*
	Genotype (G)		ns	ns	ns
	Rhizobium (R)		***	***	ns
	B*G		**	**	ns
	B*R		ns	ns	ns
	G*R		ns	ns	ns
	B*G*R		*	*	*
	CV (%)		1.2	4.3	7.4

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01), *(P<0.05) and CV (coefficient of variation).

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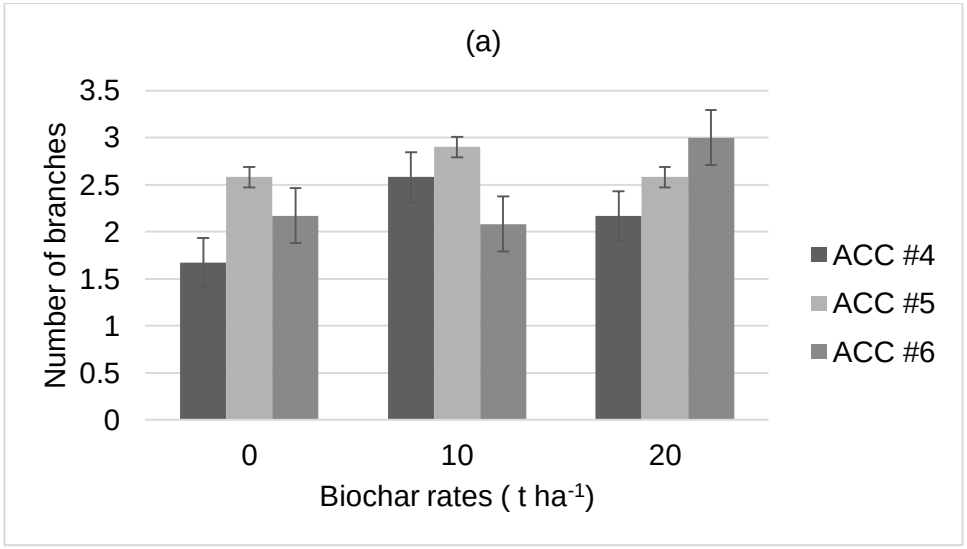

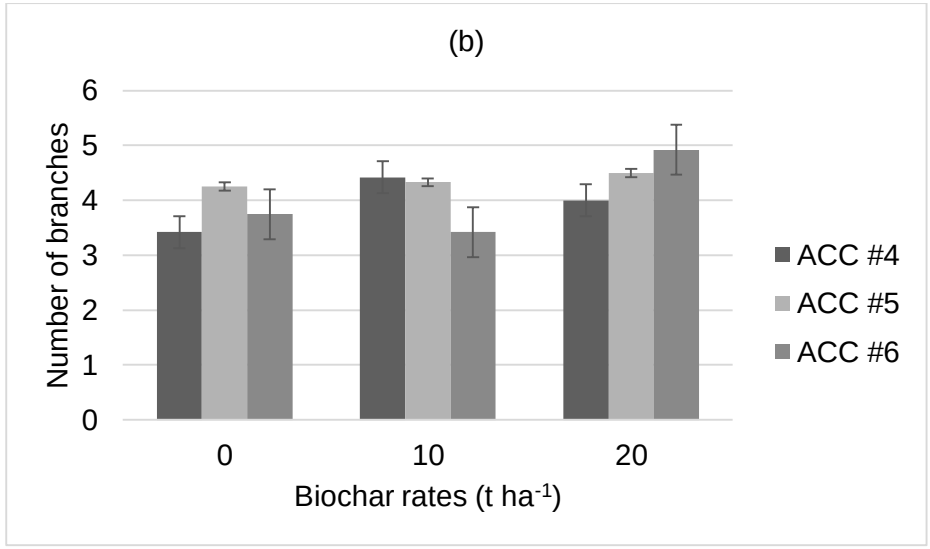

Figure 7.3: Interactive effect of biochar and genotype on number of Secondary branches at 23 **(a)** and 44 **(b)** DAE in 2015 at Thohoyandou.

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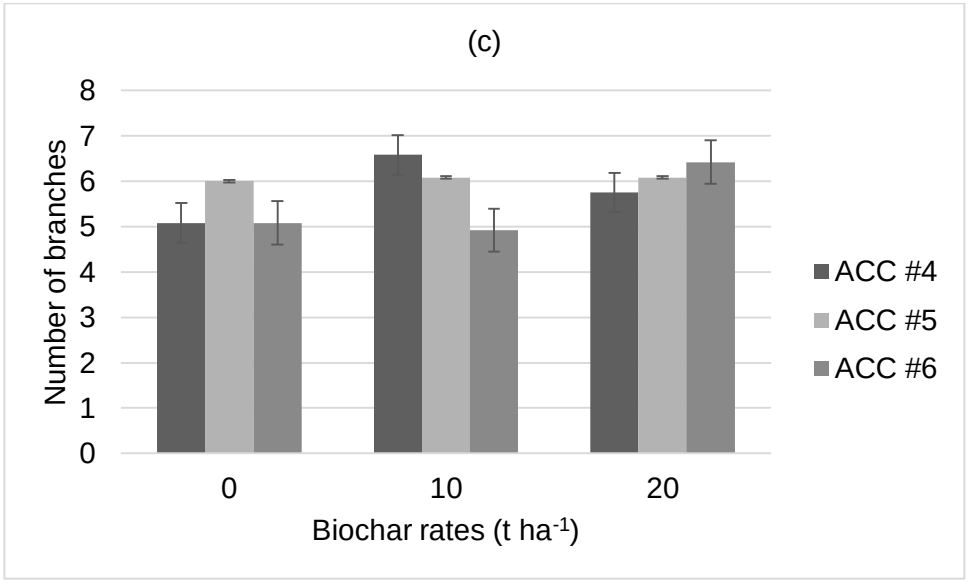

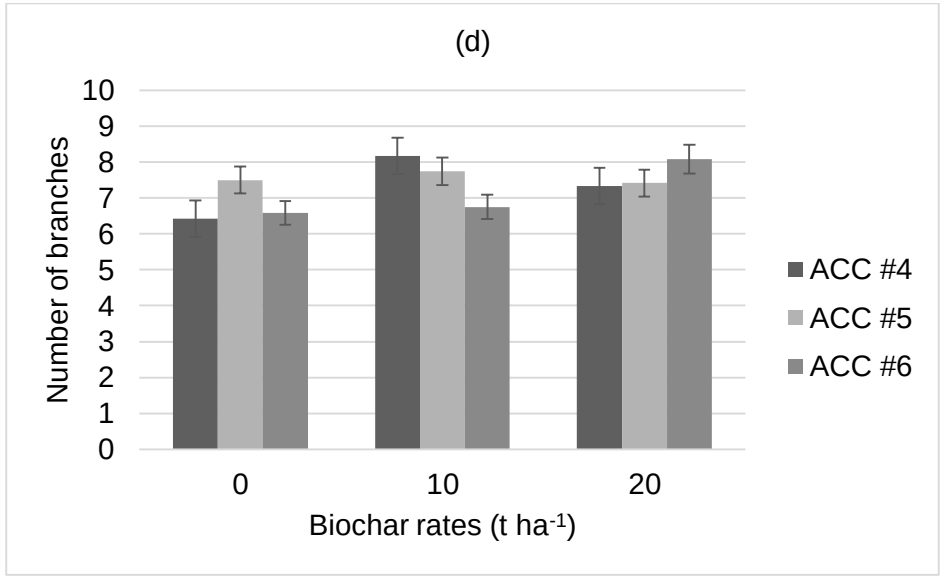

Figure 7.3: Interactive effect of biochar and genotype on the number of secondary branches at 59 **(c)** and 68 **(d)** DAE in 2015 at Thohoyandou

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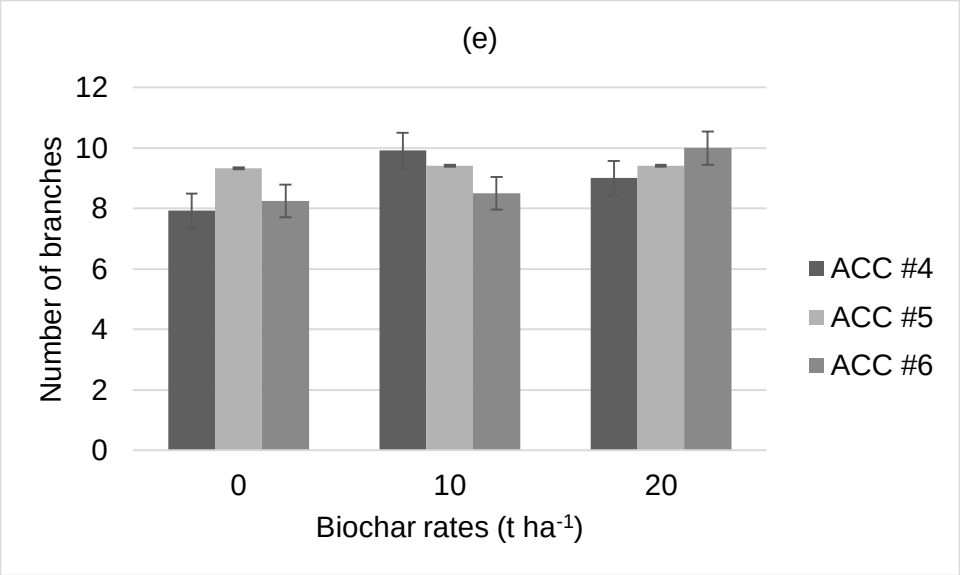

Figure 7.3: Effect of biochar and genotype on number of secondary branches at 79 **(e)** DAE in 2015 at Thohoyandou.

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Proportion of intercepted radiation

Application of biochar affected the proportion of intercepted radiation (IR) at all measurement dates in 2015 and 2016 (Table 7.8). The proportion of IR increased with biochar application rates at all measurement dates (Figure 7.4a & b). However, there was no difference in the proportion of IR between 10 and 20 t ha^{-1} biochar rates. Biochar application increased the proportion of IR by 6.5% (23 DAE), 5.3% (44 DAE), 5.08% (59 DAE), 5.4% (68 DAE) and 5.3% (79 DAE) at 10 t ha^{-1} biochar rate in 2015. Also, increased IR by 18.8% (26 DAE), 13.2% (47 DAE), 15.36% (61 DAE), 14% (75 DAE) and 12% (83 DAE) at 10 t ha^{-1} biochar rate in 2016.

Rhizobium inoculation increased the proportion of IR at all measurement dates in both seasons (Table 7.8). The proportion of IR increased with rhizobium inoculation rates (Figure 7.5a & b). Rhizobium inoculation increased the proportion of IR by 15% (23 DAE), 12% (44 DAE), 11% (59 DAE), 10% (68 DAE) and 11% (79 DAE) in 2015; and 19% (26 DAE), 23% (47 DAE), 17% (61 DAE), 14% (75 DAE) and 12% (83 DAE) in 2016.

The main effect of genotype on the proportion of intercepted radiation was significant in 2016 but not in 2015. Genotype significantly affected the proportion of intercepted radiation at 47, 61, 75 and 83 DAE (Table 7.8). The interactive effect of biochar and genotype on IR was significant at 26 and 47 DAE during winter 2016 but not in 2015. Also, three-way interaction between biochar, genotype and rhizobium inoculation on IR was significant at 26, 47 and 61 DAE in 2016 but not in 2015 (Table 7.9). At 26 DAE, Inoculation increased IR in ACC #6 at 0 (54%) and 20 (36%) t ha^{-1} but had no effect at 10 t ha^{-1} (Table 7.9). In contrast, inoculation increased IR in ACC #4 at all biochar application rates while inoculation did not affect IR in ACC #5 at all biochar rates. Rhizobium inoculation increased IR in ACC #4 at 10 (37%) and 20 (40%) t ha^{-1} but had no effect at 0 t ha^{-1}. In contrast, inoculation increased IR in ACC #5 and 6 at all biochar application rates at 47 DAE. Rhizobium inoculation increased IR at 61 DAE in ACC #5 at 0 (12%) and 20 (34%) t ha^{-1} but had no effect at 10 t ha^{-1} biochar rate. In contrast, inoculation increased IR in ACC #4 and 6 at all biochar application rates.

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Table 7.8, Effect of biochar, genotype and rhizobium inoculation on the proportion of intercepted radiation (%) during winter 2015 and 2016 growing seasons in Limpopo Province (Thohoyandou).

	2015					2016				
Treatments	**23 DAE**	**44 DAE**	**59 DAE**	**68 DAE**	**79 DAE**	**26 DAE**	**47 DAE**	**61 DAE**	**75 DAE**	**83 DAE**
Biochar (B)	***	***	***	***	***	**	***	***	***	***
Genotype (G)	ns	ns	ns	ns	ns	ns	**	*	*	*
Rhizobium (R)	***	***	***	***	***	***	***	***	***	***
B*G	ns	ns	ns	ns	ns	***	**	ns	ns	ns
B*R	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
G*R	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
B*G*R	ns	ns	ns	ns	ns	***	***	***	ns	ns
CV (%)	7.4	7	6.8	6.8	5.3	9.9	3.4	2.2	1.4	6.8

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01), *(P<0.05) and CV (coefficient of variation).

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Table 7.9, Effect of biochar, genotype and rhizobium inoculation on the proportion of intercepted radiation (%) during winter 2016 growing seasons in Thohoyandou (Limpopo Province).

