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Root Phenotyping of Soybean (*Glycine Max* (L.) Merr.) Related to Nodulation and Biological Nitrogen Fixation

By

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Submitted in partial fulfilment for the requirements for the degree

M.Sc (Agric): Agronomy

In the Faculty of Natural and Agricultural Sciences

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August 2021

UNIVERSITEIT VAN PRETORIA
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DECLARATION

I, **Nokwanda Octavia Dlamini** declare that the dissertation, which I hereby submit for the degree **Master of Science in Agricultural Agronomy** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: N.O. Dlamini Date: 31/08/20201

Nokwanda Octavia Dlamini

TABLE OF CONTENTS **PAGE**

DECLARATION i

INDEX OF FIGURES viii

INDEX OF TABLES x

ACKNOWLEDGEMENT xi

THESIS COMPOSITION xii

ABBREVIATIONS AND SYMBOLS xiii

ABSTRACT xv

CHAPTER 1 1

1.0 INTRODUCTION 1

1.1 Production of Soybeans in Africa 1

1.2 Production of soybean in the world 4

1.3 Types of Soybean cultivars 5

1.4 Nutrients supply by soybean plants 6

1.5 Symbiotic nitrogen fixation 9

1.6 Nodulation efficiency 12

1.7 Specificity in signaling for nodule development and infection 16

1. 8 Benefits of Nitrogen fixation 19

1.9 Nitrogen fixation on root growth and germination 21

1.10 Bradyrhizobium japonicum 23

1.11 Development and use of WB-74 in South Africa 25

1.12 Sinorhizobium fredii 25

1.13 Uses of Sinorhizobium fredii 26

1.12 Soybean X rhizobium compatibility and use 27

1.13 Starter Nitrogen on plant growth 27

1.14 Soybean and salinity 31

1.15 Aims and Objectives 32

1.16 Problem statement 32

1.17 Justification 33

1.18 Hypothesis 33

CHAPTER 2 34

2.0 METHODOLOGY 34

2.1 Plant material and Experimental procedure 34

2.2 Crop selection 34

2.3 Experimental Layout 35

2.4 Growing bacteria strains and culture conditions 37

2.5 Cell viability test 37

2.6 Plant material and growth 37

2.7 Root phenotyping and Cultivar choice 38

2.7.1 Cultivar choice based on germination percentage 38

2.7.2 Cultivar choice based on root length, angle and density 39

2.8 Effect of inoculating soybeans with SMH12 and WB74-1 40

2.9 The effect of starter nitrogen on nodulation 41

2.10 Preparing the roots system for analysis 42

2.11 Root architecture 43

2.12 Nodule number, size and weight 43

2.13 Ureide determination 43

2.14 Plant response to rhizobium and starter nitrogen 44

2.14.1 At cultivar choice and germination stage 44

2.14.2 When inoculated with SMH 12 and WB74-1 44

2.14.3 After the application of stater Nitrogen 45

2.15 Data analysis trials 45

CHAPTER 3 46

3.0 RESULTS 46

3.1 Root phenotyping and cultivar choice 46

3.1.1 Cultivar choice based on germination percentage 46

3.1.2 Cultivar choice based on root length, angle and density 48

3.2 Effect of inoculating soybeans with SMH1*2* and WB74-1 50

3.2.1 Plant Height 50

3.2.2 Root length 52

3.2.3 Root angle 53

3.2.4 Root density 54

3.2.5 Shoot Mass 57

3.2.6 Root Mass 58

3.2.7 Total mass **59**

3.2.8 Number of Nodules **61**

3.2.9 Nodule size **62**

3.2.10 Ureide content **64**

3.3 Effect of Starter Nitrogen (KNO_3) on soybeans inoculated with SMH12 and WB74-1 ..... **67**

3.3.1 Plant height and number of leaves **67**

3.3.2 Root length **69**

3.3.3 Root angle **70**

3.3.4 Root density **71**

3.3.5 Shoot dry mass **74**

3.3.6 Root dry mass **75**

3.3.7 Number of Nodules **77**

3.3.8 Nodule size **79**

3.3.9 Nodule weight **81**

3.3.10 Ureides Content **83**

CHAPTER 4 ..... **86**

4.0 DISCUSSION 86

4.1 Effect of inoculating soybeans with SMH12 and WB74-1 86

4.2 Effect of starter N on nodulation 92

CHAPTER 5 96

5.0 CONCLUSION 96

FUTURE PROSPECT 97

CHAPTER 6 98

6.0 REFERENCES 98

CHAPTER 7 116

7.0 APPENDIX 116

INDEX OF FIGURES

Figure 1. 1: Soybean production in SSA the leading soybean producing countries; South Africa, Nigeria, Zambia, and Uganda from 1987 to 2016 (Khojely et al. 2018). 2

Figure 1. 2: The increase in the production area and yield of soybean (Scheltema et al.). 3

Figure 1. 3: Leading soybean producing countries in the world, Argentina, Brazil, South Africa, and United States of America from 1998 to 2017 (SAGIS and FAOSTAT). 4

Figure 1. 4: The nutrient content of the seeds of soybean (National Oilseed Processors Association) 7

Figure 1. 5: Physiology of nutrient acquisition by soybean (Ohyama et al. 2017b)..... 9

Figure 1. 6: Mechanism of nodule formation in rhizobium-legume symbiosis (Singh et al. 2019). 10

Figure 2. 1: Plants growing in the brown pvc pipes. ……36

Figure 2. 2: a. The soybean seed on paper towel 1 day after planting, b. The germination paper in the growth chamber, c. Seedlings considered to have emerged, d. Seedlings 10 days after planting, ready for measurements. 39

Figure 2. 3: Protractor sketch board for measuring the root angle 40

Figure 3. 1: The mean germination percentage of five (5) soybean cultivars at 1,3 and 10 days after planting……47

Figure 3. 2: The mean germination percentage of three (3) soybean cultivars 1,3 and 10 days after planting. 47

Figure 3. 16: The effect of SMH12 and WB74-1 on ureides content of soybean cultivars..... 65

Figure 3. 18: The effect of different rates of starter N on plant height plants with A. SMH12 B. WB74-1 and C. Uninoculated Control..... 68

Figure 3. 19: The effect of different rates of starter N on root length. 69

Figure 3. 20: The effect of different rates of starter N on Root angle. 70

Figure 3. 21: The effect of different rates of starter N on root density..... 71

Figure 3. 22: The effect of different levels of starter N on root density. 72

Figure 3. 23: The effect of different levels of starter N on shoot dry mass. 74

Figure 3. 24: The effect of different levels of starter N on root dry mass. ……...75

Figure 3. 25: The effect different rates of starter N on number of nodules. 77

Figure 3. 26: The effects of different rate of starter N on nodulation in plants inoculated with SMH12..... 78

Figure 3. 27: The effect of different rates of starter N on nodule size..... 79

Figure 3. 28: The size of nodules from plants with starter N. 80

Figure 3. 29: The effect of different rates of starter N on nodule weight. 81

INDEX OF TABLES

Table 1. 1: Average soybean yield (kg ha^{-1}) in the leading producing countries in SSA year 2012-2016 (Mundi 2017, USDA 2017). 2

Table 2. 1: Cultivars used in the trial ……35

Table 2. 2: Characteristics of rhizobium strains 36

Table 2. 3: Composition of nitrogen free nutrient solution based on Hoagland..... 41

Table 2. 4: The rates of starter Nitrogen (Potassium Nitrate)..... 42

Table 3. 1: The root length, root angle and root density of soybean cultivars at germination ….49

Table 3. 2: Standard deviation for SMH12 and WB74-1 on the plant height, root length root angle and root density of soybeans 56

Table 3. 3: Standard deviation for SMH12 and WB74-1 on the shoot, root and total dry mass .. 60

Table 3. 4: Standard deviation for SMH12 and WB74-1 on the nodules/plant, nodule size and ureide content..... 66

Table 3. 5: Standard deviation for the plant height, root length angle and density 73

Table 3. 6: Standard deviation for shoot dry mass, root dry mass and number of leaves 76

Table 3. 7: Standard deviation for nodules/plant, nodule size and nodule weight 82

Table 3. 8: Standard deviation for ureide content in nodules and leaves 85

ACKNOWLEDGEMENT

I would like to thank the almighty God for all the blessing and guidance. I would like to express my sincere gratitude to my supervisor Prof JB Vorster for the guidance and patience throughout this journey. This work would have not been possible without the constant support, guidance and assistance. I would like to thank Tsholofelo Kibido for her constant support with my labwork, you have given me the gift of encouragement and hope in difficult times.

My deepest gratitude goes to the MasterCard Foundation Scholars Program for their financial support and mentorship. Thank you for providing the kind of support that I needed, I have never someone so generous as you. I would also like to appreciate Dr Mavuso for the assistance with data analysis.

Many thanks to all may lab mates (Tsholo, Molly, Ofentse, Adelaide and Thatego) thank you for your continuous encouragement, support and company. To my friends I appreciate the moral support and always being a shoulder to cry on. Your friendship made writing less of an isolation slog through hell. My sincere gratitude goes to CRC family, thanks for the prayers and words of encouragement.

A special gratidute to my family whose constant love and support keep me confident and motivated. My accomplishment and success are because they believed in me. A special thanks goes to Mr Comfort Dlamini, and his wife for always looking out for me and always being available to assist. Deepest thanks to my siblings, who keep me grounded, reminding me of what is important in life and always supporting me. To my mother, I am sure you are proud of me wherever you are. Thank you for the undying love and encouragement.

THESIS COMPOSITION

Chapter 1 of this thesis elaborately discuss the current state of soybean production in Southen Africa and in the world. Nutrient acquisition by soybean . it als extensively describes the process of symbiotic nitrogen fixation , and the condition for symbiotic nitrogen fixation. The benefits of nitrogen fixation in agriculture. The last part provides information on the impact of starter nitogen on nodulation legumes. The aims and objectives of the study and problem statement are outlined.

Chapter 2 comprises of the materials and methods used in the study. The process of bacterial growth for the strains used in the experiment. The description of the doll method for seed germination for the germination trial, cultivar selection, planting, growing and harvesting of the plants. Lastly the method for measuring different parameters and ureides analysis. **Chapter 3** presents the results obtained, and siscusing the key results and how the contributed to plant growth and nodulation. Chanes in plant growth and nodulation when different rates of starter nitrogen was applied. Chapter 4 the results obtained are deeply discussed , the ones contributing to the differences in plant growth and nodulations between *B japonicum* and *S fredii*. The changes brought by different rates of starter nitrogen on nodulation. The c**onclusion** outlines the rhizobium strain observed to be more efficient in nodulation, and the recommended starter nitrogen rate for effective nitrogen fixation. Under **references** is the literature used and cited in the thesis. **Appendices** contains figures used in analysis.

ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
ha^{-1}	Per hectare
SSA	Sub-Suharan Africa
Mt	Metric tonnes
N_2	Nitrogen
KNO_3	Potassium Nitrate
LCOs	Lipochitooligosaccharides
NFRs	Nod factor receptors
ATP	Adenosine triphosphate
0_2	Oxygen
NH_3	Ammonia
FeMoCo	Iron-Molybdenum Cofactor
NCRs	Cysteine-rich peptides
SO_4^{2-}	Sulfate
Fe^{2+}	Iron (ıı)
Mg^{2+}	Magnium
MoO_4^{2-}	Molybdate
HCL	Hydro Chloric Acid
NaOH	Sodium hydrocide
dH_2O	Sterile distilled Water
H_2O	Water
PGPR	Plant Growth- Promoting Rhizobacteria
DNA	Deoxyribonucleic acid
BNF	Biological Nitrogen Fixation
IAA	Indolyl3-Acetic Acid

CRD	Complete Randomised Design
LSD	Least Significant Difference
SAS	Statistical Analysis Software
WB-74	*Bradyrhizobium japonicum*
SMH12	*Sinorhizobium fredii*
B. japonicum	*Bradyrhizobium japonicum*
S. fredii	*Sinorhizobium fredii*
SARCC	South African Rhizobium Culture Collection
Mg/ml	Milligram per milliliter
µg/µl	Microgram per microliter
mM	Millimole
YEM	Yeast Extract Mannitol

ABSTRACT

Soybean is an important leguminous crop, mostly used for soybean cake and soybean products for human consumption. The increase in cost of fertilizers has led farmers to opt for biological nitrogen fixation. This is a process whereby atmospheric nitrogen is converted to organic form by symbiotic microorganisms in the soil. Therefore, there has been a need to identify the best cultivar X rhizobium interaction. Nitrogen application at planting may reduce N_2-fixation on soybean and yield, however results from previous researchers have been inconclusive. This trial was conducted in a glasshouse to assess the effect of *Bradyrhizobium japonicum* and *Sinorhizobium fredii* strains on nodulation efficiency of soybean cultivars. This will also help in understanding if the different cultivars perform the same or the interaction is cultivar specific. The first phase, was a germination trial with five soybean cultivars and three cultivars with the highest germination percentage were chosen. After that root length, root angle using a protractor, and root density was measured. PHB 95Y 20R had significantly high ($P \leq 0.05$) root length, root angle and root density followed by PHB 94Y 80R, there was no significant difference between Williams 82, PAN 1521R and PHB 96T 06R. In the second phase the three cultivars PHB 94Y 80R, PHB 95Y 20R and PAN 1521R, were planted in a glasshouse, each cultivar was inoculated with the two strains including an uninoculated control. Nodule parameters namely, nodulation rate, nodule number per plant, nodule volume per plant, and nodule dry weight were measured. Ureide assays were also conducted to determine the amount of nitrogen in the nodules. Cultivar PAN 1521R had the highest efficiency. After that one cultivar was planted with starter Nitrogen source KNO_3 at four rates 0mM, 5mM, 10mM and 15mM. To find the effect of starter nitrogen on nodulation as researcher have inconclusive results on whether it inhibits root growth and activity thus affects nodulation. Plants inoculated with *S. fredii* exhibited inhibition of nodulation

when N rate was more than 10 mM KNO_3 and in *B. japonicum* 5 mM KNO_3 inhibited nodulation.

Keywords: Nodulation, *Bradyrhizobium*, *Sinorhizobium* and soybean.

CHAPTER 1

1.0 INTRODUCTION

1.1 Production of Soybeans in Africa

Dry Soybean (*Glycine max* (L.) Merr.) have always been considered a good source of protein in livestock and human diet (Mpepereki et al. 2000). It is one of the essential crop in agricultural production worldwide (Lu et al. 2017). According to Ishikawa et al. (2018) nnual production of soybean was 2290 kg ha^{-1} in 2016, and being the fourth most important grain crop following maize, paddy rice, and wheat. South Africa produced the highest amount of soybean in 2016, compared to Zambia, Nigeria, and Uganda in Sub-Saharan Africa (SSA). Other soybean producers that have experienced commercial soybean expansion, included countries like Zimbabwe, Ghana, Malawi, Ethiopia, and Sudan. The increase in area planted and production of soybean for the leading countries in SSA during the period of 1987 to 2016 are shown in Figure 1.1 (Rusike et al. 2013). The average yield of soybean produced in South Africa, Nigeria, Zambia, and Uganda between 2012 and 2016 are shown in Table 1.1 (Khojely et al. 2018) .

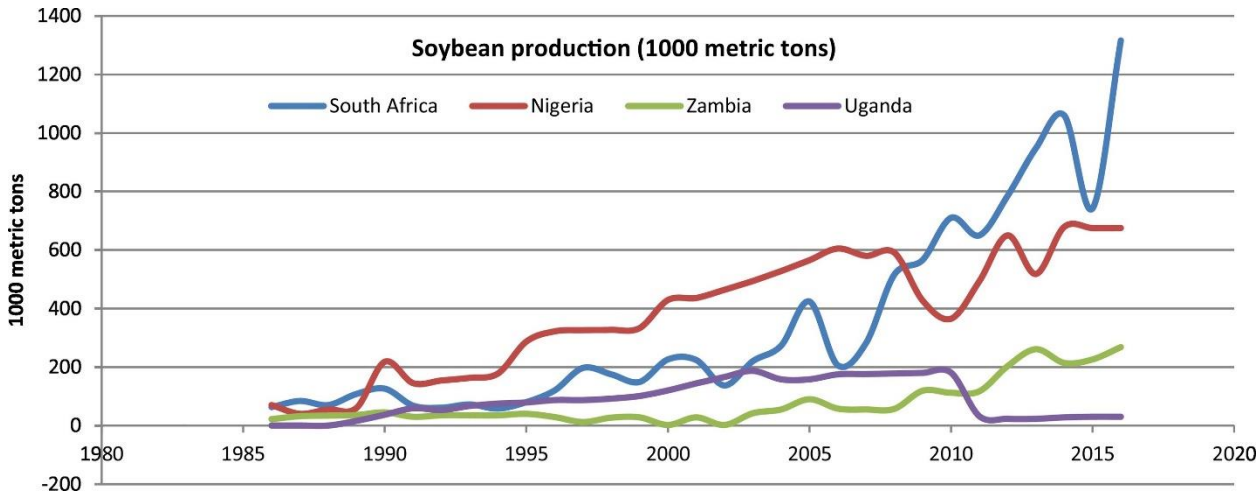

Figure 1. 1: Soybean production in SSA the leading soybean producing countries; South Africa, Nigeria, Zambia, and Uganda from 1987 to 2016 (Khojely et al. 2018).

Table 1. 1: Average soybean yield (kg ha^{-1}) in the leading producing countries in SSA year 2012-2016 (Mundi 2017, USDA 2017).

Country	2012/2013	2013/2014	2014/2015	2015/2016	2016/2017
South Africa	1300	1620	1450	1480	2290
Nigeria	1000	1000	1000	960	960
Zambia	1200	1200	1560	2020	1940
Uganda	630	650	600	600	600

The sharp increase in the production and planting of soybean over the past years, is main driven by factors such as an increase in soybean processing capacity of locally produce to reduce soybean imports (Meyer et al. 2018). Farmers have also realised the positive impact of soyabean if used in crop rotation with maize in their summer grain production plan. As a result, the planting area for soybean have doubled over the past 10 years (Figure 1.2). The annual

production of soybean was 1.5 million tons in 2019/20 with an increase of 14% from the previous season (Mbatyoti et al. 2020).

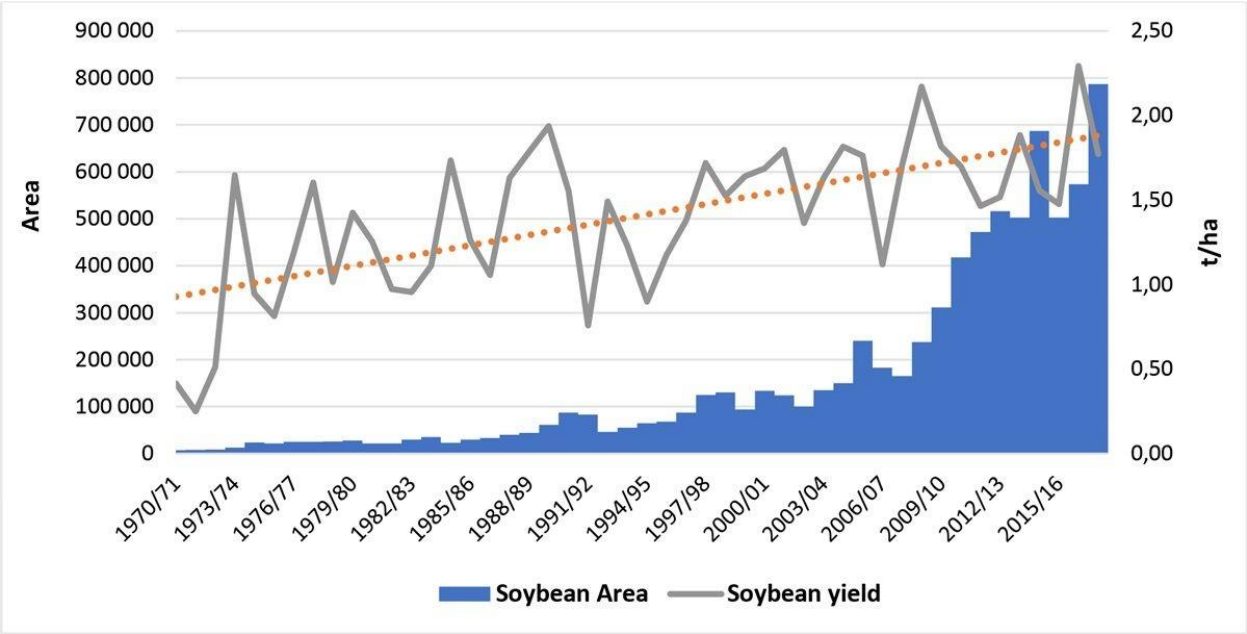

Figure 1. 2: The increase in the production area and yield of soybean (Scheltema et al.).

South Africa, Nigeria, and Uganda have retained the top ranks in the production of soybean in the SSA region. There are a number of reasons for leading in the production of soybean, these include (1) government putting policies in place for local food production instead of importing; (2) ability of improved varietis to adapt to environmental conditions; (3) inclusion of soybean in traditional recipes; (4) the use of soybean in vegetable oil, baby food and animal feed by manufactures and (5) stakeholders like hospitals encourage the use and production of soybean (Shurtleff and Aoyagi 2020).

1.2 Production of soybean in the world

The Bureau for food and Agricultural Policy (BFAP) said the producers of soybean in South Africa are currently competing with the USA, Brazil, and Argentina (Figure 1.3), this is because the yield of these countries has increase by up to 1.5% yearly over the few decade. However, the yields in South African have risen by 0.4% yearly over the same period. The area under soybean cultivation was around 111 M ha in year 2013. There has been an increase also in the world production considering what it was twenty (20) years ago (79 M t in 1983, 115 M t in 1993, 191 M t in 2003, and 276 M t in 2013) (Ohyama et al. 2017b). The leading soybean production countries in the world (annual production in 2013) includes the USA (89.5 M t), Brazil (81.7 M t), Argentina (49.3 M t), China (12.5 M t), and India (12.0 M t) (Ohyama et al. 2017b).

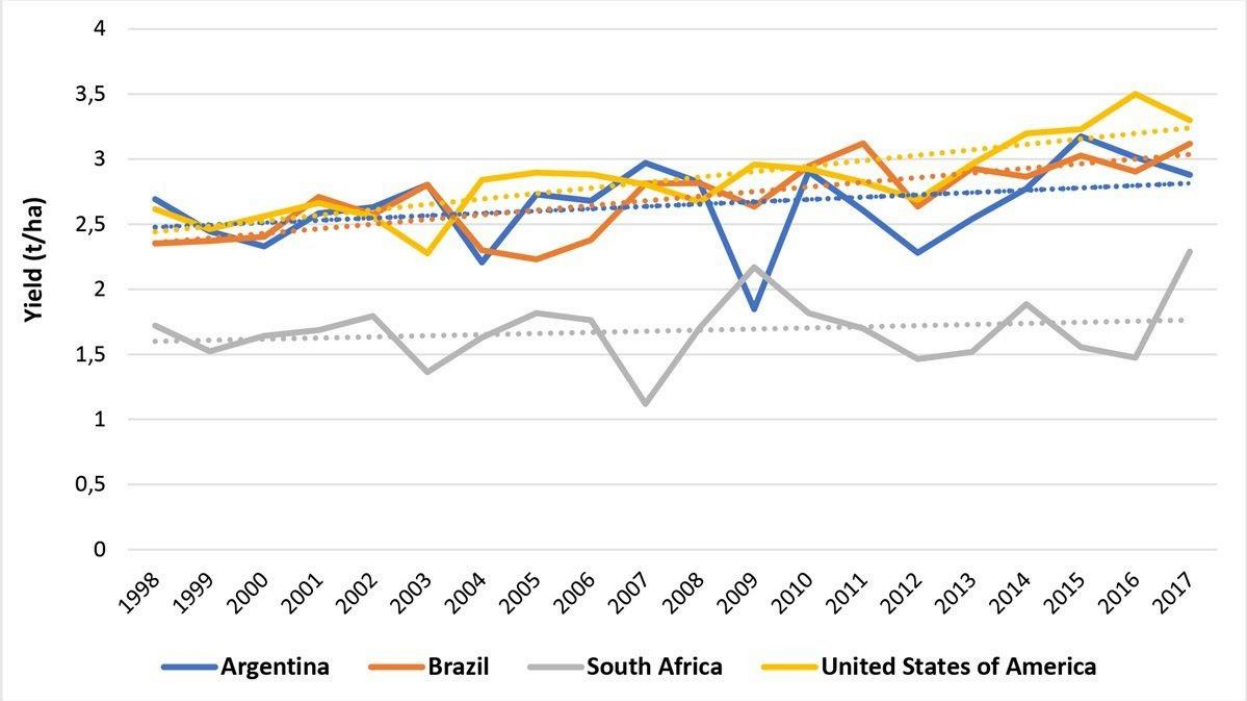

Figure 1. 3: Leading soybean producing countries in the world, Argentina, Brazil, South Africa, and United States of America from 1998 to 2017 (SAGIS and FAOSTAT).