Treatments			Growth stage		
Biochar rates	**Genotype**	**Rhizobium rates**	**26 DAE**	**47 DAE**	**61 DAE**
0	ACC #4	Uninoculated	7.38^{a}	21.77^{a}	35.85^{ab}
0	ACC #4	Inoculated	13.2^{c}	21.87^{a}	43.85^{bcd}
0	ACC #5	Uninoculated	9.15^{a}	23.10^{ab}	36.18^{ab}
0	ACC #5	Inoculated	9.60^{ab}	25.63^{c}	40.43^{abcd}
0	ACC #6	Uninoculated	12.27^{c}	21.28^{a}	33.90^{a}
0	ACC #6	Inoculated	18.86^{de}	30.37^{d}	47.80^{cde}
10	ACC #4	Uninoculated	11.90^{c}	23.17^{ab}	37.17^{ab}
10	ACC #4	Inoculated	16.19^{d}	31.76^{de}	48.95^{de}
10	ACC #5	Uninoculated	12.27^{c}	26.49^{c}	42.80^{abcd}
10	ACC #5	Inoculated	12.62^{c}	29.27^{d}	44.33^{abc}
10	ACC #6	Uninoculated	9.01^{a}	23.46^{ab}	42.53^{abc}
10	ACC #6	Inoculated	10.74^{a}	26.68^{c}	48.94^{de}
20	ACC #4	Uninoculated	8.13^{a}	20.95^{a}	35.76^{ab}
20	ACC #4	Inoculated	15.87^{d}	29.41^{d}	50.52^{de}
20	ACC #5	Uninoculated	16.00^{d}	27.35^{c}	38.00^{abc}
20	ACC #5	Inoculated	17.17^{d}	32.75^{de}	51.00^{e}
20	ACC #6	Uninoculated	12.04^{c}	26.63^{c}	44.17^{abcd}
20	ACC #6	Inoculated	16.42^{d}	31.90^{de}	50.43^{de}
	SED		2.00	1.809	2.76
	F-test probability				
	Biochar (B)		***	***	***
	Genotype (G)		ns	**	*
	Rhizobium (R)		***	***	***
	B*G		***	**	ns
	B*R		ns	*	ns
	G*R		ns	*	ns
	B*G*R		***	***	***
	CV (%)		9.9	6.8	7.9

Means followed by the same letter are not significantly different, *** (P<0.001),** (P<0.01), *(P<0.05) and CV (coefficient of variation).

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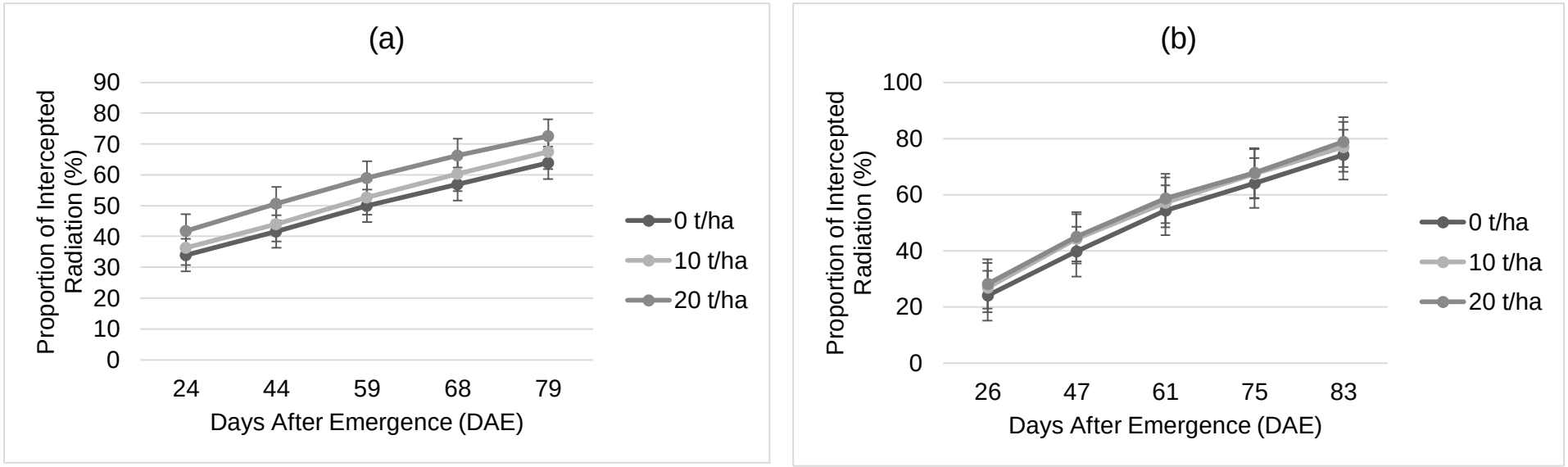

Figure 7.4: Effect of biochar on the proportion of intercepted radiation during winter 2015 **(a)** and 2016 **(b)** growing season in Thohoyandou (Limpopo Province).

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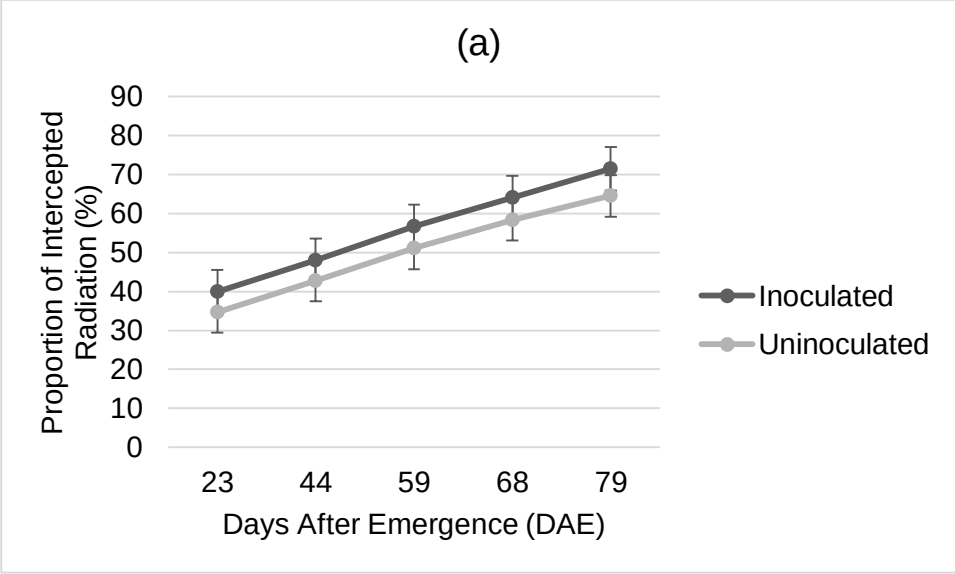

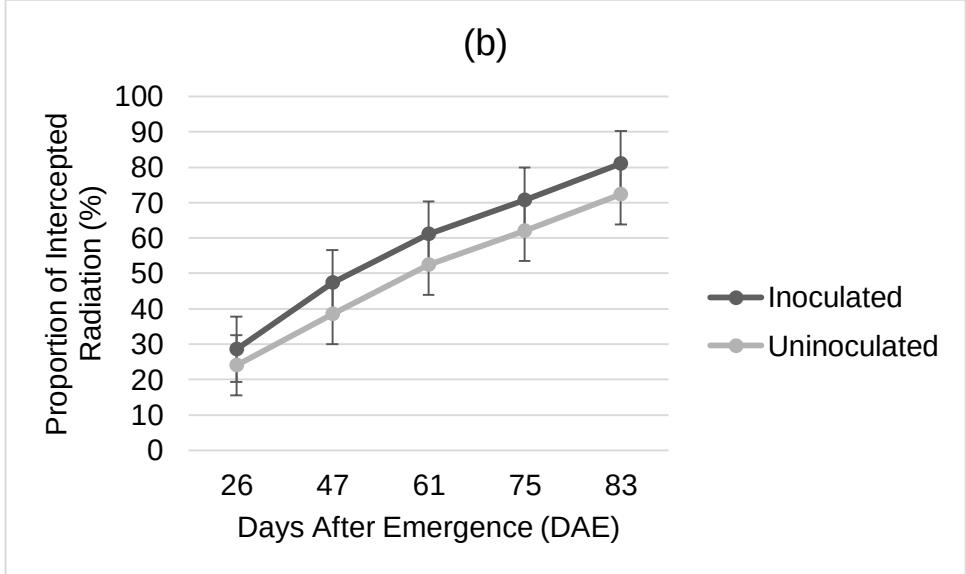

Figure 7.5: Effect of rhizobium inoculation on the proportion of intercepted radiation during winter 2015 **(a)** and 2016 **(b)** growing seasons.

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7.4 Discussion

7.4.1 Crop phenology

7.4.1.1 Days to 50% crop emergence

Biochar application rates reduced the number of days to crop emergence, probably due to the effect of biochar on soil moisture retention. Availability of water after planting is very critical for imbibition which is the first stage of germination where the seed absorb waters. If water is limited during this period, it is likely that poor germination and crop emergence may occur (Yusif *et al*., 2016). Biochar improves soil moisture retention and ensures availability of water for germination of the seed (Carter *et al*., 2013). The soils tend to dry quicker in no biochar treatments and that may be detrimental for germination, plant growth and development especially in dry areas where air temperatures are relatively high throughout the year (Namvar and Sharifi, 2011). Early emergence encourages vigorous vegetative growth which allows sufficient biomass accumulation before the onset of reproductive stage thus improving biomass production and yield. Thus the use of biochar as a soil amendment may play a crucial role in soil moisture retention and ultimately improving crop growth. However, soil moisture content was not measured in the current study.

The effect of genotype on number of days to 50% crop emergence was significant in both seasons. The variation amongst the different crop genotypes may have been due to the difference in their growth pattern and life cycle. Some crop genotypes grow relatively faster compared to others. However, these patterns are influenced by a number factors, both internal and external. Soil moisture content and temperature are some of the factors which may influence the performance of genotypes. Indeed, Gul *et al*. (2014) outlined that genotype has a prominent role in the phenotypic expression.

The interactive effect of biochar, genotype and rhizobium inoculation on crop emergence was significant in 2016 and not in 2015. The variation on the interactive effect of biochar, genotype and rhizobium inoculation on the number of days to 50% emergence may probably be due to the variation in weather conditions (temperature, rainfall, relative humidity, light) with season and year. Biochar improves both physical and chemical properties of soil hence creating conditions that will support seed germination and emergence. The decreased number of days to emergence due to biochar and rhizobium inoculation may be partly attributed to increased moisture availability with biochar application and also the production of phytohormones with inoculation which promote

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germination (Yilangai *et al*., 2014; Namvar and Sharifi, 2011). Also, some of the nutrient metabolites associated with rhizobium inoculation in the soil may trigger germination and ultimately enhance crop emergence (Saxena *et al*., 2013). The combination of biochar and rhizobium inoculation improved crop emergence through improved moisture retention and secretion of biomolecules by the rhizobia. These effects suggest that the interactive effect of biochar and inoculation may also reduce the need for seed priming especially to enhance germination. These may serve as an environmentally friendly strategy which does not only provide improved crop emergence but also enhance soil fertility and sustain crop production.

7.4.1.2 Days to 50% flowering

The effect of biochar on the number of days to flowering was not significant during both seasons although it affected some of the growth parameters. Phenological developments are affected by a number of factors including internal and external factors within crop genotypes (Namvar and Sharifi, 2011; Gul *et al*., 2014). Days to flowering is affected by the rate of crop development that is mainly driven by abiotic factors such as soil moisture, temperatures and nutrient availability. Despite the fact that biochar contain some appreciable amount of nutrients which are essential for plant growth, our result suggest that the non-response of number of days to flowering was not entirely due to biochar application rates but other factors such as temperature and crop factors (genetic attributes). The crops did not show any signs of moisture stress and nutrient deficiencies. Therefore, moisture and nutrient availability were not liming hence genetic attributes of the crop genotypes may be the factor that controlled the phenological developments in this study.

The effect of genotype on number of days to flowering was significant during winter 2016 and not in 2015. Genotypic variation and genetic attributes play an important role in distinguishing phenotypic differences amongst crop genotypes. However, environmental conditions including modification of the crop micro-environment through addition of external inputs may also influence these variations (Namvar and Sharifi, 2011; Gul *et al*., 2014). These may probably be due to the different responses of crop genotypes to different management practices. Environmental conditions play a significant role in influencing crop growth and development and in this case, the non-response of genotype in winter 2015 may have been due to both management practices and climatic factors. Progression of different genotypes to flowering may be used as a tool to select genotypes which are suitable for certain environmental conditions. Genotypes that progress to flowering early usually produce higher biomass and yield and escape drought periods.

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The effect of rhizobium inoculation on the number of days to 50% flowering was not significant in both seasons. Adequate availability of N may improve vegetative growth and delay the onset of reproductive stage, however there were no differences in number of days to flowering between inoculated and uninoculated treatments. Earlier studies showed that rhizobium inoculation reduced the number of days to flowering in chickpea compared with uninoculated treatments (Namvar *et al*., 2011). However, these responses may be affected by other factors such as environmental conditions and crop factors. Management practices that hastens crop development such as days to flowering also accelerates the period of maximum BNF.