Board and Kahlon (2011) said soybean yields in the world are increasing because of improved cultivars and genetic and cultural practices. Improved cultural practices have shown sustainability in yield gain. In the USA, cultural practices improvement increase yield anywhere from 25 to 66%. The steady increasing interest in the growing of soybean in South Africa connected to the health benefit associated with its products (Figure 1.3). The consumption of soybean in South Africa is estimated to be at 32% for oil and oilcake, 60% for animal feed (mainly broiler and egg production) and 8% for human consumption (Joubert and Jooste 2013).

On the other hand, even though the yields are increasing, but the increase is slower and some of the reasons for the slower improvement in the yield of soybean, is the shortage of new good-performing cultivars and shortage of fertilizer especially Nitrogen and lack of sustainability in rhizobial inoculant use. This is caused by a practice common worldwide of practice of using open-pollinated soybean cultivars as planting material (Dlamini et al. 2014). The average yield in the USA in 2017 was approximately 4t/ha whilst in South Africa it was below 3t/ha (Figure 1.1).

1.3 Types of Soybean cultivars

There are several soybean cultivars that are being developed. It is essential to evaluate these cultivars to determine their nodulation efficiency and adaptability to the environment (Maphosa and Maphosa 2015). Soybean genotypes are categorized into two groups: promiscuous and non-promiscuous soybean genotype. The promiscuous soybean cultivars nodulate naturally, while the non-promiscuous soybean cultivars nodulate with specific cultivars. They usually nodulate when inoculated with commercial rhizobial strains (*Sinorhizobium fredii* and *Bradyrhizobium japonicum*). The inoculation is done prior to planting in order to form active nodules. The

promiscuous soybean cultivars are more compatible with cowpea-type inoculant, the indigenous rhizobia (Bello et al. 2018). This suggest that rhizobial strains are host specific.

1.4 Nutrients supply by soybean plants

Soybean plants need N in large quantities, the reason being the seeds have a high protein content which is between 35% and 40% (Figure 1.4), and the total N content in the shoot after harvesting is proportionate to the seed yield (Saito et al. 2014). These days farmers have the advantage of applying rhizobium bacteria, into soils because in some soils the population is lower than the requirement. It is important that the rhizobium is applied effective, and efficiently in the soil for formation of nodules in the soil environment (Lim et al. 2014). The bacteria must also survive the high competition and maintain to survive in high levels through natural changes in the environment (Barakat et al. 2012). The nitrogen supplied usually do not meet all the plant's requirements. Bortolon et al. (2018) said some of N_2 is fixed by legumes of agriculturally important and some is fixed by legumes found in natural ecosystems, through symbiotic association with rhizobium (e.g., Bradyrhizobium and Sinorhizobium).

The soybean roots develop root nodules and use atmospheric N_2 that is generated in the symbiotic relationship between rhizobia and roots. This nitrogen compensate nitrogen from inorganic fertilizers to improve yields (Saito et al. 2014). It is also worth observing that the plant is the main determinant of the response of plants to nitrogen fixation, nodulation rate and the growth of the plants not the rhizobium (Van Dao et al. 2008). Crop rotation increases the rhizobia population in soils. However, Furseth *et al*. (2012) suggested that competition ability of rhizobia strains have also influenced inoculation efficiency. Factors like soil nitrate concentration, soil phosphorus content, soil pH and moisture content can also affect rhizobia

population number, biological nitrogen fixation and the nodulation efficiency (El-Sharkawi e*t al*. 2007).

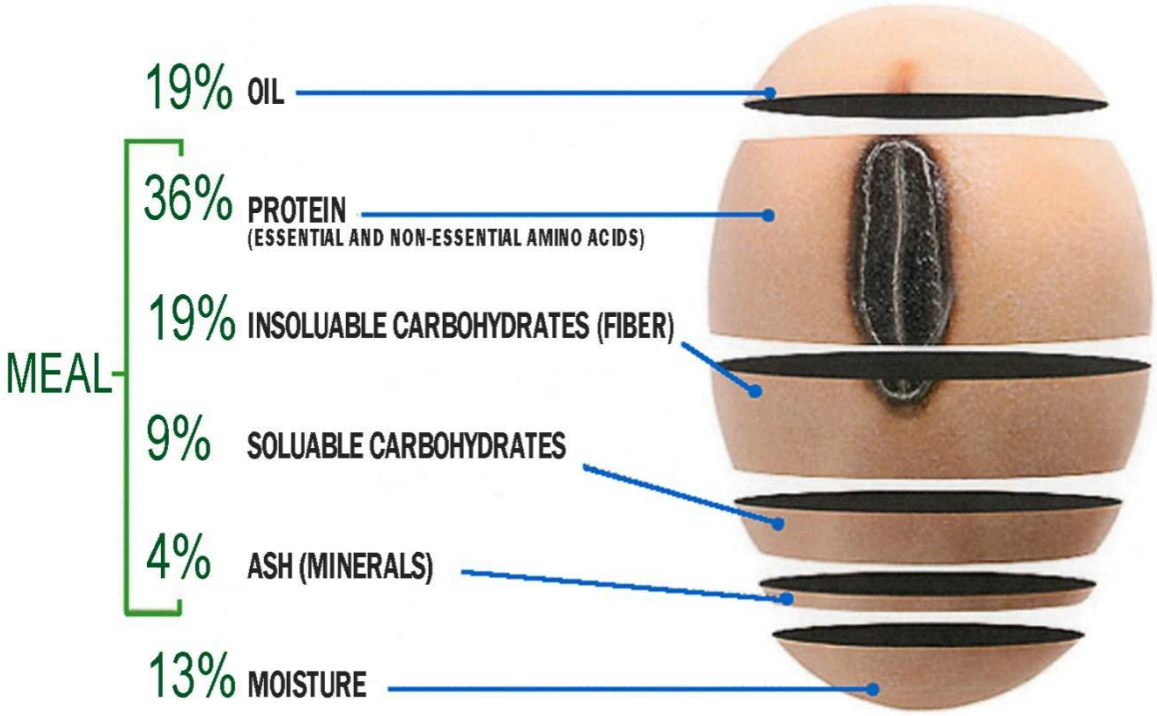

Figure 1. 4: The nutrient content of the seeds of soybean (National Oilseed Processors Association)

The effectiveness of biological nitrogen fixation is important for supplying the nitrogen requirements of soybean seed. The nitrogen content in the soil must be maintained throughout the growing season because soybean have big seeds; nutrients are stored in the cotyledon to support the growth 7-10 days after planting. The nutrient content of soybean seeds (per 100g) includes water 12,5 g, proteins 35.3 g, lipids 19.0 g, carbohydrates 28.2 g and minerals 5 g (Ohyama et al. 2017b). Soybean development is catergorised into two stages; vegetative followed by reproductive development period as described by Fehr and Caviness (Bastidas et al.

2008, Li et al. 2008). During the vegetative stage of growth, there is the development of stem, leaves and nodes with the overlap of reproductive stage up until seed initiation (R) stage. These two growth stages differs by cultivar and cultivar site (Wei et al. 2015).

The vegetative stage usually takes about 2 months and the reproductive stage 3 months in most South African cultivars. The vegetative and reproductive stage are of great importance to high soybean seed yield. High amount of photosynthesis, enough water and nutrient absorption from the soil are crucial to boost vigorous plant growth (Wall and Heiskanen 2003). In addition to supply of nutrients into the soil (Figure 1.5), soybean plants fix atmospheric nitrogen (N_2) by root nodules, which work in a symbiotic relationship with soil bacteria called rhizobium (Ohyama et al. 2017b). On the other hand, high levels of soil nitrogen have been observed to inhibit the root growth, biological nitrogen fixation and nodules initiation. Nitrate, a major form of inorganic nitrogen in upland soil, strongly inhibits nodulation and N_2 fixation activity (Ohyama et al. 2017a). Nitrate inhibitions have negative results on nodulation efficiency. It decreases the nodule number, nodule mass, and N_2 fixation activity, as well as the increase in nodule senescence or disintegration (Saito et al. 2014, Alaru et al. 2017).

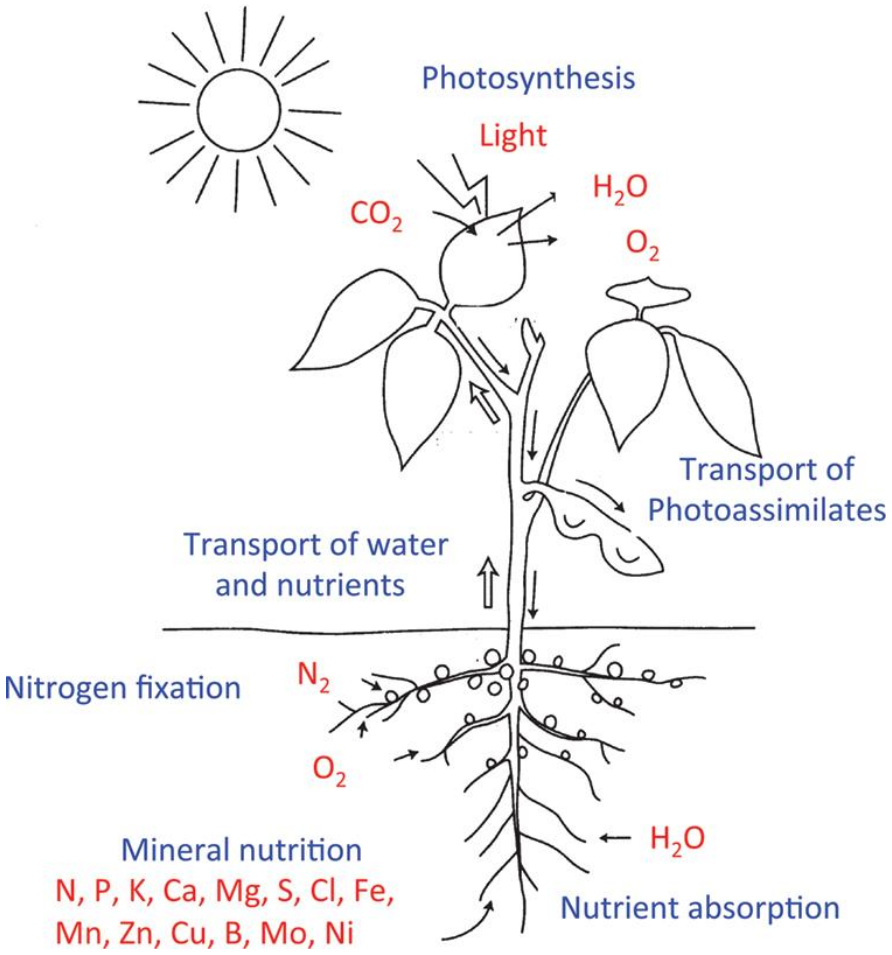

Figure 1. 5: Physiology of nutrient acquisition by soybean (Ohyama et al. 2017b).

1.5 Symbiotic nitrogen fixation

Legumes (Leguminous) family fix nitrogen in a symbiotic association with bacteria. They provide an alternative for reducing the use of nitrogen fertilisers. Legume plants are different from other plants in that they establish endosymbiotic association with soil bacteria called "Rhizobia". (Schwember et al. 2019) and (Franche et al. 2009). The nodulation process starts with the legume secreting phenolic compounds, mainly flavonoids and isoflavonoids into the soil environment. These signals are absorbed by rhizobia, which induce the synthesis of NodD proteins in rhizobia (Downie 2014, Mus et al. 2016a).

Nitrogen fixation takes place only in prokaryotes. The prokaryotes that fix nitrogen are called diazotrophs, and the most well-known symbiotic diazotrophs are called rhizobia (Mus et al. 2016a). In the legume-rhizobia symbioses, the nodulation process takes place in nodules (Figure 1.6). Legumes secrete different phenolic compound, which are responsible for triggering genes involved in the nodulation process and signal molecules like Nod factors whose function is induce organogenesis (Long 1996).

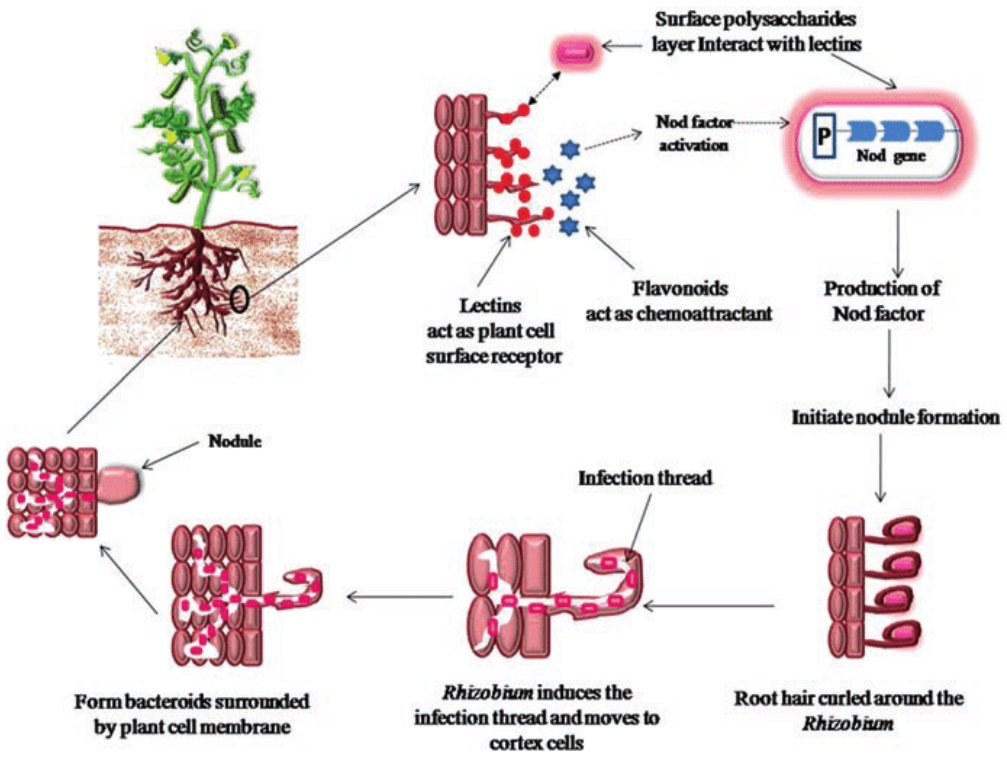

Figure 1. 6: Mechanism of nodule formation in rhizobium-legume symbiosis (Singh et al., 2019).

The nodulation genes (e.g., *nodABC)* code for the proteins essential to produce the lipochitooligosaccharides (LCOs) called Nod factor (Miyazawa et al. 2010). These signals play a significant role in the host specificity and rhizobium interaction process, Mus et al. (2016b) observed their importance also during nodule organonesis and infection process. They are very

efficient at low concentrations (nanomolar to picomolar range). Nod factors from diverse rhizobia, usually have the same structure of chitin-like N-acetyl glucosamine oligosaccharide backbone with a fatty acyl chain at the non-reducing end, but vary in their length of N-acetyl glucosamine oligosaccharide backbone and the length and the saturation of the fatty acid chain (Persson et al. 2011, Andrews and Andrews 2017).

The Nod factor core is altered by species-specific protein, which leads into a variety of substitutions, involving acetylation, methylation, glycosylation, and sulfation (Andrews and Andrews 2017). These decorations on the ends of LCOs perform a vital task in establishing whether the Nod factor will be recognizable to the specific host (Santi et al. 2013, Mus et al. 2016a). Nod Factor Receptors (NFRs) facilitates the interpretation of Nod-factor signal in legumes. These NFRs include the plasma membrane localized threonine/ serine receptor kinases in the local legumes (Berg et al. 1999, Andrews and Andrews 2017).

Nod factors are important in plant meristem formation and cell division. There are three ways in rhizobia infect legumes; through the crack entry, intercellular colonization of the epidermal cells and formation of the infection thread a well-explored mechanism (Mus et al. 2016b). The literature indicates that in the formation of infection thread mechanism; in most legume species, rhizobia gain entry into the root through the root hair infection (Svistoonoff et al. 2010, Miri 2017). In this method, the bacteria gain entry int the root hair and then the cell wall of the plant grows around the area where infection occurred (Giller 2001). This results in the development of the infection thread which eventually grow through the cortex and branch quickly (Duggan et al. 2007, De Bruijn 2015).

Mostly, the tip of the infection thread releases the rhizobia into the symbiosome, this is where they develop into nitrogen fixing structures called bacteroids. This rhizobium is usually kept in within the infection thread in almost all species of Caesalpinioideae and in a limited number of species in Papilionoideae (Sprent and Platzmann 2001). Bacteroids differ to a greater extent in their degree of differentiation and sustainability depends on the legume host. The root hair formation method can result in the development of either determinate or indeterminate nodules. The second method of rhizobial infection involves the rhizobial entering roots at the site of horizontal root emergence called the crack entry, in this method an infection thread is not used during the infection process. The last mode of rhizobial infection is where the rhizobia enter the roots through the root epidermis at the point of intersection between epidermal cells (Alexandre and Zhulin 2007, Andrews and Andrews 2017).

1.6 Nodulation efficiency

Nodulation is a complicated process involving different bacteria and plant signal (Ferguson 2013). The effective symbiotically root nodules formation includes interpretation of signal between microsymbiont (rhizobia) and host. This is followed by secretion of isoflavonoids and/or flavonoids from the of legume host root (Mathesium 2003). This induces transcription of Nod factor in compatible rhizobia, resulting in the development of lipochitooligosaccharide molecules that result to, signaling of the host to start the development of nodules (Graham and Vance 2003). One of the rhizobia strains that forms nodules in soybean is *Bradyrhizobium japonicum*. The host plant identifies nodulation genes through the LysM receptors on the root. These receptors identified in soybean are known as DmNFR1 and GmNFR5. Several changes

take place in host and bacterial gene expression during infection, and nodule formation process (Sprent and Platzmann 2001, Vance 2002).

Nodulation efficiency is described in relation of nodulation rate, size and the capability of the plant to fix atmospheric nitrogen into the roots. Wang et al. (2009) pointed out that the distribution of the nodule sizes differs in the different growth stages. The size if the nodules is attribute of partitioning of photosynthates to the roots. Nitrogen fixation can further be affected by the position of the roots in the soil and the growth stage of the plant (Tajima et al. 2006). In soybean and most legumes, nodules are formed on the taproot, however, they form on the lateral root in other crops like common beans (King and Purcell 2001).

Plants have established various mechanisms in with diazotrophs to acquire atmospheric nitrogen to increase nitrogen fixation efficiency (Elmerich 2007). Propinquity between plant host and bacterial symbiont is crucial in translocation of nutrient between them. This is categorized into three modes, based on the level of intimacy and interdependency of the plant and rhizobium: loose associations with free-living nitrogen fixers, intercellular endophytic associations, and endosymbioses (Li et al. 2011). Interaction between plants and rhizobia are the most common method of symbiotic relationship. These symbiotic bacteria react to root exudes via chemotaxis to, and colonization of the root environment of several plants. The proximity of the symbiotic nitrogen-fixing bacteria to the roots benefit the plant in nutrient absorption, growth and yield (Mus et al. 2016a).

The formation and effectiveness of symbiosis are also determined by the genetic make-up of both plant and bacteria. The compatibility of bacteria and host plant starts from recognition, penetration, stimulation of host cell division, and differentiation of the endosymbiont (Mus et al.

2016a). During morphogenesis of nodule in soybean, rhizobia differentiate into bacteroids are enveloped by a plant membrane (Soyano et al. 2019). The structures formed during this process are called symbiosomes and acts as plant organelles. During the symbiosis process there is reduction of N_2 instead of O_2 or CO_2 like in the mitochondria or chloroplasts of all plants.

Rhizobial nitrogenase is a multifaceted process, it consists of six protein subunits (two each of NifH and NifK and contain [two 4Fe-4S] and two (Fe_8S_7) iron-sulfur clusters and two iron-molybdenum cofactors (Fe_7MoS_9N) called FeMoco, which is the site of N_2 reduction. The nitrogenase metallo-centres are all very oxygen labile and the microaerobic niche is maintained at low levels of free oxygen (Smith et al. 2016). This is because nitrogen fixation takes place under anoxic conditions in strict anaerobes and only during periods of anaerobic growth (Santi et al. 2013). Respiration is needed to generate 16 ATP for reduction of N_2. Legumes maintain the low oxygen levels by expressing high affinity oxygen binding protein called leghemoglobin. This strategy facilitates rapid transfer of O_2 to energize nitrogen fixation (Udvardi and Poole 2013). Leghemoglobin are O_2 buffering proteins like myoglobin and hemoglobin that provides oxygen to mitochondria in the muscles. The difference with leghemoglobin is that the O_2 maintains a much lower concentration.

The effectiveness of symbiosis needs reduced growth of bacteria in the nodules. Legumes regulate this growth in various methods (Elmerich 2007). Firstly, rhizobia make ammonia (NH_3), which is toxic to the plant and cannot use it for growth. It is then quickly changed to compounds like glutamate and ureide compounds that are less toxic and are carefully translocated in the different parts of the plant (Emerich and Krishnan 2009). The NH_3 generated go to the plant because rhizobia need require amino acids. Rhizobia lack the gene needed for the production of homocitrate and depend on the nodule expressed plant gene product (FEN1) to synthesize this

important sign for FeMoco production from acetyl-CoA and α-ketoglutarate (Franche et al. 2009).

Legumes control the nitrogenase process in rhizobia (Ferguson 2013). Legumes from Galegoid group of legumes (peas, clover) have different degree of restriction, they synthesize nodule-specific, cysteine-rich peptides (NCRs) that are moved throughout the symbiosomal membrane and are directed to the bacteroid membrane (Downie 2014, Schwember et al. 2019). These peptides trigger bacterial endoreduplication without cell division and some permeabilization of the bacteroid membrane. Although these enlarged bacteroids can successfully fix nitrogen, they have lost the capacity to be restore and so pose no threat. The infecting bacteria still benefit, because some bacteria within nodule infection threads survive and grow when plant dies.

There are several internal and external factors that negatively regulate nodulation. These regulators result reduced nodulation rate, thereby balancing the total N-fixation with plant growth (Elmerich 2007). It also balances the nitrogen absorbed by the plant against its ability establishment and maintaining nodulation. This mechanism is called autoregulation of nodules (AON) pathway. However, super nodulating legume species lack AON and develop slow even with compatible rhizobia strain and exhibit poor yields. It relies on the signal generated throughout the development of nodule primordia (Downie 2014). This signal is transported to leaves and stems, and it is believed to stimulate the production of a signal that is then transported to the roots to reduce nodules development. Mutation in the leucine-rich repeat receptor kinase known as the Nodulation Autoregulation Receptor Kinase (NARK) in soybean, results in increased nodulation (Achom 2018).

There is also the development of Shoot-Derived Inhibitor (SDI). The SDI signal is translocated from the shoot back to roots through the phloem, where it also inhibits nodulation. SDI is in low quantities in soybean, and it is dependent on Nod-factor and NARK. In addition, CLE peptides act as regulators that suppress nodulation similar to Nitrogen- Induced CLE1 (NIC1), but are induced by nitrate not rhizobia and show less mobility and is perceived by roots, whereas CLE induced by rhizobia travel long distance and so are responsible for a translocation of signal from root to shoot (Desbrosses et al. 2009, Board 2013).