The interaction between biochar and rhizobium inoculation affected the number of days to 50% flowering. Rhizobium inoculation decreased number of days to 50% flowering at all biochar application rates, however the effects were greater at 10 t ha^{-1} compared to 0 and 20 t ha^{-1}. These results may suggest that increasing biochar rates above 10 t ha^{-1} may not be useful for crop growth and development. Biochar effects are affected by biochar application rates and soil properties. Therefore, other types of soils may require lower application rates in order to enhance fertility and productivity hence increasing the rate above the required amount may not be necessary. Namvar and Sharifi, (2011) observed that number of days to flowering were reduced with rhizobium inoculation and delayed with the addition of chemical N fertilizers. Days to flowering is affected by nutrient and moisture availability and application of biochar and rhizobium inoculation provide nutrients and retain moisture thus promoting plant growth and development (Macdonald *et al*., 2014; Major *et al*., 2010b).

7.4.1.3 Days to 50% podding

The effect of biochar application on the number of days to 50% podding was not significant in both growing seasons. These results suggest that phenological developments are not only dependent on the environmental modifications but also internal and external factors within crop genotypes (Namvar and Sharifi, 2011). Abiotic factors such as temperatures, nutrients and moisture availability affect crop development. Therefore, the effect of biochar on crop development may be altered by environmental conditions.

The effect of genotype on the number of days to 50% podding was not significant despite having an effect on both number of days to 50% emergence and flowering. This may imply that the genotypes only differed in their vegetative growth with longer flowering duration for early flowering genotypes. Thangwana and Ogola (2012) reported that the phenological stages of chickpea are

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mainly affected by photoperiod and temperature. Even though the differences among the crop genotypes on the number of days to podding were not significant, ACC #6 had shorter days compared ACC #4 and 5. Even though there were significant difference in the number of days to podding amongst the crop genotypes, the effect of genotype on the number of pods per plant was significant. Early flowering genotypes produced greater number of pods compared to the late flowering genotypes.

Rhizobium inoculation did not have an effect on the number of days to 50% podding in both 2015 and 2016. Namvar *et al*. (2011) reported shorter growing period of chickpea with rhizobium inoculation. In contrast, the results of the current study showed that the effects of rhizobium inoculation were more visible at vegetative than at reproductive stage. The current findings are not in line with the reports by Yusif *et al*. (2016) and Namvar and Sharifi, (2011) who reported a significant effect of rhizobium inoculation on the number of days to podding. However, the effect of rhizobium inoculation on number of days to 50% podding in the current study may have been affected by genetic attributes of the crop genotypes and the environmental conditions.

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7.4.2 Crop growth

Plant height

Plant height did not respond to biochar application rates in contrast to previous studies at the current site (Macil *et al*., 2017). It is not clear why biochar did not affect plant height. However, there may be other factors such as the genetic attributes of the crop genotypes and environmental conditions which may have contributed.

Effect of genotype on plant height was significant at all measurements taken during winter 2015 but did not affect plant height in 2016. 2015 was a relatively dry and hot (weather data chapter 3) growing season compared to 2016 and hence ability of different genotypes to respond to such environmental conditions may vary. It is likely that the genotypes used in the current study respond differently to varying temperature regimes. Although chickpea is regarded as drought tolerant, different chickpea genotypes may vary with regard to their ability to thrive well under adverse environmental conditions (Khan *et al*., 2014).

Rhizobium inoculation did not affect plant height in both seasons. It is not clear why rhizobium inoculation did not affect plant height even though it affected other growth parameters. Earlier, Ogola (2015) reported no response of plant height to rhizobium inoculation in the same study location. Similar results were reported by Namvar and Sharifi, (2011) and further outlined that plant height responses are more when chemical N fertilizers are used than rhizobium inoculation. The period of maximum BNF occur at flowering which is the stage where maximum height has been reached, hence the effect of inoculation may not be visible.

The three-way interaction between biochar, genotype and rhizobium inoculation on plant height was significant at 73 and 80 DAE in 2015. Greater plant height was observed at 10 t ha^{-1} on ACC #6 with rhizobium inoculation. This response was partly attributed to effect of both biochar and rhizobium inoculation on plant growth. The period of maximum BNF occur during flowering and biochar may take time to mineralize in the soil. Therefore, these may explain the interaction of biochar, genotype and rhizobium inoculation at the advanced stage of growth (podding).

7.4.2.2 Number of branches

Application of biochar increased the number of secondary branches in both seasons probably due to the effect of biochar on moisture and nutrient availability. Biochar application increased

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soil pH in the current study and that is likely to influenced nutrient availability in the soil. Although no soil nutrient analyses were done in the current study, a number of studies have reported an increase in plant growth with biochar application which was associated with availability of macronutrients such as N, P and K (Saxena *et al*., 2013; Nigussie *et al*., 2012; Oram *et al*., 2014). The biochar used in the study contains appreciable amount of macro nutrients such as magnesium, phosphorus, carbon and calcium. Schulz *et al*. (2014) outlined that biochar effects on plant growth are depended on application rate, soil properties, crop type and trial conditions. The effects of biochar varied with application rates and season on the different genotypes used. Therefore, it is clear that application rates, type of crop and trial conditions do affect the effect of biochar on plant growth.

The effect of genotype on the number of secondary branches was observed only at vegetative growth in both seasons. The rate of crop development is affected by a number of factors including genetic attributes and environmental conditions. Crops that emerged earlier may have greater number of branches than the late emerging crops. However, slow growing genotypes may catch up with the fast growing genotypes at a later stage of growth which may contribute to lack of variation in some parameters at a later growth stage. Biomass production at flowering and harvest maturity was not affected by genotype. Therefore, that may explain the difference in the number of secondary branches only at an early growth stage (Namvar and Sharifi, 2011).

Rhizobium inoculation affected the number of branches per plant in both seasons. This response was probably due to high leaf N status (reflected by high chlorophyll content in the leaves, which serves as an indirect indicator of plant nutrient status). Similarly, Saxena *et al*. (2013) reported an increase in the number of branches which was associated with increased growth rate due to application of biofertilizers such as *rhizobia* and *bacillus spp.* Improved root architecture due to colonization by root bacteria result in improved water and nutrient uptake by the plant and ultimately increasing plant growth (Benidire *et al*., 2017). Also, Namvar and Sharifi (2011) reported an increase in number of branches per plant in inoculated treatments compared to uninoculated ones.

The interactive effect of biochar and genotype on number of secondary branches was observed in 2015 at all measurements dates. ACC #4 produced greater number of branches at 10 t ha^{-1}, ACC #5 at 0 t ha^{-1}, and ACC #6 at 20 t ha^{-1}. These results suggest that the response of different genotypes to biochar application may vary with application rates. Some genotypes may respond

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positively with lower application rates while some may require higher rates. Other than application rates, genetic attributes play an important role in the expression of genotype. Therefore, the response of different genotypes to varying biochar rates may be due to genetic factors and the environmental conditions.

The significant three-way interaction between biochar, genotype and rhizobium inoculation was observed in 2015 at 44 and 59 DAE and 2016 at 26 DAE. Biochar and rhizobium inoculation increased number of secondary branches of all genotypes with greater increase on ACC #6 at 20 t ha^{-1} with rhizobium inoculation. The increase in number of secondary branches with biochar and inoculation may be associated with nutrient uptake and availability. Biochar is a nutrient rich soil amendment and incorporation with N sources such as rhizobium inoculation may improve crop growth (Yusif *et al*., 2016; Koskey *et al*., 2017). Addition of soil amendments such as biochar may influence crop growth indirectly through improved soil fertility. The result of the current study showed that as much as addition of external inputs such as biochar and rhizobium inoculation affect crop growth, genotypic differences also contribute to the performance of genotypes under different environmental conditions. Increase in the number of branches, leaves and other growth parameters have been reported (Namvar *et al*., 2011; Yusif *et al*., 2016; Koskey *et al*., 2017). The observations were associated with the availability of growth limiting factors such as soil moisture and plant nutrients.

7.4.2.3 Proportion of intercepted radiation

Biochar application increased the proportion of intercepted radiation in both 2015 and 2016. Biochar application improved number of branches (number of leaves) and canopy size which increased the proportion of radiation intercepted. An increase in the proportion of intercepted radiation in chickpea due to biochar application was reported in Thohoyandou (Macil *et al*., 2017). Also, an increase in the proportion of IR has similar been reported on other crops such as lettuce and groundnut (Carter *et al*., 2013; Yusif *et al*., 2016). The increase in number of leaves and size may lead to early canopy closure hence increasing the proportion of intercepted radiation. Moreover, increase in nutrients availability with biochar application may lead to increased crop growth including leaf area and leaf area duration (LAD) and therefore increasing the proportion of radiation intercepted. Biochar application of biochar increased chlorophyll content and that may improve the light trapping ability of the crop hence greater proportion of IR intercepted.

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The effect of genotype on the proportion of intercepted radiation was significant in winter 2016 but not in 2015. The response of genotype on IR was partly attributed to differences in the growth patterns of the genotypes used in the current study. Genotypes with more lateral growth intercept more radiation due to early canopy closure. Also, the high chlorophyll b (CHLb), a (CHLa) and total chlorophyll content was observed on the genotypes with high IR. Greater chlorophyll content improves the efficiency of the crop in terms of light harvesting (Sumanta *et al*., 2014). Also, high chlorophylls indicate positive plant health status and that reduce the chances of early senescence hence more IR intercepted. Biomass was higher in ACC #6 which had greater chlorophyll contents and proportion of IR. These results imply that the crop was able to intercept more IR due to longer leaf area duration and translated that to biomass production (Namvar and Sharifi, 2011).

The effect of rhizobium inoculation on the proportion of intercepted radiation was significant in both seasons. This could have been due to the availability of nutrients associated with inoculation. Rhizobium inoculation as a source of N fertilizer has a number of benefits for plant growth and development including biological nitrogen fixation and provision of growth promoting substances. Increase in the number of leaves, leaf area and leaf area index are associated with availability of mineral N which is one of the growth limiting minerals. Therefore, adequate supply of N will improve crop growth and ultimately increase the proportion of intercepted radiation (Yusif *et al*., 2016; Khaitov *et al*., 2016). Leaf area duration (LAD), canopy size and cover directly affect the proportion of IR. Availability of N mineral prevent early or premature senescence which will therefore lead to longer LAD thus increasing the amount of radiation intercepted. Earlier, Ogola (2015) reported a non-response of IR to rhizobium inoculation in chickpea in Thohoyandou, however inoculated crops intercepted a slightly higher radiation compared to uninoculated crops. The findings of the current study suggest that the higher proportion of IR may have been due to greater number of branches (which led to early canopy closure) and higher chlorophyll content. Greater biomass was produced which is associated to longer leaf area duration, high proportion of IR and chlorophyll content (reduced premature senescence).

The interactive effect of biochar, genotype and rhizobium inoculation on IR was significant in winter 2016 at 26, 47 and 61 DAE. Biochar and rhizobium inoculation increased IR of all genotypes. Although biochar application and rhizobium inoculation increased the proportion of IR in all genotypes, greater IR was observed on ACC #6 and 5 at all biochar rates with inoculation. The response of IR to biochar, genotype and rhizobium inoculation was observed at vegetative and reproductive growth stages and that was associated with rapid growth rate. This was also

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reflected on the significant correlation between number of nodules and chlorophyll content. Biochar application improves soil properties, availability and retention of nutrients. Also inoculation as a source of N reduces the chances for N deficiencies hence promoting crop growth and development. Namvar *et al*. (2011) outlined that mineral nutrition is one of the major factors affecting plant growth and recommended the use of biofertilizers due their effects on soil fertility and crop growth while sustaining the environment.

7.5 Conclusions

The effect of biochar and rhizobium inoculation on the number of days to 50% crop emergence, flowering and podding varied with seasons. The effect of biochar and rhizobium inoculation on plant height was not significant but affected number of branches and proportion of intercepted radiation. The effect of biochar and rhizobium inoculation on crop phenology and growth were not consistent. Therefore, more research is required to assess the effect of rhizobium inoculation and biochar application on crop growth and phenology under different environmental conditions.

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CHAPTER 8. EFFECT OF BIOCHAR AND RHIZOBIUM INOCULATION ON CHLOROPHYLL CONTENT OF CHICKPEA

8.1 Introduction

The green pigment in leaves is composed of different constituents including chlorophyll a, b and total carotenoids that play a role in light trapping and carbon acquisition by plants during the process of photosynthesis (Sumanta *et al*., 2014). Chlorophyll content may vary with crop species and also amongst different genotypes within the same species. Pigment measurements may be used as direct or indirect indicators for plant nutrient status and health. Low content of pigments on plant leaves may indicate mineral deficiencies and disease symptoms, for example, the total leaf chlorophyll content decreases when plants are exposed to stress conditions (Latrach *et al*., 2014). The ratios of chlorophyll a to b may be indicators of the efficiency of photosynthetic process; lower ratios are indicators of plant stress (Sumanta *et al*., 2014). Chlorophyll b is a constituent of peripheral light-harvesting complexes hence lower content of this component may adversely affect the light interception by plants which may consequently reduce crop yield (Eggink *et al*., 2001). The variation in chlorophyll content may be influenced by a number of internal factors and environmental conditions, hence modification of the environment to improve plant growth and development may be reflected on the chlorophyll content.