The relationship between autoregulation and high soil nitrogen (nitrate) and ethylene is the fact that they suppress nodulation, and this also takes place in autoregulatory mutants. Legumes acquire and translocate nutrients such as malate, SO_4^{2+}, Fe^{2+}, Mg^{2+} and MoO_4^{2-} across the peribacteriod membrane and this is a potential level of control; the legume transporter for SO_4^{2-} and Fe^{2+} (Sst1 and Dmt1, respectively) have been characterized, but others are still unknown (Saikia and Jain 2007). On the other hand, chemicals including phytoalexins and antimicrobial metabolites such as canavanine are produced by legumes, these limit bacterial growth during infection (Downie 2014, Pan et al. 2018)This show that legumes regulate the symbiosis and the bacteria only trigger nodule morphogenesis, induce infection, and fix nitrogen if the environment is favorable to the host plant (Ikeda et al. 2010).

1.7 Specificity in signaling for nodule development and infection

The successful formation of the legume-rhizobium symbiosis is dependent on two developmental programmes, one resulting to nodule morphogenesis in the plant cortex and the the infection threads formation (Downie 2014). Rhizobia are excellent in attaching to legume root hair, where they will deliver signals for the formation of nodules. Rhizobia have different hosts, which they

nodulate (Dénarié et al. 1992). There are several genetic and biochemical mechanisms by which regulatory and structural nodulation genes control host specificity (Roche et al. 1996). Legumes release flavonoids, isoflavonoids and other related compounds that binds to rhizobial transcriptional activators (NodD), they induce transcription of nodulation genes. The different NodD proteins are adapted to identify different flavonoids and isoflavonoids generated by the legumes. The *nod* genes are the major determinants of legume host specificity. These *nod* genes comprise both of species-specific and genes that are common to all rhizobium species (Andrews and Andrews 2017).

Differences in the Nod factors are recognized by membrane receptor-like kinases, conferring specificity in signaling. This induces root hair deformation, activation of nodule formation and initiation of the first step of infection. Some rhizobia are highly specific, nodulating a small number of hosts or only some closely related legumes species, while others are promiscuous have much wide host ranges (Iyer and Rajkumar 2017). There is aprominent rhizobium strain belonging to *Sinorhizobium* , that is capable of nodulating 112 genera.

The specificity in the Nod-factor is important for recognition between the symbiotic bacteria and the host plant, adding to the layers of specificity related to root attachment and rhizobial *nod* gene induction (Downie 2014). The regulatory *nodD* genes encode DNA-binding proteins that activate the transcription of the other nod operons (Long 1996). The specificity in some legumes can be determined by proteins secreted by rhizobial Type I, Type II, Type III and Type IV secretion system. These secreted proteins normal complement Nod-factors-induced signaling. *Bradyrhizobium japonicum* is one of the few rhizobial strains that induce nodulation without producing Nod factors. This strain is fully dependent on Type III secretion system.

The level of specificity between legumes and rhizobia differs. Some rhizobia have very narrow host ranges, composed of only one or a few closely related legumes species, whereas others have much broader host ranges (Ardley et al. 2013). Two rhizobia that nodulate the same plant may secrete different Nod factors. For instance, *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum*, Rhizobium sp. strain NGR234, and *Sinorhizobium fredii* strain USDA257 have several common hosts, but their Nod factors differ considerably (Wang et al., 2012). Rhizobia with a wide host rage are said to be promiscuous.

Although the most promiscuous rhizobial strain, NGR234, is a poor symbiont on many of its hosts, there are many instances in which, for example, a Bradyrhizobium strain is very efficient on several disparate hosts (Mania et al. 2020). It is striking how often members Bradyrhizobium, or closely related bacteria, are found as the dominant symbionts of legumes in the Caesalpinioideae and Mimosoideae, as well as in the diverse taxa of the Papilionoideae (Pessi et al. 2007).

Some strains may incite nodules but may not provide nitrogen fixation. Some combinations of plant–rhizobia are efficient, others not, and the nitrogen fixation effectiveness may vary even between closely related plant cultivars/bacterial strains. the host range of nitrogen fixation (‘effective’ symbiosis) is narrower than the host range of nodulation. Since soils may lack matching and/or effective rhizobia for certain legumes, especially when newly introduced to an area (Rodríguez-Echeverría et al. 2012), seeds of many legume crops need to be inoculated with compatible rhizobia. There are variant individuals within a host species that resist nodulation by a rhizobial strain that does well on most other races, cultivars, and varieties of that host. This

type of incompatibility can be strain dependent and sometimes has been traced to presence or absence of specific genes in the rhizobial strain (Andrews and Andrews 2017).

Another class of bacterial components that can interact directly with the host is bacterial surface polysaccharides: exopolysaccharides (succinoglycans and galactoglucans), lipopolysaccharides, capsular polysaccharides, and cyclic glucans. They have been reported in numerous studies as being symbiotically important, and depending on the system, a defect in surface polysaccharides may cause failure of symbiosis at either an early or late stage. The presence of proteins from Type III and Type IV secretion system can act as plant defense system, causing inhibition of nodulation (Downie 2014).

1. 8 Benefits of Nitrogen fixation

There is a heavy reliance on nitrogen for agricultural production to achieve high yields (Jensen et al 2012). This comes with increased risks of environmental pollution. Incorporation of legumes in farming system in modern agriculture, is highly beneficial to humanity and the environment. The process is importance as it decreases the dependence of agricultural crops on nitrogen fertilisers. Predictions show that symbiotic nitrogen fixation generates close to 200 million tonnes of nitrogen per year (Saikia and Jain 2007). Symbiotic nitrogen fixation is sustainable in agriculture, more especially in intercropping and crop rotation. Nitrogen fixing plants have lesser N fertilizers requirements and therefore consume less fossil fuel (Jensen et al. 2012) they work in association with bacteria. It reduces reliance on inorganic fertilizers and increase utilizing on biological nitrogen fixation in farming systems (Herridge et al. 2008).

It is predicted that soybeans generate at least 200 Kg N ha^{-1} in the dry mass per growing season. It is estimated that more than 68% nitrogen in soybean is from symbiotic nitrogen fixation. The

plant material remaining after harvesting contains 30-60% nitrogen, if left on soil surface or ploughed back into the soil, it adds to the soil organic matter. The N generated is basically free N for use by the crops in that soil environment. Substituting with inorganic fertilizer increase annually costs. Additionally, inorganic fertilizers in the soil most of the time are not easily available for plant absorption, the only option is to depend on N fixing organisms or N- fixation by legumes (Herridge et al. 2008). Soybeans are an important source of proteins in human diet and the combat malnutrition .

Agricultural sustainability is important in managing the N in the environment (Graham and Vance 2003). There has been an increase in the amount of nitrogen fertilizers used in recent years, it has been estimated to be around 10-fold to 90 million Mt (Raymond et al. 2004) with the increase in energy used for N fertilizer manufacturing and application to plants. It has been projected that the N needs will increase for agriculture for the period to 2030 (Fornara and Tilman 2008), and these requirements cause pollution to the natural environmental. It is important for farmers to practices more economically viable and environmentally prudent methods of N_2 fixation (Graham and Vance 2003).

Legumes also sequester Carbon, and has also been identified as a method to counteract escalations in levels of atmospheric CO_2 while improving soil structure and texture. Binkley (2005) found that 0.11 ± 0.07 kg m^2 $year^{-1}$ of soil organic carbon was sequestrated from tree that fix nitrogen, compared to *Eucalyptus* spp., where there was no significant difference (Graham and Vance 2003, Joubert and Jooste 2013). Carbon sequestration under *Prosopis* spp. has also been reported (Vance 2001).

The soil condition needs to be managed well for symbiotic nitrogen fixation to occur. Factors like drought, N fertilization, soil acidity and nutrient limitations suppress or lead the process not taking place. Some cultivars have inadequate capacity to fix symbiotic nitrogen- fixation due to lack of compatibility (Mohammadi et al. 2012). Acid soil management is of great significance in agriculture. (Udvardi and Poole 2013). Acid-tolerant legumes cultivars and rhizobia determine the management strategy of soil acidity (Liu et al. 2011). The soil acidity is controlled by the application of lime until a pH at which Al and Mn are no longer toxic to plant roots. The benefits from N_2 fixation are dependent on the availability of P in soils, with 33% of the world's arable land lacking P. Tropics and subtropics soils are acid-weathered and are more prone to P deficiency. Even the application of adequate P fertilization, <15% of the applied P will be readily available for plants uptake (Graham and Vance 2003).

1.9 Nitrogen fixation on root growth and germination

Rhizobia a diverse bacterial species of phylum Proteobacteria, have the capability to create effective symbiosis with leguminous plants (Leite et al. 2017). Legumes have the benefit of entering a symbiosis relationship with bacteria (Downie 2014). When bacteria come into contact with conditions suitable for biological nitrogen fixation, nodules develop on the stem and roots of plants (Graham and Vance 2003). The development of these nodules is a highly controlled method, the effectiveness is controlled by the exchange of particular signaling molecules amongst the micro and the macro-symbiont (Oldroyd et al. 2011, Leite et al. 2017). There are hundreds of bacterial species (Peix et al. 2015) that have been isolated from different nodule of legumes (Leite et al. 2017).

The application of inoculum on legume seeds is considered the oldest agro-biotechnological process (Laranjo et al. 2014). The key objective of inoculating soybean seeds with rhizobia is to induce nitrogen fixation which release nitrogen to the plant. This helps in the improvement of crop yields through their exceptional capability to establish a symbiotic nitrogen-fixing in legumes (Hungria et al. 2015). This decreases utilization of inorganic fertilizers in cropping systems which are very costly. Nitrogen fertilizers causes conservation threats such as pollution of water bodies, eutrophication and emission greenhouse gases into the atmosphere, leading to global warming and climate change (Clúa et al. 2018). Biological nitrogen fixation results in suppression of ecological pollution (Tauro et al. 2009).

Inoculant strains needs to be compatible with the cultivars of the host legume, environmental conditions and rhizosphere (Laranjo et al. 2014). Another important attribute is the ability of the strain to persist in soils over a long time (Dixon and Kahn 2004). When the conditions are ideal inoculation rates, sufficient inoculant strains always dominate in nodulation surface (Prasanna et al. 2012).

The effective usage of nitrogen fertilizers in crop production is of great significance, it has the potential to increase food security and nutritional safety (Samudin and Kuswantoro 2018). Soybean seed is composed of 40% protein, 20% oil and 30% carbohydrates (Maphosa and Maphosa 2015). Nitrogen fertilizers are considered essential for optimal plant growth and development, deficiencies result in yield losses. Plants have a high nitrogen demand to maintain the required number of proteins in the seeds (Arora 2015). Conley et al. (2008) said the nitrogen demand is usually achieved with the application of nitrogen fertilizers, which are often too expensive for small scale farmers. Fortunately, soybeans can develop nodules with rhizobium.

The soybean roots and rhizobium in the soil always have a symbiotic relationship (Iijima et al. 2011). This relationship is an advantage to the farmer, it reduced the costs and the need for nitrogen fertilizers. The rhizobium bacteria fix atmospheric nitrogen into the soil and make it accessible for plant uptake in the form of ammonium (Khojely et al. 2018).

Rhizobia are saprophytes, they have the capability to live in the root environment for a several years, approximately 15 years after inoculation (Zhang et al. 2020). Inoculum strains survive in the nodules after inoculation (Lindström et al. 2010). Several studied have revealed that rhizobia stimulate plant growth and development through different mechanism other than fixation of nitrogen (Chernin and Glick 2012). (Duan et al. 2009) described how the presence of ACC deaminase activity in rhizobia strain encourage plant growth and development through reducing the amount of ethylene in the plant. Furthermore, (Avis et al. 2008) reported an increase in resistance against plant pathogens when inoculated with rhizobia.