For example, the use of biochar as a soil amendment and rhizobium inoculation to improve soil fertility may also affect the chlorophyll content. Macil *et al*. (2017) assessed the effect of biochar on chlorophyll content and observed an increase in chlorophyll content with biochar application rates. Rhizobium inoculation improves the availability of N mineral which is the major constituent in green pigments. Inoculated plants tend to have high chlorophyll content compared to uninoculated ones and therefore, chances of N deficiencies are minimal unless the method used for inoculation was not appropriate (Yusif *et al*., 2016; Nyoki and Ndakidemi, 2014). Increase in chlorophyll content with inoculation has similarly been reported in bush beans (Mfilinge *et al*., 2014). However, there is no evidence in literature on the interactive effect of rhizobium inoculation and biochar on chlorophyll content of chickpea. The hypothesis tested was biochar and rhizobium inoculation affect chlorophyll content of three different chickpea genotypes. Therefore, the objective of the study was to assess the effect of biochar and rhizobium inoculation on chlorophyll content.

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8.2 Materials and methods

Full experimental details are given in chapter 3, but a brief summary is described below. Field experiments were conducted at the University of Venda's Experimental Farm, Thohoyandou (Limpopo Province) during winter 2015 (experiment I) and 2016 (experiment II). The experiments consisted of three levels of biochar (0, 10 and 20 t ha^{-1}), two rhizobium inoculation rates (inoculated and uninoculated) and three chickpea (desi) genotypes (ACC #4, 5 and 6) in a factorial combination arranged in a randomised complete block design replicated three times. Biochar was applied according to treatments a week before planting. Seeds were inoculated at planting at a rate recommended by the manufacturer. Uninoculated seeds were planted first to avoid contamination. Chlorophyll content (CHLa and CHLb) was determined at flowering in 2015 and 2016 using the extraction method as described by Lichtenthaler (1987). Also, chlorophyll content meter (CCM-200 PLUS, Opti-Sciences, Tyngsboro, Massachusetts) was used to measure chlorophyll content in the fields at 26, 47, 61, 75 and 83 DAE in 2016. The ratio of chlorophyll a to b (CHLa/CHLb) was calculated. The data obtained was subjected to ANOVA using the general linear model of Genstat 17th Edition. The significant differences among the treatment means were compared using standard error of difference (SED) of the means at 5% level. Correlation analyses for chlorophyll content and nodulation were done.

8.3 Results

The effect of biochar on chlorophyll a (CHLa) and b (CHLb) content was significant ($P< 0.001$) in both 2015 and 2016 (Table 8.1). CHLa and b content increased with biochar application rates. CHLa content was greater by 2% (10 and 20 t ha^{-1}) in 2015, and 15% (10 t ha^{-1}) and 21% (20 t ha^{-1}) in 2016 compared with the control. Similarly, CHLb was greater by 47% (10 t ha^{-1}) and 114% (20 t ha^{-1}) in 2015 compared to 0 t ha^{-1}, and 5% (10 t ha^{-1}) and 11% (20 t ha^{-1}) in 2016 compared to the control. Application of biochar increased the total chlorophyll content (Table 8.3). Total chlorophyll content was greater by 18.6% (26 DAE), 13.9% (61 DAE), 20.1% (75 DAE) and 22.8% (83 DAE) at 10 t ha^{-1} compared to 0 t ha^{-1}, and 26.4% (26 DAE), 27.4% (61 DAE), 31.5% (75 DAE) and 32.8% (83 DAE) at 20 t ha^{-1} compared to 0 t ha^{-1}.

(CHLa and b content varied with genotypes with greater CHLa on ACC #6 (0.5 and 0.8%) in 2015 compared to ACC #4 and 5 in 2015, and ACC #4 (10 and 2%) in 2016, respectively. Greater CHLb was observed on ACC #5 (25 and 277%) compared to ACC #6 and 4 in 2015, and ACC #6 (16 and 1%) compared to ACC #4 and 5 in 2016, respectively Table 8.1). The differences in total

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chlorophyll content amongst the genotypes appeared later during reproductive stage (75 and 83 DAE) in the growing. Total chlorophyll content was greater by 18 and 6% (75 DAE), and 11 and 6% (83 DAE) on ACC #6 compared to ACC #4 and 5, respectively.

CHLa and b content increased with rhizobium inoculation rates in both seasons. Rhizobium inoculation increased CHLa by 11% in both 2015 and 2016 seasons. CHLb content was greater by 44 and 18% in inoculated compared with the control 2015 and 2016, respectively. Rhizobium inoculation increased total chlorophyll content by 23.6% (26 DAE), 18% (61 DAE), 14% (75 DAE) and 15% (83 DAE) compared to uninoculated treatments. The ratios of CHLa to b were relatively high in both inoculated and uninoculated treatments.

The two-way interactive effect of biochar and genotype on CHLa and b content was significant in 2015 but not in 2016 (Table 8.1). CHLa was greater on ACC #5 (0 t ha^{-1}), ACC #4 (10 t ha^{-1}) and ACC #6 (20 t ha^{-1}) with inoculation in 2015 and 2016, respectively (Table 8.3). The two-way interaction of genotype and inoculation was significant in 2015 but in 2016 (Table 8.1). Inoculated ACC #5 had greater CHLa content (29 and 7%) in 2015, and (26 and 6%) in 2016 at 0 t ha^{-1} compared to ACC #4 and 6, respectively. Inoculated ACC #4 had greater CHLa content (29 and 23%) in 2015, and (37 and 17%) in 2016 at 10 t ha^{-1} compared to ACC #5 and 6, respectively. Inoculated ACC #6 had greater CHLa content (27 and 18%) in 2015, and (13 and 16%) in 2016 at 20 t ha^{-1} compared to ACC #4 and 5, respectively. Also, the three-way interactive effect of biochar, genotype and rhizobium inoculation on CHLa and b content was significant in both seasons (Table 8.3). Biochar application and rhizobium inoculation increased CHLa and b content of all genotypes. In 2015, inoculated ACC #6 gave the highest CHLa while the uninoculated had the lowest at 20 t ha^{-1} biochar rate. Inoculated ACC #6 gave the highest CHLb at 20 t ha^{-1} while the uninoculated had the lowest at 10 t ha^{-1} biochar rate. In 2016, inoculated ACC #6 at 20 t ha^{-1} had the highest CHLa while the lowest was observed at 10 t ha^{-1}. Inoculated ACC #6 had higher CHLb at 20 t ha^{-1} while the lowest was in ACC #5 at 0 t ha^{-1} biochar rate.

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Table 8.1, Effect of biochar, genotype and rhizobium inoculation on chlorophyll a (CHLa) and b (CHLb) content (µg/ml) during winter 2015 and 2016 in Thohoyandou, Limpopo Province.

	YEAR/SEASON					
TREATMENTS	**2015**			**2016**		
Biochar (t ha^{-1})	**CHLa**	**CHLb**	**CHLa/b ratio**	**CHLa**	**CHLb**	**CHLa/b ratio**
0	9.52^{a}	3.70^{b}	2.57^{b}	8.29^{a}	5.94^{a}	1.51
10	9.69^{b}	2.52^{a}	3.80^{c}	9.52^{b}	6.23^{b}	1.53
20	9.68^{b}	5.38^{c}	2.00^{a}	10.03^{c}	6.59^{b}	1.52
SED	0.02	0.05	0.03	0.60	0.42	0.03
Genotypes						
ACC #4	9.62^{a}	1.49^{a}	6.45^{c}	9.63^{b}	5.88^{a}	1.64^{b}
ACC #5	9.59^{a}	5.61^{c}	1.71^{a}	8.79^{a}	6.68^{b}	1.32^{a}
ACC #6	9.67^{b}	4.50^{b}	2.15^{b}	9.42^{b}	6.77^{b}	1.39^{a}
SED	0.03	0.03	0.02	0.60	0.42	0.30
Rhizobium						
Inoculated	10.12^{b}	4.57^{b}	2.21^{a}	9.76^{b}	6.55^{b}	1.49
Uninoculated	9.13^{a}	3.17^{a}	2.88^{c}	8.80^{a}	5.57^{a}	1.58
SED	0.03	0.03	0.02	0.49	0.80	0.10
F-Test probability						
Biochar (B)	***	***	**	**	**	ns
Genotype (G)	***	***	**	*	*	**
Rhizobium (R)	***	***	*	*	*	ns
B*G	***	***	**	ns	ns	ns
B*R	***	***	**	ns	ns	ns
G*R	***	***	**	ns	ns	ns
B*G*R	***	***	**	**	**	*
CV (%)	0.8	2.0	6.5	9.2	9.5	8.9

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01), * (P<0.05) and CV (coefficient of variation).

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Table 8.2, Interactive effect of biochar, genotype and rhizobium inoculation on chlorophyll a (CHLa) and b (CHLb) content (µg/ml) during winter 2015 and 2016 in Thohoyandou, Limpopo Province

			Year/season					
			2015			2016		
Treatments			CHL a	CHL b	CHLa/b ratio	CHL a	CHL b	CHLa/b ratio
Biochar (t ha^{-1})	Genotypes	Rhizobium inoculation						
0	ACC #4	uninoculated	7.69^{ab}	4.14^{de}	1.86^{a}	8.69^{b}	3.06^{a}	2.84^{ab}
0	ACC #4	inoculated	8.81^{cd}	7.82^{l}	1.13^{a}	9.89^{bc}	6.74^{abc}	1.47^{a}
0	ACC #5	uninoculated	9.41^{f}	3.76^{ab}	2.50^{ab}	10.56^{d}	2.68^{a}	3.94^{b}
0	ACC #5	inoculated	11.36^{j}	4.44^{f}	2.56^{ab}	12.45^{d}	3.36^{a}	3.71^{b}
0	ACC #6	uninoculated	9.18^{e}	5.96^{h}	1.54^{a}	10.29^{bc}	4.88^{ab}	2.11^{ab}
0	ACC #6	inoculated	10.63^{j}	7.01^{j}	1.52^{a}	11.72^{d}	7.08^{bc}	1.66^{a}
10	ACC #4	uninoculated	8.69^{cd}	4.24^{e}	2.05^{ab}	9.55^{ab}	5.31^{ab}	1.80^{a}
10	ACC #4	inoculated	12.22^{k}	7.76^{k}	1.57^{ab}	13.75^{e}	8.83^{d}	1.56^{a}
10	ACC #5	uninoculated	8.71^{cd}	8.48^{m}	1.03^{a}	8.71^{b}	10.73^{d}	0.81^{a}
10	ACC #5	inoculated	9.51^{f}	9.25^{n}	1.03^{a}	10.05^{bc}	11.50^{d}	0.87^{a}
10	ACC #6	uninoculated	9.57^{f}	3.31^{a}	2.89^{b}	7.99^{a}	5.56^{ab}	1.44^{a}
10	ACC #6	inoculated	9.96^{i}	4.25^{de}	2.34^{ab}	8.76^{b}	6.50^{abc}	1.35^{a}
20	ACC #4	uninoculated	9.74^{g}	3.89^{bc}	2.50^{ab}	9.09^{ab}	4.14^{ab}	2.20^{ab}
20	ACC #4	inoculated	9.89^{gh}	6.29^{i}	1.57^{a}	12.24^{d}	8.26^{bc}	1.48^{a}
20	ACC #5	uninoculated	9.89^{gh}	3.45^{a}	2.87^{b}	10.24^{bc}	4.42^{ab}	2.32^{ab}
20	ACC #5	inoculated	10.67^{i}	4.25^{e}	2.51^{ab}	12.02^{d}	7.53^{bc}	1.60^{a}
20	ACC #6	uninoculated	7.31^{a}	4.84^{g}	1.51^{a}	8.60^{b}	8.12^{bc}	1.06^{a}
20	ACC #6	inoculated	12.59^{l}	9.54^{o}	1.32^{a}	13.88^{e}	12.82^{e}	1.08^{a}
SED			0.061	0.065	1.72	1.09	1.12	1.65
F-test probability								
	Biochar (B)		***	***	*	**	**	**
	Genotype (G)		***	***	**	**	**	*
	Rhizobium (R)		***	***	**	**	**	*
	B*G		***	***	ns	ns	ns	*
	B*R		***	***	ns	ns	ns	ns
	G*R		***	***	ns	ns	ns	ns
	B*G*R		***	***	**	**	**	*
CV (%)			0.8	2	5.9	4.8	3.9	10.2

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01), * (P<0.05), CV-coefficient of variation

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Table 8.3, Effect of biochar, genotype and rhizobium inoculation on total chlorophyll content (mmol cm^{-2} s^{-1}) during winter 2016 growing season in Thohoyandou, Limpopo Province.

Treatments	26 DAE	61 DAE	75 DAE	83 DAE
Biochar (t ha^{-1})				
0	1.40[a]	3.17[a]	2.73[a]	2.59[a]
10	1.66[b]	3.61[b]	3.28[b]	3.18[b]
20	1.77[b]	4.04[c]	3.59[c]	3.44[c]
SED	0.121	0.2	0.18	0.179
Genotypes				
ACC #4	1.45	3.41	2.92[a]	2.99[a]
ACC #5	1.67	3.61	3.24[b]	3.12[a]
ACC #6	1.7	3.81	3.43[c]	3.32[b]
SED	0.121	0.22	0.18	0.179
Rhizobium				
Inoculated	1.78[b]	3.91[b]	3.41[b]	3.29[b]
Uninoculated	1.44[a]	3.31[a]	2.99[a]	2.86[a]
SED	0.099	0.7	0.15	0.146
F-Test probability				
Biochar (B)	*	***	***	***
Genotype (G)	ns	ns	*	*
Rhizobium (R)	**	***	***	***
B*G	ns	ns	ns	ns
B*R	ns	ns	ns	ns
G*R	ns	ns	ns	ns
B*G*R	ns	ns	ns	ns
CV (%)	1.8	7.7	5.5	5.4

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01), * (P<0.05) and CV (coefficient of variation).