Plants inoculated with rhizobium perform better than when the only source of N_2 is obtained only from inorganic nitrogen fertilizers. It is necessary to inoculate with rhizobia to obtain maximum seed and N yields. This is because inoculation increase seeds of soybean plants and the total dry matter content, which is a good tool for measuring yield.it also improves the quality and quantity of soybean seeds (Sogut 2006). The symbiotic association also promote the uptake of other nutrients besides nitrogen like phosphate (Downie 2014).

1.10 Bradyrhizobium japonicum

Bradyrhizobium japonicum was described by Lindeque (2007) as slow growing bacteria that is different from other symbionts, as they produce alkaline reactions and are slow growing. *B japonicum* is a rod shaped and gram-negative nitrogen fixing member of the rhizobia

(Sameshima et al. 2003, Board 2013). The strain was first isolated from soybean nodules from Florida, USA, in 1957 (Ramírez-Bahena et al. 2009a). It is a large and diverse group of bacterial species. It includes members that possess accessory genes for nitrogen fixation, photosynthesis and/ or symbiotic interaction with legume plants (Wasai-Hara et al. 2020). *B japonicum* WB74-1 is a biological nitrogen fixing soil bacteria natural found in soil (Itakura et al. 2009). It forms nodules on soybean plants. It is also an oligotrophic bacterium since its cell density is high around 10^6 CFC (Okubo et al. 2012). *Bradyrhizobium japonicum* is under the family of *Bradyrhizobiaceae*, which falls under the order of *Rhizobiales* in the Alphaproteobacteria. Bradyrhizobium harbors several biochemical functions, these include photosynthesis, nitrification, sulfur oxidation, aromatic degradation, and oligotrophy (Itakura et al. 2009).

B japonicum is important in determination of molecular genetics, physiology, and ecology. It is regarded to be one of the of the superior symbiotic nitrogen fixing strain. *B japonicum* synthesizes several carbohydrates, which are involved in the BNF symbiosis, these includes lipopolysaccharides, capsular polysaccharides, exopolysaccharides (EPS), nodule polysaccharides, lipo-chitin oligosaccharides, and cyclic glucan (Mateos et al., 2001). *B japonicum* absorb sugars trehalose for energy, which is then converted to CO_2, UDP- glucose is absorbed in huge amounts but slowly broken-down. These sugars are important for plant growth (Board 2013, Kusakabe et al. 2020).

B japonicum contains a group of fix genes (*fix_chr*) discovered in the chromosome and a cluster of (*nif-fix_chr*) and the plasmid (*nif-fix_ pla*). The *fix_chr* cluster include *fixK_fixLJ*, which translates a component responsible for the control of biological nitrogen fixation. There is also f*ixNOQP* which encodes a micro aerobically stimulated cytochrome oxidase complex and *fixGHIS* that encodes a membrane-bound complex containing a cation pump involved in nitrogen

fixation (Okazaki et al. 2015). *B japonicum* when inoculated into seeds it induces root nodulation, with an average of 44 nodules/ plant. They form root nodules on legumes and establish nitrogen fixation. Most of the genes needed for symbiotic nitrogen such as nod, for nitrogen fixation and for secretion systems are located on the incorporated genome island called the symbiosome island (Okazaki et al. 2015).

1.11 Development and use of WB-74 in South Africa

Bradyrhizobium is a soil bacterium that is used by most farmers in South Africa for inoculation when growing soybeans. There is a diversity of Bradyrhizobium species in South Africa. *B japonicum* is one of the most important rhizobia in agriculture, it can develop nodules on soybeans. It promotes plant growth and carries out nitrogen fixation in the plant. The rapid growth of plants in turn is beneficial to the environment because, plants release oxygen which is inhaled by animals (Ramírez-Bahena et al. 2009b). They are adapted to different geographical area, climate, eater regime, soil pH, salinity, soil potassium content and geographical location (Grönemeyer and Reinhold-Hurek 2018). *B japonicum* strive well in the temperate region of the Northern Hemisphere, they are less viable in soybean nodule formation under high temperatures (Suzuki et al. 2014). It grows best in temperature of 28 ^{O}C.

1.12 Sinorhizobium fredii

Sinorhizobium is a fast growing and is one of the fast-growing rhizobial strains, with generation time of less than 6 hours. It produces nodules and fix nitrogen on soybean roots. It is believed to improve nodulation efficiency and symbiotic nitrogen fixation in different cultivars (Sameshima et al. 2003). It was first isolated from soils collected in numerous of provinces of the People's

Republic of China. *S fredii* cells are rod shaped, gram-negative, and non-spore forming. It is genetically different from another rhizobium. The genetic make-upof *Sinorhizobium* is one chromosome and six plasmids. It produces five distinctive surface polysaccharides: exopolysaccharides (EPS), liposaccharides (LPS) two different types of capsular polysaccharides (KPS [K- antigen polysaccharides]), and cyclic glucans (CG). The poly-PseAc called KPS is essential in symbiosis of *S. fredii* since mutants affect the genes of *rkp-1* and *rkp-3* region are symbiotically weakened (Rodriguez-Navarro et al. 2014).

Motility is only exhibited by a small proportion in *Sinorhizobium*; this percentage is enhanced when the bacterial cells are grown on soft agar. Cells tend to turn out to be large and pleomorphic from the log phase to stationary phase in YEM culture. *S fredii* grow best at 28 ^{o} C on YEM agar consisting of 2% NaOH, when the concentration of NaOH is 3% growth is inhibited. The strain also grows well in pH of 4.5 and growth is inhibited when the pH is 9.5 (Scholla and Elkan 1984).

1.13 Uses of Sinorhizobium fredii

S fredii form effective nodules on soybeans, and other herbaceous, shrubs and tree legumes, that are able to form determinant and indeterminant nodules (Jarvis et al. 1992). The bacteroids in the nodules fix atmospheric nitrogen and leave excess nitrogen behind to the benefit of the plant (Webb et al. 2014). The *S fredii* genome contains flagellin genes *fliC1C2-fliC3C4* which works as a unique autoregulatory protein responsible for exclusive motility during exponential growth. Inoculating seed with *S fredii* increase height and improve general growth of the plants. There are advantages of using *S fredii* as an inoculant since it is fast growing strain. This reduces bacterial cultivation high costs and the risk of contamination of the culture, because of the short

generation time. *S fredii* is also better adapted to different ecological conditions, such as alkaline soils when compared to other rhizobial strains such as *B japonicum* (Albareda et al. 2008).

1.12 Soybean X rhizobium compatibility and use

The efficiency of biological nitrogen fixation is controlled by several factors biotic, abiotic and anthropogenous. These are associated with the plant host, to rhizobia and to the symbiosis and the soil related constrains such as soil acidity, low soil fertility, poor aeration, high temperature, salinity, presence of pesticides, and drought (Campo et al. 2009, Sytnikov 2013). Virulence and activity of root nodule bacteria are also important factors in effectiveness of legume_rhizobia compactability (Sytnikov, 2013). The establishment of rhizobia symbiotically relationship with legume plants in the assistance of symbiosis genes which are in the horizontal transferable plasmid or a genomic island. Nitrogen fixation was reported in hundreds of alphas and beta-proteobacteria. In the 122 complete genome sequences from 12 genera of rhizobia available in GenBank database, 107 genomes from 11 genera have two or more DNA molecules, a genome structural design called as multifaceted genome. Transcription studies have shown that *Sinorhizobium* strain have exhibited specific up-regulation of various genes of the symbiosis plasmid within legume nodules. Genes located in the chromosome and chromid contribute to the integration and optimization of symbiotic roles in different rhizobia (Jiao et al. 2018).

1.13 Starter Nitrogen on plant growth

Nitrogen is an vital nutrient needed by in larger quantities, for the development and growth of soybean. There are main sources of nitrogen; there is nitrogen supplied to the plant by biological nitrogen fixation by root nodules, nitrogen from the soil (Gai et al. 2017). However, high levels

of soil nitrogen reduce biological nitrogen fixation because the nitrogen needs of the plant will have already been supplied. When the nitrogen concentration is low in the soil symbiotic nitrogen fixation will met the requirement. The third supply of nitrogen is from inorganic fertilizers. Nitrate hinders the formation of viable nodules on legumes inoculated with rhizobium strains (Osborne and Riedell 2006).

Zhang et al. (2013) said that around 63-65% of plant nitrogen is obtained from symbiotic nitrogen fixation for agricultural important crops like soybeans (Herridge et al. 2008). The outstanding N requirement is supplied by the by inorganic fertilizer applications and soil pool. It is of great significance to utilize the two nitrogen sources; nitrogen from symbiotic nitrogen fixation and inorganic sources, to improve the overall yield. The amount of nitrogen supplied is on the bases of soil analysis, carried out during seedling stage before the development of nodules (Valinejad et al. 2013).

Soils with small amount of N prior to nodule formation, a time of 'N hunger' prior can have a adverse effect on the yield of legumes (Van Kessel and Hartley 2000). Gai et al. (2017) said effective plant bacteria symbiosis starts 14 days from day of planting in soybean grown under ideal environmental conditions, it is therefore observed that small amount of inorganic fertilizer at sowing contribute positively to early plant growth. In the study by (Starling et al. 1998), it was reported that the application of nitrogen prior to sowing improved growth of soybean, due to the fact that the plant root structure and nodules are not well developed at this phase to meet the crop's N demand (Salvagiotti et al. 2008, La Menza et al. 2019) .

In addition, the plant used the supplied nitrogen source up until the initiation of symbiotic nitrogen fixation. Supplying the plants with starter n eradicates the lack of N phase in the early

stage of growth (Starling et al. 1998). It is also important for mitigating the N shortage during the time when the soil N is limited. A study by Huang et al. (2017) showed that application of NO_3-N < 10 kg ha^{-1} improved plant growth and development. However, residual soil NO_3-N that is higher 44 kg ha^{-1}, resulted in inhibition of plant growth (Osborne and Riedell 2006).

High level of inorganic fertilizer at the point of planting may inhibit effective symbiosis in soybean and its yield. Furthermore, excessive N may have detrimental effects on seedling establishment, vigor, and plant nodulation. (Gai et al. 2017) found that soybean plant height, shoot fresh weight, leaf area, or grain yield are mostly likely not influence by starter nitrogen. However, plant growth and grain yield were increased by the application of nitrogen fertilizer before planting. In addition to soybean grain yield, the plant height and biomass were higher after application of starter fertilizer-N (Starling et al. 1998). McKenzie et al. (2001) indicated that a large quantity of N fertilizer improved soybean grain yield for soybean plants whether irrigated and not irrigated.

(Huang et al. 2017) reported a number of advantage of starter N fertilizer on biological nitrogen fixation. Starter N improve nodulationof plants by increasing the efficiency of indigenous soil rhibium. It also improves yield and overall protein content. The concentration of seed protein decreases if there was not enough N during seed filling stage (Valinejad et al. 2013) and nitrogen fertilizers increase seeds quality. Inorganic fertilizer like urea release nitrogen slowly, thus will be avaible to the plants throughout the growing season (Baral et al. 2016). Hence, fertilizers have a positive impact on the protein content in legumes and yield potential.

Yield potential is mainly reduced both by the availability of nutrients and water for plant uptake. The germination of seeds is negatively affected by the application of starter nitrogen in the

furrow where the seeds is planted. This leads to rotting of the seed, reducing germination percentage. (Dixon and Kahn 2004) recommended that starter N needs to be applied 5 cm deeper than the seed and covered with small soil layer. The rate of the N applied may also be reduced to avoid cases of seed burn from the fertilizer.

Saito et al. (2014) said it is important to use inorganic nitrogen fertilizers and the one obtained from biological Nitrogen fixation to increase yields in soybean. Nevertheless, it is evident that high level of combine nitrogen leads in the reduced creation of actively nitrogen fixing nodules. (Barbulova et al. 2007) observed characteristics such as reduced nodule dry mass, number of nodules per plant and the nitrogen fixation activity and the increase in nodule degradation or senescence as a sign of nitrate inhibition.