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8.4 Discussion

The effect of biochar, rhizobium inoculation, and genotype on CHLa and CHLb were significant in both seasons and total chlorophyll content in 2016. The response of chlorophyll content to biochar application could be due to the effect of biochar on plant nutrients status. The biochar used in the current study contained appreciable amount of nutrients such as carbon, magnesium, phosphorus and calcium (Lusiba *et al*., 2017). Also, the response of chlorophyll content to biochar application could partly be attributed to the effect of biochar on biological nitrogen fixation. Biochar application improves soil pH which will therefore improve the availability of macro nutrients such as N and P which are important for the BNF process and also the overall plant growth and development. Although BNF was not quantified in the current study, it is likely that biochar application led to improved BNF. Correlation analyses showed a high and significant correlation between chlorophyll content and nodulation [number of nodule (r = 0.60) and nodule dry weight (r = 0.56)] which may lead to improved BNF (Appendix 3). Similarly, William and Qureshi (2015) observed that biochar addition to soils increased the chlorophyll content of okra grown in the greenhouse plants. Milla *et al*. (2013) reported an increase in chlorophyll content of water spinach content due to biochar application. The reporters associated high chlorophyll content to availability of mineral N. These results show that environment and crop type may influence the response of chlorophyll content to biochar applications. Most recently, Macil *et al*. (2017) reported an increase in chlorophyll content of chickpea with biochar in the current study site. Higher soil N was also observed in the same study (Lusiba *et al*., 2017).

Rhizobium inoculation increased average chlorophyll content by 20% and ratio of CHLa/b probably due to an increase in leaf and soil N content. Inoculating legumes such as chickpea with suitable rhizobium strains increased N content in the leaves, shoots and also in the soil and ultimately increased the chlorophyll content of chickpea (Khattak *et al*., 2006; Solaiman *et al*., 2010). Nitrogen is one of the major components of the chlorophyll and addition of N mineral through inoculation with biofertilizers will reduce the risks of N deficiencies and improve crop yield (Ullah *et al*., 2016). Nyoki and Ndakidemi, (2014) attributed an increase in chlorophyll content of cowpea with adequate availability of nitrogen with inoculation. Crops which are properly inoculated with rhizobia rarely show symptoms of N deficiency, the colour of the leaves is usually dark green compared to uninoculated plants which may be pale green. The difference in the colour of the leaves was observed in the field in 2015 growing season

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The effect of genotype on chlorophyll a and b content was significant in both seasons. The effect of genotype on total chlorophyll content were only observed at reproductive stage which may be partly attributed to nodulation. The differences in chlorophyll content with regard to genotype may be visible when the N derived from BNF is beneficial for plant growth hence they may be minor during the early crop growth stages (Vollmann *et al*., 2011). Nodulation was high at reproductive stage which may indicate the availability of N due to BNF. Greater chlorophyll content may indicate high plant nutrient status, especially for the N mineral. A decrease in chlorophyll content may suggests a need for N fertilization. However, seasonal differences and crop growth stage may influence chlorophyll content. Inoculated ACC #6 showed greater chlorophyll content at 20 t ha^{-1} compared to ACC #4 and 5. The variation in chlorophyll content among the crop genotypes may be more when plants are subjected to stress conditions (Nyoki and Ndakidemi, 2014).

Increase in chlorophyll ratios (CHLa/b) with biochar and rhizobium inoculation was observed in the current study. Higher chlorophyll ratios are indicators of good plant health status. These results may also suggest that N was not limiting and that was also observed on higher proportion of radiation intercepted which was attributed to bigger canopy size, and greater biomass accumulation. Lower CHLa/b ratios are used as indicators for senescence which may be associated with N deficiencies. Loss of photosynthetic pigments due to senescence and N deficiencies have been reported and were associated with the chloroplast degradation which was also correlated with yellowing of leaves (Martins *et al*., 2016). However, growth stage may influence chlorophyll content. Chlorophyll content may be high at early growth stages (vegetative to flowering) were the plants metabolic and biochemical processes are at maximum and decline as the crop matures (from podding to Physiological maturity).

The increase in chlorophyll content (CHLa, b and total chlorophyll content) with biochar, genotype and rhizobium inoculation may indicate the efficiency of photosynthesis and BNF (Eggink *et al*., 2001). Higher proportion of IR and biomass yield was observed which may suggest the efficiency of the light trapping pigments. Quantification of these pigments in crop production may be important for assessing the need for fertilization and maximization of crop yields. Therefore application of biochar and rhizobium inoculation may be crucial for improving crop productivity by improving chlorophyll content which may also enhance harvesting of light by green plants.

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8.5 Conclusions

Application of biochar and rhizobium inoculation increased CHLa, b and total chlorophyll content across different genotypes in both seasons. Chlorophyll content increased with biochar application rates and rhizobium inoculation. Variation in genotypes affected total chlorophyll content at reproductive stage where nodulation was also high. It is clear that the addition of biochar and rhizobium inoculation improves chlorophyll content of different chickpea genotypes which may lead to increased crop yields hence ensure higher crop productivity.

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CHAPTER 9. EFFECT OF BIOCHAR AND RHIZOBIUM INOCULATION ON YIELD AND YIELD COMPONENTS OF CHICKPEA

9.1 Introduction

The increasing demand for production of food for the rapidly growing population has led to an interest and necessity for the use of resource-efficient and cost-effective inputs such as biochar and rhizobium inoculation to improve crop yields and sustain the environment. Chickpea (*Cicer arietinum* L.) is one of the newly introduced grain legumes in South Africa. Incorporation of this crop into the existing cropping systems may not only improve soil fertility but ensure provision of nutritious diet for human health since it is high in proteins and other minerals such as potassium and phosphorus (Khan *et al*., 2014).

The use of biochar as a soil amendment has gained popularity due to its benefits on soil health, fertility and crop productivity and hence biochar application may be an option in ensuring sustainable crop production especially in poor soils (Milla *et al*., 2013; William and Qureshi, 2015). However, contradicting results have been reported on the effect of biochar on crop yields (Saxena *et al*., 2013; Abrishankesh *et al*., 2015; Natalie *et al*., 2014; Major *et al*., 2010) probably because the effects of biochar on crop yield may be influenced by a number of factors such as soil and biochar properties. Therefore, selection of biochar that is suitable for specific soils, climate conditions and crop species may be important.

Biofertilizers such as rhizobium inoculation may be a useful and alternative type of N fertilizer which is relatively affordable and environmentally friendly for improving yield of legumes (Nishita and Joshi, 2010). Improved crop yields with application of biofertilizers is associated with increased availability of N mineral (Solaiman *et al*., 2010: Namvar *et al*., 2011). Nitrogen is a major constituent of chlorophyll and enzymes, and adequate supply of N may enhance physiological processes such as photosynthesis which play a significant role in yield formation. Adequate availability of N may lead to increased biomass accumulation and grain yield by increasing the proportion of radiation intercepted due to higher leaf area, longer leaf area duration and extinction coefficients. Lusiba (2015) reported an increase in chickpea biomass due to application of biochar at the site of the current study. However, Ogola (2015) reported a non-significant increase of yield and yield components of chickpea to rhizobium inoculation at the current site of study. Clearly, the effect of biochar and rhizobium inoculation vary greatly with environment. Moreover, it is not clear how the interaction between biochar application and rhizobium inoculation may affect yield

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and yield components of chickpea. However, there is scant information in literature on the interactive effect of biochar and rhizobium inoculation on chickpea yield and yield components. Therefore, the objective of the study was to assess the effect of biochar and rhizobium inoculation on yield and yield components of chickpea in Mpumalanga and Limpopo provinces.

9.2 Materials and methods

Full experimental details are given in chapter 3, but a brief summary is described below. Two field experiments were conducted at two sites with contrasting soil types in winter 2015 (experiment I) and 2016 (experiment II); the University of Venda's Experimental Farm, in Thohoyandou, Limpopo Province, and at the University of Mpumalanga's Experimental Farm, in Nelspruit, Mpumalanga Province, South Africa. The soils at the University of Venda are classified as clay and those at University of Mpumalanga as loamy sand. The experiments consisted of a 2 x 3 x 3 with two inoculum rates (inoculated and uninoculated), three biochar rates (0, 10 and 20 t ha^{-1}) and three chickpea (desi) genotypes (ACC #4, 5 and 6) in a factorial combination arranged in a randomised complete block design replicated three times. Biochar was applied according to treatments a week before planting. Seeds were inoculated at planting at a rate recommended by the manufacturer. Uninoculated seeds were planted first to avoid contamination. At maturity, all plants from 0.3 m^2 of two inner crop rows from each plot were cut at ground level. The pods were manually removed from all the harvested plants and counted to determine number of pods per plant. The pods were threshed by hand and number of seeds per pod was determined. All the seeds were air dried and weighed to determine the grain yield (kg ha^{-1}). The sub-samples of the seeds were used to determine 100 seed weight (100-SW). Harvest index was determined as the ratio of grain yield to biomass. The data obtained was subjected to ANOVA using the general linear model of Genstat 17th Edition. Cross location analysis were also done using site as a factor. Significant differences between treatment means were compared using standard error of difference (SED) at 5% level. Correlation analyses for yield and yield components were also determined to assess the relationship between parameters.

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9.3 Results

Above ground biomass

Application of biochar did not affect above ground biomass in 2015 but had a significant effect in 2016 in Nelspruit (Mpumalanga Province) (Table 9.1). Biomass increased by 20% and 30% at 10 t ha^{-1} and 20 t ha^{-1}, respectively. Effect of genotype on biomass was not significant in both 2015 and 2016 seasons. The effect of rhizobium inoculation on biomass was significant in both 2015 and 2016 seasons. Rhizobium inoculation increased biomass by 53% in 2015 and 21% in 2016 growing seasons. The interaction between biochar, genotype and rhizobium inoculation on biomass was not significant in both seasons. Biomass was greater (by 118%) in 2015 compared to 2016 growing season.

The effect of biochar application on above ground biomass was significant in 2015 but not in 2016 in Thohoyandou (Table 9.2). Biochar application increased biomass by 17.4% (10 t ha^{-1}) and 12% (20 t ha^{-1}). Similarly, genotype affected biomass in 2015 but not in 2016. ACC #6 produced greater biomass (31 and 9%) compared to ACC #4 and 5, respectively. Also, ACC #5 produced greater (by 42%) biomass compared to ACC #4. The effect of rhizobium inoculation on above biomass was significant ($P< 0.01$) in both 2015 and 2016 seasons. Rhizobium inoculation increased biomass (31 and 23%) in 2015 and 2016, respectively. The interactive effect of biochar, genotype and rhizobium inoculation on biomass was not significant in both seasons. Above ground biomass was greater by 110.53% in 2016 compared to 2015. Also, biomass was greater by 15% (2015) and 426% (2016) in Thohoyandou compared to Nelspruit (Table 9.1& 9.2).

Grain yield

Biochar application and genotype did not affect grain yield in 2015 in Nelspruit (Table 9.1). Rhizobium inoculation increased grain yield by 81% compared to uninoculated plots (Table 9.1). The interactive effect of biochar, genotype and rhizobium inoculation on grain yield was not significant.

The effect of biochar application on grain yield was not significant in Thohoyandou in both seasons (Table 9.2). In contrast, genotype affected grain yield in 2015 ($P< 0.001$) but not in 2016. ACC #6 produced greater grain yield (695 and 124.6 kg ha^{-1}) compared to ACC #4 and 5, respectively. Also, ACC #5 produced greater grain yield (by 570.4 kg ha^{-1}) compared to ACC #4. Rhizobium inoculation increased grain yield by 337.5 kg ha^{-1} (2015) and 671.4 kg ha^{-1} (2016). The interactive

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effect of genotype and rhizobium inoculation on grain yield was significant only in 2015 (Figure 9.1a). Rhizobium inoculation increased grain yield of all genotypes but the increase was greater in ACC #6 (52%) compared to ACC #5 (17 %) and ACC #4 (4%). The interactive effect of biochar, genotype and rhizobium inoculation was not significant in both seasons. Grain yield was greater (117%) at Thohoyandou in 2016 compared to 2015. Also, grain yield was greater (52%) in Thohoyandou compared to Nelspruit in 2015.

Harvest Index

The effect of biochar, genotype and rhizobium inoculation on harvest index was not significant in Nelspruit (Table 9.1). Application of biochar did not affect harvest index in both seasons in Thohoyandou (Table 9.2). In contrast, the effect of genotype and rhizobium inoculation on harvest index was significant ($P< 0.001$ and $P< 0.05$, respectively) in 2015 but not in 2016. Harvest index was greater by 16% in ACC #5 and 6 compared to ACC #4. Also, inoculation increased harvest index by 8% (Table 9.2). The interactive effect of genotype and rhizobium inoculation on harvest index was significant in 2015 but not in 2016 (Figure 9.1b); inoculation increased harvest index (22 and 3%) in ACC #4 and 6, respectively. In contrast, there was no difference in harvest index between inoculated and uninoculated ACC #5. Overall harvest index was greater (3%) in 2016 compared to 2015 at Thohoyandou. Harvest index was also greater (by 54%) in Thohoyandou compared to Nelspruit in 2015 (Table 9.1 & 9.2).

Number of pods per plant

The effect of biochar and genotype on the number of pods per plant was not significant in Nelspruit (Table 9.1). In contrast, rhizobium inoculation increased the number of pods per plant by 54% (8.9 nodules) (Table 9.1). The interactive effect of biochar, genotype and rhizobium inoculation on number of pods per plant was not significant.

The main effects of biochar did not affect the number of pods per plant in both seasons in Thohoyandou (Table 9.2). However, the effect of genotype on the number of pods per plant was highly significant ($P<0.001$) in 2015 but not in 2016 (Table 9.2). Number of pods varied from 18.8 (ACC #4) to 31.5 (ACC #6) Number of pods per plant were greater (10.8 and 67.7%) in ACC #6 compared to ACC #5 and 4, respectively (Table 9.2). Also ACC #5 produce greater number of pods by 52% compared to ACC #4. Rhizobium inoculation increased the number of pods by 18%

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(4.3) in 2015 and 31% (8.6) in 2016 (Table 9.2). The interactive effect of biochar and genotype on number of pods was significant in 2015 (Table 9.2); application of biochar at 20 t ha^{-1} increased number of pods in ACC #5 and 6 while ACC #4 had greater number of pods at 10 t ha^{-1} (Figure 9.2a). Number of pods were greater on ACC #5 (11 and 1%) compared to ACC #4 and 6, respectively at 0 t ha^{-1}, ACC #4 (9 and 12%) compared to ACC #5 and 6, respectively at 10 t ha^{-1}, and ACC # 6 (26 and 6%) compared to ACC #4 and 5, respectively at 20 t ha^{-1}. Also, the interactive effect of genotype and rhizobium inoculation was significant in 2015 but not in 2016 (Table 9.2). Rhizobium inoculation increased number of pods of all genotypes but the increase was greater in ACC #6 (40%) compared to ACC #5 (9%) and ACC #4 (4%) (Figure 9.2b). The interactive effect of biochar, genotype and rhizobium inoculation was not significant in both seasons. Number of pods per plant was greater (by 24%) at Thohoyandou in 2016 compared to 2015. Similarly, number of pods was greater by (26%) in Thohoyandou compared to Nelspruit I 2015 (Table 9.1 & 9.2).

Number of seeds per pod

The effect of biochar and genotype on the number of seeds per pod was not significant in Nelspruit (Table 9.1). In contrast, the effect of rhizobium inoculation on number of seeds per pods was significant (Table 9.1). Rhizobium inoculation increased number of seeds per pod by 89% (Table 9.1). There was no interactive effect of biochar, genotype and rhizobium inoculation on the number of seeds per pod (Table 9.1).

Application of biochar and rhizobium inoculation did not affect number of seeds per pod in both seasons in Thohoyandou (Table 9.2). In contrast, the effect of genotype on number of seeds per pod was significant ($P< 0.05$) in 2015 but not in 2016. ACC #4 produced greater number of seeds per pod (3.8 and 2.4%) compared to ACC #5 and 6, respectively (Table 9.2). The interactive effect of biochar, genotype and rhizobium inoculation on number of seeds per pod was not significant in both seasons. Number of seeds per pod was greater by 23.5% in 2015 compared to 2016 in Thohoyandou, and greater by 12.4% in Nelspruit compared to Thohoyandou in 2015 (Table 9.1 & 9.2).

100 Seed weight (100-SW)

The effect of biochar, genotype and rhizobium inoculation on 100 seed weight was not significant in Nelspruit (Table 9.1).

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The effect of biochar on 100-SW was highly significant (P<0.01) in Thohoyandou in both seasons (Table 9.2). Application of biochar increased 100 seed weight by 9% (10 t ha^{-1}) and 7% (20 t ha^{-1}) in 2015, and 4% (10 t ha^{-1}) and 11% (20 t ha^{-1}) in 2016. In contrast, the effect of genotype on 100-SW was significant in 2016 but not in 2015 (Table 9.2). ACC #6 produced greater 100-SW (2 and 4%) compared to ACC #5 and 4, respectively. Also, ACC #5 produced greater 100-SW by 3% compared to ACC #4. Rhizobium inoculation increased 100-SW (by 10 and 3%) in 2015 and 2016, respectively (Table 9.2). The interactive effect of biochar and genotype on 100-SW was significant in 2016 but not in 2015 (Table 9.2). Application of biochar at 10 t ha^{-1} (10%) and 20 t ha^{-1} (14%) increased 100- SW in ACC #4. In contrast, only 20 t ha^{-1} biochar rate increased 100-SW in ACC #5 and 6. In average, 100-SW was greater by 11% in Thohoyandou in 2016 compared to 2015, and also by 16% in Nelspruit compared to Thohoyandou in 2015.

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Table 9.1 Effect of biochar and rhizobium inoculation on yield and yield components of chickpea in Nelspruit, Mpumalanga during winter 2015 and 2016 growing seasons

Year	2015							2016
Treatments	Above ground biomass (kg ha^{-1})	Grain yield (kg ha^{-1})	Harvest index	Number of pods per plant	Number of seeds per plant	Number of seeds per pod	100 seed weight (g)	Above ground biomass (kg ha^{-1})
Biochar (t ha^{-1})								
0	1712	770	0.38	19.81	9.5	0.95	24.75	793.89^{a}
10	1981	902	0.4	20.94	11.49	1.15	24.65	954.21^{b}
20	2370	1216	0.46	23.25	14.53	1.45	24.46	1031.9^{c}
SED	330.6	275	0.074	2.631	3.292	0.329	1.24	82.69
Genotypes								
ACC #4	1771	777	0.4	20.01	9.72	0.97	25.31	919.77
ACC #5	1970	994	0.43	20.84	11.96	1.2	24.68	919.95
ACC #6	2321	1117	0.42	21.83	13.83	1.38	23.87	940.28
SED	330.6	275	0.074	2.631	3.292	0.329	1.24	82.69
Rhizobium								
Inoculated	2447^{b}	1240^{b}	0.46	25.33^{b}	15.51^{b}	1.55^{b}	24.71	1013.55^{b}
Uninoculated	1595^{a}	686^{a}	0.39	16.47^{a}	8.17^{a}	0.82^{a}	24.53	839.78^{a}
SED	270	224.6	0.06	2.149	2.688	0.269	2.15	67.52
F-Test probability								
Biochar (B)	ns	ns	ns	ns	ns	ns	ns	*
Genotype (G)	ns	ns	ns	ns	ns	ns	ns	ns
Rhizobium (R)	**	*	ns	***	**	**	ns	*
B*G	ns	ns	ns	ns	ns	ns	ns	ns
B*R	ns	ns	ns	ns	ns	ns	ns	ns
G*R	ns	ns	ns	ns	ns	ns	ns	ns
B*G*R	ns	ns	ns	ns	ns	ns	ns	ns
CV (%)	9.3	25.3	27.2	12.6	30	30	7.5	16.6

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01) and * (P<0.05), CV- Coefficient of variation

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Table 9.2, Effect of biochar, rhizobium inoculation and genotype on yield and yield components of chickpea in Thohoyandou, Limpopo during winter 2015 and 2016.

Year	2015						2016					
Treatments	**Above ground biomass (kg ha^{-1})**	**Grain yield (kg ha^{-1})**	**Harvest index**	**Number of pods per plant**	**Number of seeds per pod**	**100 seed weight (g)**	**Above ground biomass (kg ha^{-1})**	**Grain yield (kg ha^{-1})**	**Harvest index**	**Number of pods per plant**	**Number of seeds per pod**	**100 seed weight (g)**
Biochar (t ha^{-1})												
0	2107.74^{a}	1333.15	0.63	25.11	1.05	20.14^{a}	4655.93	2973.85	0.64	30.2	0.85	22.29^{a}
10	2475.00^{c}	1553.80	0.62	26.61	1.05	22.01^{c}	4821.22	3186.74	0.65	33.5	0.88	23.28^{b}
20	2359.98^{b}	1502.87	0.63	27.00	1.04	21.46^{b}	5139.46	3363.87	0.65	33.7	0.82	24.76^{c}
SED	172.34	99.631	0.023	1.165	0.022	0.503	486.29	249.67	0.033	3.73	0.061	0.331
Genotype												
ACC #4	1860.89^{a}	1041.48^{a}	0.57^{a}	18.78^{a}	1.07^{b}	21.01	4729.22	3097.19	0.64	32.3	0.85	22.92^{a}
ACC #5	2437.43^{b}	1611.85^{b}	0.66^{b}	28.44^{b}	1.04^{a}	21.21	5204.74	3360.98	0.65	34.9	0.84	23.50^{b}
ACC #6	2644.41^{c}	1736.48^{c}	0.66^{b}	31.50^{c}	1.03^{a}	21.38	4882.65	3066.30	0.65	30.3	0.86	23.91^{c}
SED	172.34	99.631	0.023	1.165	0.022	0.503	486.29	249.67	0.033	3.73	0.061	0.331
Rhizobium												
Inoculated	2628.14^{b}	1632.04^{b}	0.65^{b}	28.41^{b}	1.05	22.18^{b}	5376.74^{b}	3510.53^{b}	0.65	36.8^{b}	0.89	24.92^{b}
Uninoculated	2000.35^{a}	1294.51^{a}	0.60^{a}	24.07^{a}	1.05	20.22^{a}	4367.67^{a}	2839.11^{a}	0.65	28.2^{a}	0.81	21.97^{a}
SED	298.5	81.35	0.019	0.95	0.038	0.411	331.73	203.86	0.027	3.04	0.05	0.27
F-Test probability												
Biochar (B)	*	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	***
Genotype (G)	***	***	***	***	*	ns	ns	ns	ns	ns	ns	*
Rhizobium (R)	***	***	*	***	ns	***	**	*	ns	**	ns	***
B*G	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	**
B*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
G*R	ns	**	*	***	ns	ns	ns	ns	ns	ns	ns	ns
B*G*R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CV (%)	15.9	13.3	3.7	13.3	2.1	0.9	25	33.4	15.2	16.5	21.4	4.2

Means followed by the same letter are not significantly different, *** (P<0.001), ** (P<0.01) and * (P<0.05), CV- coefficient of variation

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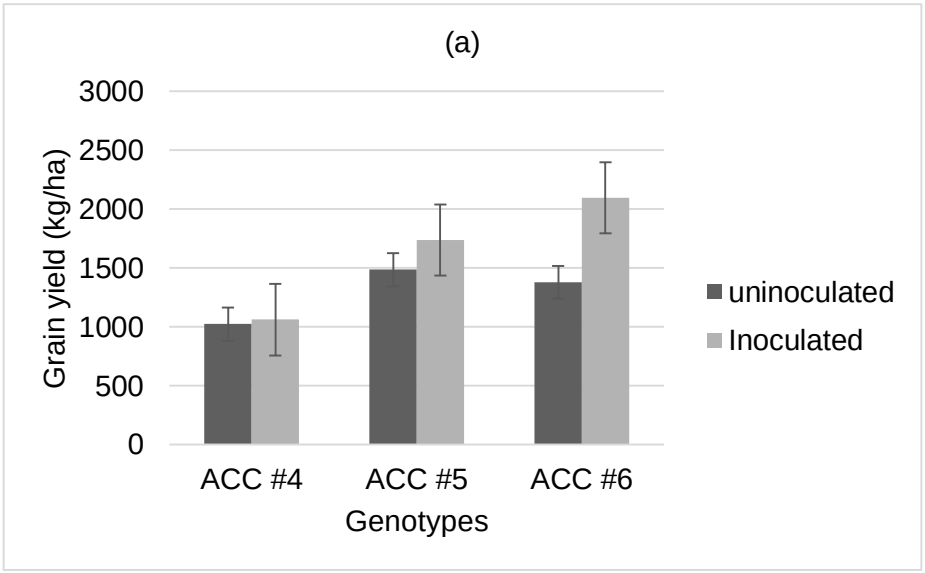

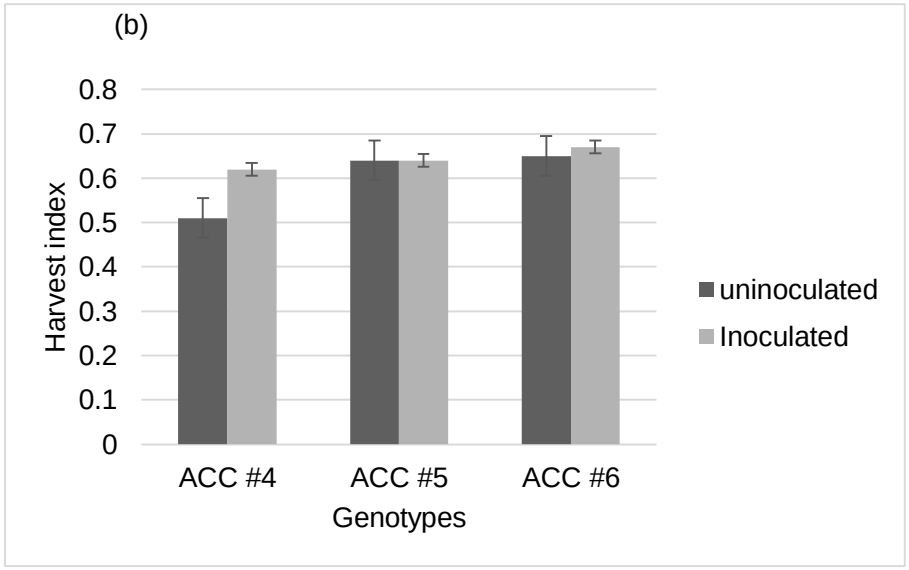

Figure 9.1: Effect of genotype and rhizobium inoculation on grain yield **(a)** and harvest index **(b)** during winter 2015 in Thohoyandou, Limpopo Province.