Nitrates inhibit the development of nodules; the effect is inscribed to the reduction of root hair infection. There is usually production of Indolyl 3-acetic acid (IAA), which is secreted by rhizobia from tryptophan in the root exudations, possibly engaged in root-hair infection and nodule initiation (Angle 1990). Pagan et al. (1977) observed that most rhizobia can convert nitrate to nitrite. The inhibitory action is based on the catalytic destruction of IAA by nitrite. Nitrate is also accountable for the reducing levels of fixed nitrogen in legumes. The impact is attributed to a diminishing photosynthate readily available to the nodules, following its use in the assimilation of nitrate in the shoots and roots. Mantelin and Touraine (2004) explained that the decrease in amount of photosynthates translocated to nodules could be explained if the added nitrate acted within the nodules to inhibit nitrogen fixation, this is through reducing the role of the nodules as a sink for photosynthate.

The inhibition could also be triggered by nitrite produced because of nitrate-reductase activity in the nodules. Pagan et al. (1977) highlighted that nitrite is an inhibitor of nitrogenase in bacteroids an in N_2- fixation cultures of rhizobia. Barbulova et al. (2007) proposed that two mechanisms explain the nitrate-induced depression of nitrogenase activity by rhizobia. It was suggested that nitrate form a NO- compound with leghemoglobin, thus destroying its function as an O_2-supplier to the bacteroids (Ohyama et al. 2011). Nitrite is also a potent inhibitor of nitrogenase activity, in bacteroids extracted from nodules, in N-fixing cultures of rhizobia and of the nitrogenase enzyme. Meakin et al. (2007) said the presence of nitrate reductase in nodules, and the absence of nitrite reductase could be the source of nitrite that could interfere with nitrogenase activity by affecting leghemoglobin or the enzyme nitrogenase, separately or simultaneously.

1.14 Soybean and salinity

Soil salinity is a main factor reducing the growth and development of many agricultural crops (Machado and Serralheiro 2017). Salinity causes major decrease in cultivated areas, crop production and quality (Jamil et al. 2011). A soil is generally characterized as saline when the electric conductivity (EC) of a saturation extract (EC_e) in the root zone exceeds 4 dS m^{-1} at 25 ^{O}C and has a sodium saturation of 15% (Shrivastava and Kumar 2015). Soil salinity reduces nodulation efficiency in soybeans and subsequently reduces yields (Maas and Grattan 1999).

In a study by (Kibido et al. 2020), two rhizobia strains were investigated. The results show *Sinorhizobium fredii* strains to be more tolerant to salinity compared to *Bradyrhizobium japonicum*. *Sinorhizobium* exhibited growth even under high salt conditions. It was also observed that *Sinorhizobium* strain more tolerated treatment PEG 6000, which was used as a medium with low osmotic potential. The cell water potential and available water were lowered PEG. In saline

soils bacterial cells accumulate osmolytes that have reduced-molecular weight organic solutes to prevent dehydration. These osmolytes include glycine, betaine, proline, and trehalose. Osmo-tolerant rhizobial strains persist in the soil, they achieve this by maintaining a positive turgor. *S. fredii* was more competitive and effective in nodulating soybeans compared to *B. japonicum*.

1.15 Aims and Objectives

The study aim was to investigate and compare the interaction of different soybean cultivars with the two different species and strains of rhizobium specifically related to root phenotypic characteristics and nodulation efficiency. The following objectives were set out in the study:

1. To quantify phenotypic differences on root patterns among different soybean cultivars.
2. To determine the nodulation efficiency of the different rhizobium strains in different soybean cultivars, in soybean inoculated with different rhizobium strains, in different cultivars.
3. To evaluate the effect of using starter nitrogen application on soybean nodulation.

1.16 Problem statement

Soybean is one of the most importance agricultural crops in South Africa. Soybean is mainly grown as a source of vegetable oil and protein used for animal feed. Commercially only *Bradyrhizobium japonicum* strain WB-74 has been used in South Africa, this is because the genetic background of the cultivars used in South Africa have traditionally traced back to the US and is more compatible with slow growing *B. japonicum* strains. However, with the introduction of new cultivars as well as increasing incidences of drought and increasing levels of soil salinity there is a possibility that more salt tolerant species like *Sinorhizobium fredii* might offer benefits

to farmers in the future. We therefore need to investigate the compatibility and performance of these two inoculants.

1.17 Justification

The study quantified phenotypic differences on root patterns of different soybean cultivars to choose the cultivar that is more efficient in nodulation when inoculated with SMH12 and WB74-1. The study also evaluated soybean strains to choose a strain that will promote production in South Africa. The use of starter N was also done to determine the rate that is promote symbiotic nitrogen fixation.

1.18 Hypothesis

The hypothesis was that there will be cultivar X rhizobium interactions that influence nodulation efficiency and nitrogen fixation capacity.

CHAPTER 2

2.0 METHODOLOGY

2.1 Plant material and Experimental procedure

This study was conducted during the cropping season of October 2019 to March 2020 in a phytotron (FABI), located at the University of Pretoria in Hatfield, Pretoria. The location has coordinates 25.754 S, 28.231 East and altitude of 1372m Annandale et al. (1999). The Plant Science laboratory at the University of Pretoria was used to analyze samples and laboratory work.

2.2 Crop selection

Five soybean cultivars were planted in this experiment (Table 2.1). The experiment was conducted in three stages; the germination stage was conducted in a growth chamber, to study the root patterns and germination of the five soybean cultivars. The second stage was for evaluating the effect of rhizobial strains on three soybean cultivars. The third stage was for determining the effect of starter nitrogen on nodulation and plant growth. The last two stages of the experiment were conducted in the phytotron. The phytotron was fumigated to control pests and diseases.

Table 2. 1: Cultivars used in the trial

Varieties	Description	Source
Variety 1	Williams 82	Monsanto
Variety 2	PAN 1521R	Pannar Seed
Variety 3	PHB94Y 80R	Pioneer Hi-Breed RSA
Variety 4	PHB95Y 20R	Pioneer Hi-Breed RSA
Variety 5	PHB96T 06R	Pioneer Hi-Breed RSA

2.3 Experimental Layout

The experimental design was Complete Randomised Design (CRD). The treatments were five soybean cultivars (Table 2.1) and two strains of rhizobium (Table 2.2). The treatments were replicated six times. Brown PVC pots (Figure 2.1) were used in this experiment. The pots were kept in the phytotron at a temperature of 28 °C, light was provided at night for 12 hour per day. There were 18 pots per cultivar. The seeds were planted in sterile vermiculite and irrigated three times a week. The seedlings were inoculated with the two strains of rhizobium on the day of planting. A fungicide was applied two weeks after planting, this is because the plants were affected by stem rot caused by Phytophthora. After the development of the first trifoliate leaf the seedlings were irrigated with nitrogen free half-strength Hoagland solution and a week after nitrogen free full-strength Hoagland solution was used. The plants were harvested 7- 8 weeks after planting.

Table 2. 2: Characteristics of rhizobium strains

Rhizobium strain	Optimum temperature (°C)	Optimum pH (H_2O)
Sinorhizobium Fredii SMH12	28	6.8
Bradyrhizobium japonicum WB74-1	28	6.8

Figure 2. 1: Plants growing in the brown pvc pipes.

2.4 Growing bacteria strains and culture conditions

The bacteria that were used in this experiment were *Bradyrhizobium japonicum* (WB74-1) and *Sinorhizobium fredii* (SMH12). *Sinorhizobium fredii* strain was obtained from Prof Ruiz Sainz (Universidad de Sevilla, Spain) and *Bradyrhizobium japonicum* strain is a local strain, was obtained from South African Rhizobium Culture Collection (SARCC) (Kibido et al. 2020). The bacteria were grown in plates with yeast mannitol in the incubator at 28°C and its quality was checked with streaking on yeast mannitol congo red medium (25 mg/ml). Pure colonies were white and however in the presence of contamination the congo red was abserved. The bacteria grown in yeast extract mannitol (YEM) slant was stored at 4°C and the yeast extract mannitol (YEM) broth was stored at -80°C (Batista et al. 2015).

2.5 Cell viability test

After removing the yeast mannitol (YEM) slants from the incubator, the optical density was measured at 600 nm, using a spectrophotometer. If the absorbance reading was greater than 1A the slants were diluted to make a 10-fold dilution series (Wang et al. 2004). This was done in order to be able to count the number of cells and the range must be between 30-300.

2.6 Plant material and growth

Soybean seeds (*Glycine max* L. Merr.,) Williams 82, PAN 1521R, PHB94Y 80R, PHB95Y 20R and PHB96T 06R were obtained from Pannar Seeds in Greytown, South Africa. Before planting the seeds from each cultivar were surface sterilized with sodium hypochlorite (2.5%) for 15 minutes and after that were rinsed five times with distilled water and left to imbibe for 3-5 hours. The seeds were then pre-germinated in petri-dishes, containing sterile water-agar for 2 days in

the growth chamber at 28 ^{0}C. The whole experiment was divided into three phases, discussed below.

2.7 Root phenotyping and Cultivar choice

2.7.1 Cultivar choice based on germination percentage

The germination percentage was determined by using the paper roll method. The rolls were prepared by using three 30 cm^2 brown paper sheets with two absorbent white paper sheets on top of the brown paper (Figure 2.2a). Before seeds placement, rolls were soaked in water and gently wrung out to get rid of excess water. There were replicates per cultivar with 50 seeds in each roll (Figure 2.2a). These seeds were placed uniformly, in straight rows and columns, on the white sheets and then rolled up, sealed in a plastic bag, and placed in a growth chamber. The chamber was kept at a constant temperature of 25 °C (Figure 2.2b).

The paper rolls were left undisturbed for 24 hours. The number of seeds germinated were counted after the first 24 hours and 36 hours to track germination rate and percentage and was placed back in the growth chamber for a further eight days. After 10 days the final germination percentage was determined (Figure 2.2d). This was done by counting the number of seeds that successfully germinated and divided by the total number of seeds in the roll. A seed was counted as successfully germinated when the radicle has emerged from the seed and the radicle length exceeds 1cm (Figure 2.2c).

Figure 2. 2: a. The soybean seed on paper towel 1 day after planting, b. The germination paper in the growth chamber, c. Seedlings considered to have emerged, d. Seedlings 10 days after planting, ready for measurements.

2.7.2 Cultivar choice based on root length, angle and density

The plant's roots were then captured, and the root length, angle and density were acquired. The root length was measured by placing a ruler next to the from the root base to the root tip. The root angle was determined by laying the whole root on a 180° protractor sketch board (Figure 2.3) with the stem at 0° (Oyanagi et al. 1993). The average lateral root angle on both sides was measured as the root angle. The root density of both the tap and lateral root was determined by

counting the number of roots emerged from lateral and tap roots within a two cm root segment of either the tap or three randomly selected lateral roots (Fenta et al. 2014).

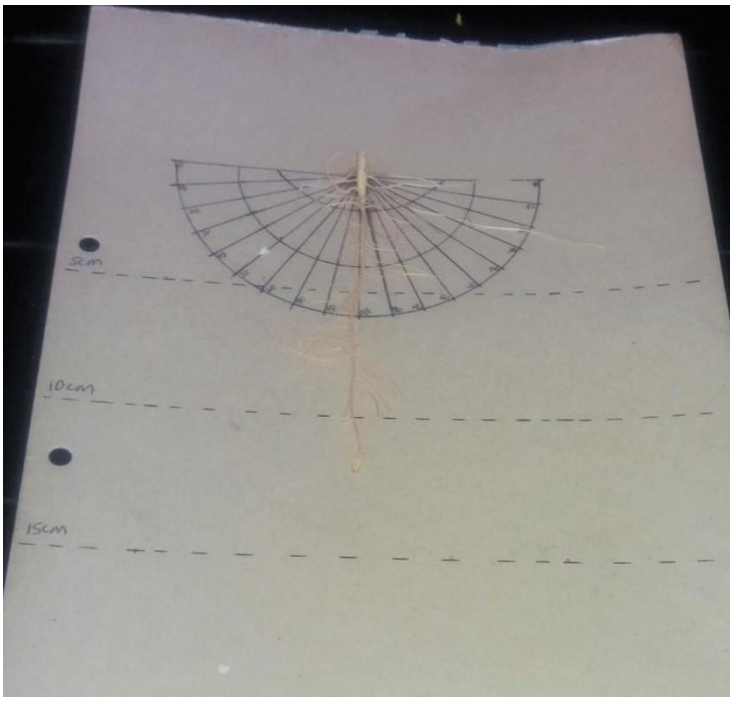

Figure 2. 3: Protractor sketch board for measuring the root angle

2.8 Effect of inoculating soybeans with SMH12 and WB74-1

This experiment was conducted in cylindrical tubes. The brown pvc pots were 40 cm in height with a diameter of 10 cm. The pots were closed with a stopper at the bottom and four holes were made on the stopper to allow for water movement. The pots were filled with vermiculite and six pots were placed in each tray. The vermiculite was watered three days before planting. On the day of planting the vermiculite was inoculated with 1ml rhizobium strain (Table 2.2), covered with a bit of vermiculite. Then one seed was planted in each pot. The plants were watered every second day and water was poured on the tray, it reached the plants through capillary action. After the development of the first trifoliate leaf, the plants were watered with Hoagland solution (Table

2.3), half strength was used at the beginning up until the plants got used to it. Then full strength was used.