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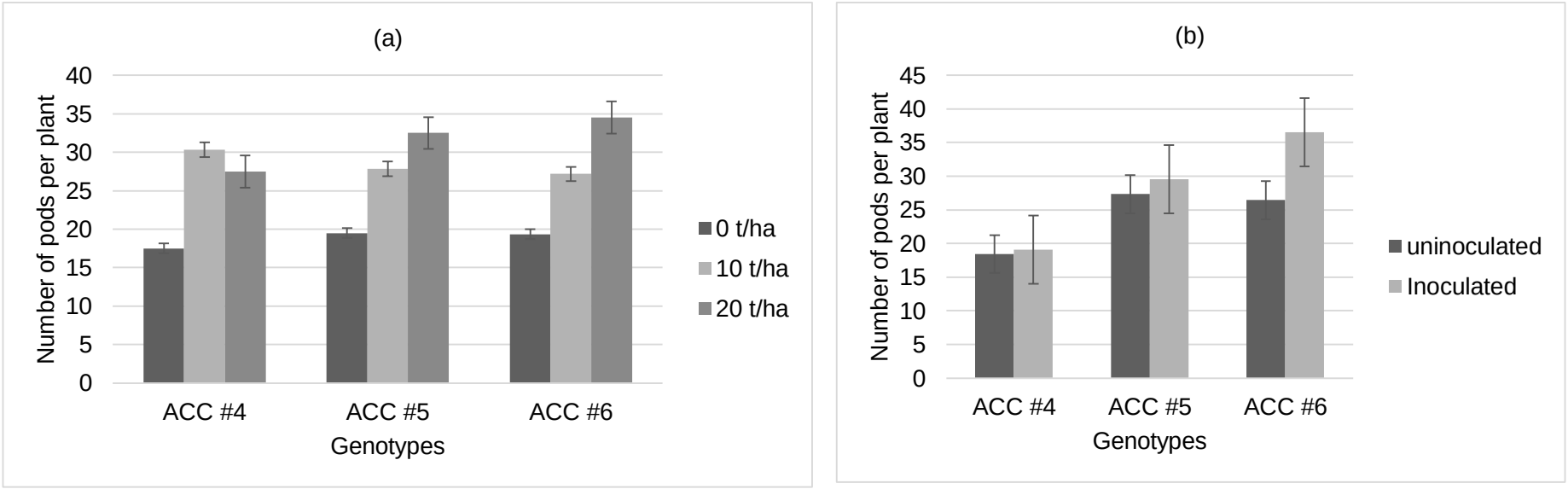

Figure 9.2: Effect of biochar and genotype **(a)** and rhizobium inoculation and genotype **(b)** on the number of pods per plant during winter 2015 in Thohoyandou, Limpopo Province.

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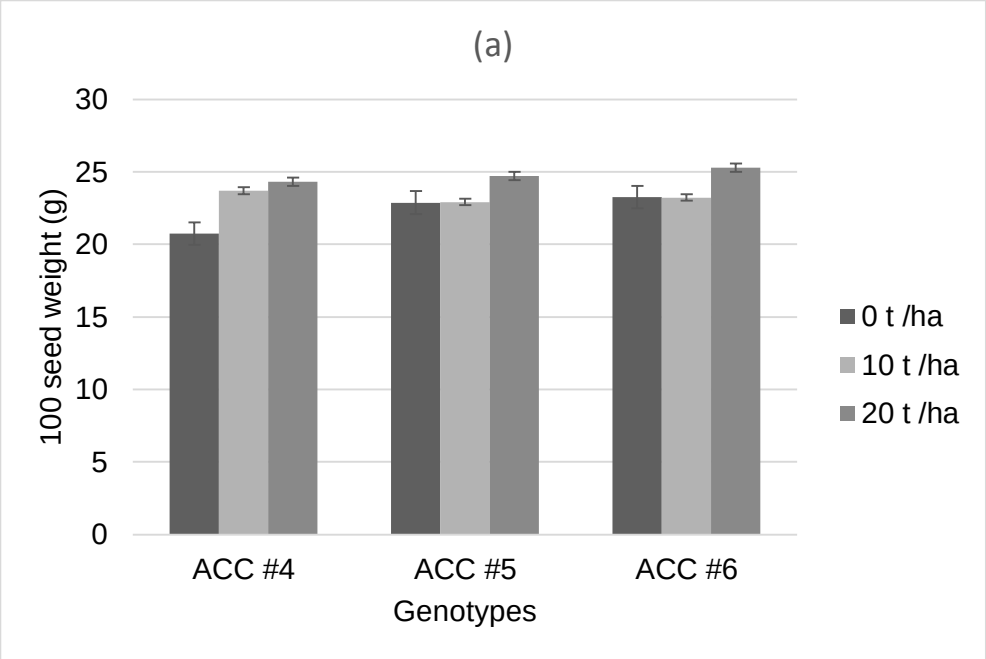

Figure 9.3: Effect of biochar and genotype on 100 seed weight in Thohoyandou during winter 2016

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9.4 Discussion

Above ground above ground biomass

Biochar, rhizobium inoculation and genotype affected above ground biomass in 2015 but not in 2016. There was an increase in biomass with biochar application rates. The response of biomass to biochar application could be associated with an increase in soil pH from 6.36 to 7.08 due to biochar application and hence improving the availability of important plant nutrients such as phosphorus in the soil. Increase in biomass could also be associated with increase in nodulation which was observed in the study. Correlation analyses showed a significant correlation between biomass, number of nodules and nodule dry weight (r = 0.54 and 0.58, respectively) (Appendices). The ability of biochar amended soils to retain and increase nutrient availability could be the driver for improved crop growth and biomass accumulation (Chan *et al*., 2007). Recently, Lusiba (2015) reported an increase in biomass with biochar application in the current study location which was associated with increased soil N, P and organic carbon. Furthermore, biochar application is likely to result in positive response in plant growth in acidic soils compared to neutral and alkaline soils (Macdonald *et al*., 2014). This could explain the positive response of biomass to biochar application in the current study due to slightly acidic pH of the soils used. Wood biochar tend to be more suitable for soils with low capacity to buffer chemical changes and hence improving crop growth (Yilangai *et al*., 2014).

Rhizobium inoculation increased the total above ground biomass. The response of biomass to rhizobium inoculation could be associated with higher nodulation (number of nodules and nodule dry weight which showed a significant correlation with chlorophyll content (r = 0.60 and 0.56) and soil pH (r = 0.53 and 0.57). Higher nodulation and chlorophyll content with rhizobium inoculation could serve as an indicator of increased BNF in inoculated plots hence higher biomass production. Similarly, inoculation increased grain yield, number of pods, and harvest index. Also, the increased biomass with inoculation could be partly attributed to high N content in the leaves which was reflected by the high chlorophyll content measured. Nitrogen is one of the most important plant nutrients responsible for plant growth and development and any practice that improves availability of N may lead to increased crop growth. Similarly, Nishita and Joshi (2010) reported that crops inoculated with rhizobia produced higher stover yield compared to uninoculated crops. Uninoculated plants may often show symptoms of N deficiencies especially where there is no source of mineral N provided to the plants. Greater biomass with rhizobium inoculation was reported in the current study location (Ogola, 2015).

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The effect of genotype on biomass was significant. The three genotypes used in the study showed variation in a number of crop growth parameters such as height and proportion of intercepted radiation. These results suggest that the variation in the biomass production by the different genotypes used could be associated with factors affecting crop growth. This was evident with ACC #6 performing best in almost all parameters across sites and seasons and produced greater biomass compared to other genotypes. Imran *et al*. (2015) reported that increased biomass produced by certain genotypes is associated with high nodulation, chlorophyll content and other growth parameters. The ability of different genotypes to grow and produce higher yields is often limited by environmental conditions (Namvar *et al*., 2011). This suggests that the response of different genotypes may not be driven by internal factors only. Environmental conditions influences both genotypic and phenotypic responses since the ability of various genotypes to utilize available resources such as nutrients and water and translate that to higher yields may vary. It is clear that ACC #6 may be the most suitable genotype for the region of the current study.

The effect of biochar, genotype and rhizobium inoculation on biomass production was greater in Thohoyandou compared to Nelspruit. The greater biomass in Thohoyandou was probably due to the effects on growth parameters. Seasonal differences in temperature may have partially attributed to lower biomass in 2015 compared to 2016. Warmer and dry seasons may lead to lower biomass production especially when soil moisture content is low. Management practices play an important role in influencing growth and development of crops. Weeding and irrigation greatly affect biomass accumulation through reduced weed competition and water stress. The timing for weeding and irrigation in Nelspruit (due to distance) may have contributed to lower biomass obtained. It is likely that the crops had competition with weeds and also exposed to water stress as a result biomass accumulation was lower. The interactive effects of biochar, genotype and rhizobium inoculation on biomass was not significant in both seasons. Biochar application and rhizobium inoculation both improve plant growth through improved nutrient availability and moisture retention. However, it is not clear why the interactions were not significant although the main effects were significant.

Grain yield

The effect of biochar on grain yield was not significant in Thohoyandou and Nelspruit in both seasons despite the significant effect on above ground biomass. Similarly, the number of pods per plant and number of seeds per pod in plant was not affected by biochar application. Non-response of chickpea grain yield to biochar application has been reported in the current study location and

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was associated with no effect of biochar on water use efficiency of grain formation (Lusiba, 2015). The response of crop yields to biochar application may vary with the type of crop. Different crops respond differently to biochar application. Despite the type of crop, biochar and soil properties play a vital role in plant response to biochar applications. Agegnehu *et al*. (2015) reported that legumes respond positively to biochar application simply because biochar improves biological functions of soil biota thus improving the relationship between legumes and root colonizing bacteria thus enhancing absorption of water and nutrients. These result suggest that the increase in biomass with biochar application may have been due to biochar effects (on chlorophyll content, nodulation and soil pH) and other factors affecting partitioning of assimilates to biomass and grain yield.

Rhizobium inoculation increased grain yield probably due to increased nodulation with moderate correlation between grain yield and nodulation [number of nodules (r = 0.41) and nodule dry weight (r= 0.5)] (Appendix 2). The Addition of root colonizing bacteria through inoculation improves biological nitrogen fixation by the crop which enables the plant to fix adequate amount of N for growth and development. Improved nodulation also enhances water and nutrient acquisition by plants through improved root architecture. The high and significant correlation (r = 0.60) between nodulation and chlorophyll content may indicate sufficient availability of N. Increased grain yield was observed in inoculated plots compared to uninoculated plots which was associated with increased number of pods per plant (r = 0.85), seeds per pod (r= 0.04) and 100 S-W (r= 0.31) (Appendix 4). The increase in number of pods with rhizobium inoculation in legumes was reported in cowpea, peanut and chickpea (Agegnehu *et al*., 2015; Saxena *et al*., 2013; Namvar and Sharifi, 2011). The response of pod number to rhizobium inoculation was associated with increased branching, nutrient availability and uptake. Greater grain yield was also associated with the production of growth promoting substances produced by root colonizing bacteria such as rhizobia (Saxena *et al*., 2013). Similar findings were reported elsewhere (Namvar *et al*., 2011: Khan *et al*., 2014). Inoculation with root colonizing bacteria may lead to well developed and established root system which may improve nutrients and water uptake and ultimately leading to increased biomass and grain yield. Ogola (2015) reported the non-significant of grain yield to rhizobium inoculation which was partly attributed to low soil pH.

The interactive effects of rhizobium inoculation and genotype on grain yield, harvest index and number of pods per plant were significant only in 2015. Response to inoculation was greater in the high yielding ACC #6. Although no interaction of genotype and rhizobium inoculation was observed on nodulation, nodulation was greater in ACC #6 compared to rest of the genotypes. This may have

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contributed to the high yield produced by ACC #6. Compatibility of different rhizobium strains varies greatly with genotypes and environment. Selection of suitable strains for inoculation for specific genotypes and locations may be important for improving yield. The genotype that showed higher nodulation, chlorophyll content and plant height ultimately produced higher grain yield compared to other genotypes.

The effect of biochar, genotype and rhizobium inoculation on grain yield, harvest index, number of pods per plant, number of seeds per pod and 100-SW varied with site and cropping season. Yield and yield components were generally greater in Thohoyandou compared to Nelspruit. The different responses may partly be attributed to the difference in climatic conditions such as temperature. Singh *et al*. (2015) reported an increase in chickpea yield under irrigation which was attributed to warmer temperatures with no frost during flowering and podding. The higher altitudes in Nelspruit shows that winter temperatures are relatively low with high chances of frost which may result in reduced crop growth and then yield of chickpea. Photoperiod plays an important role in influencing productivity of crops (Thangwana and Ogola, 2012). Amongst other crops, chickpea is known to be sensitive to short daylength which reduces the rate of growth and development. Phenological developments are driven by photoperiod and that affect biomass accumulation and yield. The loamy sand soil in Nelspruit may be prone to leaching of nutrients hence resulting to lower yield. The greater yield in Thohoyandou could be attributed to better management (weeding and irrigation) and close monitoring of the experimental field throughout the seasons. The lower soil pH in Nelspruit indicates low nutrient availability and that may lead to reduced yield. Other than environmental factors, lower grain yields in Nelspruit may be partly attributed to management practices such as weeding and irrigation since close monitoring of the site was not possible. Therefore, possibilities of water stress and weed competition are likely to have contributed to lower grain yield in Nelspruit. Grain yield varied with cropping season with greater yield obtained in 2016 compared to 2015. There was a high rainfall at post flowering to harvesting during winter 2015 compared to 2016 and that may have contributed to lower yields in 2015. Higher moisture content at the advanced crop growth stage may lead to reduced yield. Similar observations were reported in the current site of study (Lusiba, 2015).

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9.5 Conclusions

Biochar application increased biomass and 100-SW in Thohoyandou. However, rhizobium inoculation increased biomass, grain yield, number of pods per plant, number of seeds per pod and 100-SW in both locations. The effects of biochar and rhizobium inoculation on yield and yield components were greater in 2015 than 2016 in Thohoyandou. Yield and yield components were greater in Thohoyandou compared to Nelspruit. ACC #6 performed best across all the locations and the parameters measured and may be a suitable genotype for the region of the current study. The results of the study suggest that application of biochar and rhizobium inoculation may be an option to improve chickpea productivity since it increased yield and yield components in these regions. However, the effects may be limited to specific location depending on the type of soil and climatic factors. Therefore, long term research on the effect of different biochars and rhizobium strains on yield and yield components in different locations may be necessary.

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CHAPTER 10: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

10.1 General discussion

Poor soil fertility remain one of the major constraints affecting crop productivity in arid and semi-arid regions such as the North Eastern region of South Africa. Incorporation of drought tolerant crop genotypes such as chickpea may improve crop productivity and ensure food security. Biological nitrogen fixation enables legume plants such as chickpea to fix atmospheric nitrogen which may reduce the need for N fertilizer. However, lack and poor nodulation of chickpea in these regions may lead to poor crop productivity. The lack of chickpea nodulation was partly attributed to lack of native rhizobia, low populations of infective rhizobium strains and low soil pH in these regions (Ogola, 2015). Application of biochar as a soil amendment and rhizobium inoculation may improve soil pH and add adequate populations of rhizobia to ensure nodulation and ultimately increasing crop productivity. Therefore, this study assessed the availability of native rhizobia and the effect of biochar and rhizobium inoculation on nodulation, growth, chlorophyll content yield and yield components of chickpea in two representatives of dry environments.

Biochar and rhizobium inoculation increased soil pH, nodulation, yield and yield components of chickpea in both locations. It also improved growth, chlorophyll content and phenology of three chickpea genotypes in Thohoyandou. The results of the study showed that biochar application increased soil pH of clay and loamy sand soils in Thohoyandou and Nelspruit, respectively in both seasons. Soil pH increased with biochar rates in Nelspruit. In contrast, there was no difference between 10 and 20 t ha^{-1} in Thohoyandou. Therefore, if biochar may be used primarily for increasing soil pH, then only 10 t ha^{-1} may be applied in Thohoyandou. Also, biochar application provided other benefits apart from the effects on soil pH. The increase in soil pH with biochar application was associated with the high pH and ash content of the biochar used in the study (Appendices 1). Biochar effects on soil pH depend primarily on the soil type and biochar application rates (Macdonald *et al*., 2014). The initial soil pH before biochar application in both locations was slightly acidic; acidic soils respond positively to additions of soil amendments compared to alkaline and neutral pH soils (Macdonald *et al*., 2014). Although biochar application rate had an effect on soil pH in the current study, cropping history may also have contributed to the results obtained. In Nelspruit, the experimental site was fallow for more than two years with grass species growing in the field and in Thohoyandou there were seasonal vegetables (cabbage and spinach) which were grown. This reflected on the initial soil pH as Nelspruit had a slightly lower soil pH 6.30 compared

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to 6.36 in Thohoyandou. The type of vegetation growing on the soil affects the soil pH with grass species generally reducing soil pH to acidic (Macdonald *et al*., 2014; Paneque *et al*., 2016).

Also, the results from the current study show that the soils from agricultural fields in Nelspruit and Thohoyandou lack native rhizobia. These findings suggest that the lack of nodulation without inoculation in these regions could be attributed to lack of native rhizobia. Moreover, rhizobium inoculation may not improve nodulation in extremely acidic and alkaline soils. These findings concur with the results by Ogola (2015) who reported lack of nodulation without inoculation and poor nodulation with inoculation which was partly attributed to low soil pH in Thohoyandou. Also, Khattak *et al*. (2010) reported that in soils that lack native rhizobia, inoculation with commercial inoculants may be useful in improving root nodulation and crop productivity if other factors such as soil moisture, pH and nutrients should are not limiting. The isolation and identification of *Ochrobactrum spp, Klebsiella variicola*, *Burkholderia cenocepacia*, and *Bacillus subtilis* from the experimental sites suggest that inoculation of chickpea with commercial inoculants may be necessary to improve nodulation in these regions. Lower soil pH in semi-arid regions may be one of the reasons these soils have low or lack sufficient populations of rhizobia. Indeed, the initial soil pH was lower (6.0-6.3) than the optimum pH range (6.9-7.2) for proliferation of bacteria (Laureen et al., 2016). Despite the lack of nodulation without inoculation, the identified strains are known as associate nitrogen fixers. The identified strains are able to form nodules with legume trees such as acacia (Ngom *et al*., 2004). It is likely that the increased nodulation with inoculation may be partly due to interaction of both the commercial inoculant and the resident bacteria. Increase in soil pH with biochar and rhizobium inoculation did not only affect nodulation but also number of days to 50% emergence. Similar observations were made by Paneque *et al*. (2016) who reported an increase in germination with biochar application rates which was associated with alterations in soil pH.

Biochar application and rhizobium inoculation improved nodulation in both locations and seasons. Number of nodules per plant and nodule dry weight increased with biochar and rhizobium inoculation rates. Greater nodule number was observed in Thohoyandou compared to Nelspruit. In contrast, nodule dry weight was greater in Nelspruit despite the lower nodule number. The increase in number of nodules per plant and nodule weight with biochar and rhizobium inoculation have also been reported (Gul *et al*., 2014; Yusif *et al*., 2016; Nishita and Joshi, 2010). The response of nodulation to both biochar and rhizobium inoculation could be due to increased soil pH which had

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a positive correlation with number of nodules (r = 0.53) and nodule dry weight (r = 0.57 thus improving the nitrogen fixing capacity of legumes (Gul *et al*., 2014).

Plant height, number of branches and the proportion of intercepted radiation were also high with application of biochar and rhizobium inoculation. The improved growth parameters led to higher grain and biomass yields which was attributed to greater number of pods, 100-SW and number of seeds per pod. Increase in soil pH with biochar and rhizobium inoculation improved number of nodule per plant and nodule dry weight which also varied amongst the chickpea genotypes and location. In Thohoyandou, ACC #6 had higher number of nodules per plant and nodule dry weight. In contrast, ACC #5 in Nelspruit showed higher number of nodules per plant and nodule dry weight. Despite the higher nodulation on ACC #5 in Nelspruit, ACC #6 produced greater yield in both locations. This results show that the performance of different crop genotypes may be affected by the environment.

The effect of biochar, genotype and rhizobium inoculation on total above ground biomass, grain yield, number of pods per plant, number of seeds per plant and number of seeds per pod was significant in Thohoyandou. Biochar contains important minerals which are important for plant growth and developments. Increase in availability of macro-nutrients such as N, P and organic carbon was reported on biochar amended soils (Lusiba et al., 2017; Nigussie *et al*., 2012). These results are comparable with the results reported by Macdonald *et al*. (2014) who observed an increase in grain yield with biochar application rates which was associated with an increase in the total biomass, number of pods per plant and number of seeds per plant. The increase in leaf yield and biomass of cabbage and lettuce was reported in biochar amended soils (Carter *et al*., 2013). The increase in yield of crops due to biochar application is influenced by the effect of biochar on soil properties. The increase in soil pH as a result of biochar application improves the availability of important plant minerals. The productivity of sunflower increased with application of biochar at 15 t ha^{-1} and this increase was attributed to availability of both water and nutrients in biochar amended soils (Paneque *et al*., 2016). The effect of biochar on yield and yield components is influenced by both biochar and soil properties. Also, environmental condition plays a role in influencing crop growth and development. Therefore the outcomes of biochar application may differ with environmental conditions. In the current study, the application of biochar did not affect yield and yield components of chickpea in Nelspruit.

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10.2 General conclusion

Biochar and rhizobium inoculation had a positive effect on soil pH of loamy sand and clay soils in Mpumalanga and Limpopo Provinces, respectively. The increase in soil pH with biochar application led to improved nodulation, crop phenology, growth and yield of chickpea. It is evident from the results of this study that the effects of biochar as a soil amendment are location and soil specific as the effect was more pronounced in Limpopo than Mpumalanga due to difference in environmental factors. Wood biochars are highly stable and recalcitrant due to their aromatic structures, therefore their agronomic benefits may not be seen over a short term period if applied alone, however the effects may be apparent when combined with growth and yield promoting inputs such as rhizobium inoculation. This was observed in a number of parameters in the current study. Moreover, biochar effects on soil properties and crop yield may be influenced by the nutrient composition of the biochar used which is determined by the feedstock type and the pyrolysis conditions. The results of this study show that the biochar used has the potential to improve chickpea productivity, but may give more benefits when incorporated with other fertilizer sources which provide essential nutrients for plant growth. Also, ACC #6 may be the suitable genotype for the region due its best performance in all parameters measured across all the locations. Therefore, biochar and rhizobium inoculation may be used as a resource-efficient and cost-effective strategy for improving chickpea productivity in dry environments.

10.3 Recommendations

- Molecular characterization of the strains isolated from soils collected in agricultural fields at Thohoyandou and Nelspruit showed the lack of native rhizobia. However, there is a need to assess the diversity of rhizobia in both agricultural and natural fields for better indication on the availability of native rhizobia in dry environments.
- Application of wood biochar and rhizobium inoculation increased growth, nodulation, yield and yield components of chickpea in Thohoyandou and Nelspruit. However, the effects varied with soil type and season. Therefore, more research is required to assess the effects of biochar and inoculation in these over time
- Amongst the three genotypes used, ACC #6 performed better across the locations and seasons and may be suitable for dry environments such as the site of the current study.

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- Biochar application and rhizobium inoculation improved soil pH in Thohoyandou (clay) and Nelspruit (loamy sand). However, the mechanisms which rhizobium inoculation affect soil pH are not well documented and further research is necessary.

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APPENDICES

APPENDIX 1. Chemical composition of pine wood biochar used in the experiments

Characteristics	Biochar
pH (H_2O)	9.23
EC (mS m^{-1})	40
Ash (mg kg^{-1})	28.40
C in ash (mg kg^{-1})	4.5
Moisture (%)	6.66
Total solids (%)	93.3
Organic Matter (g kg^{-1})	905
Organic -C (g kg^{-1})	547
Total C (g kg^{-1})	549
Total N (g kg^{-1})	0.70
C:N Ratio	776
Available P (mg kg^{-1})	4.89
Exchangeable cations cmol(+) kg^{-1}	
K	3.38
Na	0.53
Mg	0.89
Ca	5.23
CEC (cmol(+) kg^{-1}	1.94

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APPENDIX 2. Correlation analyses for number of nodules, nodule dry weight, total above ground biomass and grain yield.

	Number of nodules	Nodule dry weight	Total above ground biomass	Grain yield
Number of nodules	1			
Nodule dry weight	0.934228	1		
Total above ground biomass	0.542445	0.579282	1	
Grain yield	0.408711	0.492451	0.915263	1

APPENDIX 3. Correlation analyses for chlorophyll content, soil pH, number of nodules and nodule dry weight.

	Chlorophyll content	Soil pH	Number of nodules	Nodule dry weight
Chlorophyll content	1			
Soil pH	0.608712	1		
Number of nodules	0.600218	0.527549	1	
Nodule dry weight	0.560796	0.569759	0.934228	1

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APPENDIX 4. Correlation analyses for 100 seed weight (100-SW), total above ground biomass (TBM), grain yield (GY), harvest index (HI), pods per plant (P/P), seeds per plant (S/Plant), and seeds per pod (S/Pod)

	100-SW	TBM	GY	HI	P/P	S/Plant	S/Pod
100-SW	1						
TBM	0.29	1					
GY	0.31	0.91	1				
HI	0.14	0.06	0.44	1			
P/P	0.13	0.85	0.85	0.23	1		
S/plant	0.26	0.81	0.9	0.39	0.85	1	
S/Pod	-0.24	0.17	0.04	-0.24	0.33	0.28	1

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