Table 2. 3: Composition of nitrogen free nutrient solution based on Hoagland

Source	**Nutrient**	**Solution concentration (g)**
Macronutrients (g/500ml)	1M $MgSO_4$ $7H_2O$	123.24
	1M KH_2PO_4	68.045
	FEDTA	16.384
	1M $CaCl_2$ H_2O	73.51
	50mM KCl	1.85
Micronutrients (g L^{-1})	H_2BO_3	1.43
	$MnSO_4$. $4H_2O$	0.446
	$ZnSO_4$	0.575
	$CuSO_4$ $2H_2O$	0.124
	Na MO_4. H_2O	0.0125

2.9 The effect of starter nitrogen on nodulation

This experiment was also conducted using brown pvc pots. The pots were filled with vermiculite and placed in trays. The soil was watered prior to planting. On the day of planting when the soil moisture content was at field capacity, the soil in each pot was inoculated with 1ml rhizobium (Table 2.2). Starter nitrogen in the form of Potassium Nitrate (KNO_3) (Barbulova et al. 2007) was applied according the rates in Table 2.4. The KNO_3 was then covered with soil and one seed

was planted in each pot. The plants were irrigated every second day. After the development of the first trifoliate leaf the plants were irrigated nitrogen free half-strength Hoagland solution, and thereafter nitrogen free full-strength Hoagland solution was applied, to provide the nutrients needed by the plants except for nitrogen (Table 2.3). The experiment had nine treatments replicated four times. The trays with the different treatments were rotated every week to make sure the plants were exposed to similar environmental conditions.

Table 2. 4: The rates of starter Nitrogen (Potassium Nitrate)

Treatment	**Rates of Nitrogen**
1	0 mM KNO_3
2	5 mM KNO_3
3	10 mM KNO_3
4	20 mM KNO_3

2.10 Preparing the roots system for analysis

On the day of harvesting the plants were grouped according to treatment. The samples from the same treatments were washed from the root crown back and forth to remove all the vermiculite but making sure not to destroy the delicate nodules and lateral roots. One plant from each treatment was imaged for report purposes. The shoots were then cut using pruning shears just above the cotyledons. They were then oven-dried for shoot dry mass and discarded.

The root system was then disentangled so that the first order lateral roots fell naturally into the three planes of branching caused by the triarchy pattern on the root stele. The root system was

then placed at the center of a black cloth and a photo of the nodulation zone was taken. A white label sticker was placed on the top right side of the black cloth for identification. One photograph was also taken for the whole plant for record purposes. Roots that were below the nodulation zone were imaged individually as they do not bear nodules.

2.11 Root architecture

Eighteen plants per cultivar for each rhizobium were sampled. The roots after being carefully washed root traits such as root angle, were measured using a 180° sketch board. This was achieved by determining the average lateral root angle on both planes of the primary roots. The root density on both sides of the tap root will be determined by counting the number of roots that are 15 cm from the root top.

2.12 Nodule number, size and weight

The three cultivar were sampled for root phenotyping including nodule parameters. Nodule sizes were determined by placing the multiple nodules on a board with a sketch of the diameter of nodules. The number of nodules was found by counting the number of active nodules per plant. Then weight of the nodules was determined by measuring the weight of the nodules in milligrams using a balance scale, for all the plants according to treatments and later calculating the average (Ikeda 1999).

2.13 Ureide determination

For determining biological nitrogen fixation, the ureide content was measured as allantoin production of nodules assay. After determining the weight of the nodules and leaf tissues (100 mg), ureide was extracted with 100 µl of 0.2 M NaOH. Samples were then boiled for 20 minutes

to convert allantoin to allantoic acid. Samples were cooled and centrifuged at 10 000 g for 10 minutes where after 5 µl of the supernatant together with 35 µl of water H_2O will be used for further analysis according to (Young and Conway 1942). The diluted plant extract (40 µl) was boiled together with 8 µl of 0.5 M NaOH for a further 10 minutes thereafter 16 µl of mixture of a 1:1 ratio of 0.33% phenylhydrazine (Sigma, USA) and 0.65 M HCL was added and boiled for another 2 minutes. A 40 µl solution of 1.67% potassium ferricyanide (Sigma, USA) and HCL (36.5-38.0%, used for molecular biology) were incubated together with the plant mixture for 10 minutes before the absorbance was measured at 525 nm. A standard curve was set up with the 1µg, 2µg, 4µg, 6µg and 8µg of allantoin (Sigma, USA) to calculate the ureide content(Kibido et al. 2020).

2.14 Plant response to rhizobium and starter nitrogen

The following parameters were determined during the experiments:

2.14.1 At cultivar choice and germination stage

- Germination percentage 1, 3 and 10 days after planting.
- Root length, angle and density

2.14.2 When inoculated with SMH 12 and WB74-1

- Germination percentage
- Plant height, root length, angle, and density
- Shoot and root dry mass
- Nodulation rate, nodule size
- Ureide content

2.14.3 After the application of stater Nitrogen

- Germination percentage
- Plant height, root length, angle, and density
- Shoot, root and total dry mass
- Nodulation rate, nodule size, number of leaves
- Ureide content

2.15 Data analysis trials

Linear models will be used to find the differences in nodulation between SMH12 and WB74-1, on the three cultivars of soybeans and the interactions between all the growth parameters. If interactions are significant, Tukey's multiple comparison test were used to test the differences between treatments. If the interaction was not significant, analysis was rerun without the interaction term. The response data were transformed prior to analysis where required to meet model assumptions. Data collected was analyzed using Microsoft Excel, GenStat 18th edition software, GraphPad Prism and Statistical Analysis Software (SAS).

CHAPTER 3

3.0 RESULTS

3.1 Root phenotyping and cultivar choice

3.1.1 Cultivar choice based on germination percentage

The germination percentage of five soybean cultivars (Figure 3.1) was observed over a period of 10 days. The highest germination percentage (16%) on 1 day after planting was recorded in PHB 96T O6R and lowest (3%) was recorded in Williams 82. The same trend was observed 3 days after planting. However, 10 days after planting the highest germination percentage (97%) was recorded in PHB 96T 06R and the lowest germination percentage (90%) was recorded in PHB 94Y 80R. These results mean that PHB 96T 06R have a high seed vigor when compared to all the other cultivars. PHB 96T 06R shown superior germination percentage, this is probably due to gernetic factors that enhanced germination and PHB 96T 06R is a fast germinator.

In the second stage of the experiment, three soybean cultivars (Figure 3.2) were planted to determine the effect of *Bradyrhizobium japonicum* and *Sinorhizobium fredii*, on the germination percentage 10 days after planting. The seeds were inoculated with SMH12 and WB74-1. The results show the highest germination percentage was recorded in PHB 94Y 80R and the lowest was recorded in PAN 1521R at all stages. The superior germination percentage is a result of physiological quality of the seeds, which was observed during the experiment. The seeds from PHB 94Y 80R were generally good quality seeds, they germinated faster that all other cultivars (Figure 3.2), tand they exhibited high seed vigour and the seedlings were elite.

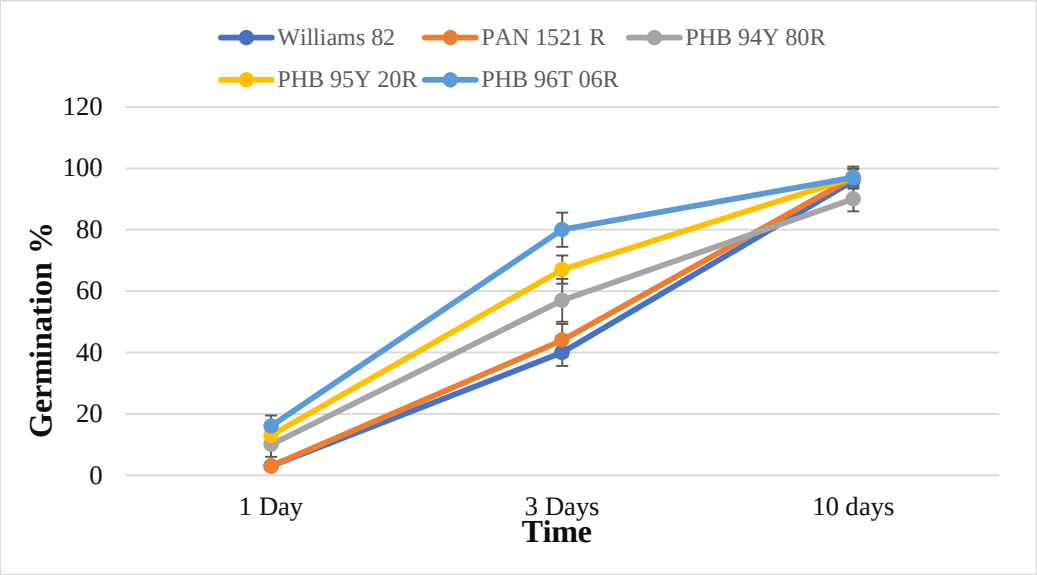

Figure 3. 1: The mean germination percentage of five (5) soybean cultivars at 1,3 and 10 days after planting.

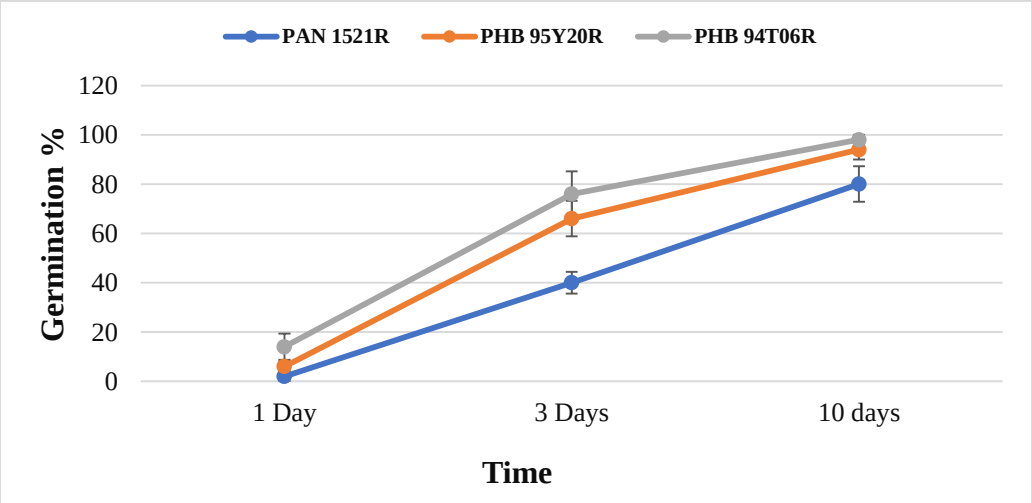

Figure 3. 2: The mean germination percentage of three (3) soybean cultivars 1,3 and 10 days after planting.

3.1.2 Cultivar choice based on root length, angle and density

The mean root length, angle and density for each cultivar are shown in Table 3.1. This was measured in cultivars with different root characteristics to evaluate if it played a role in nodulation. The root growth angle was the largest in PHB95Y 20R (40°) and the smallest was in Williams 82 (24°). The difference in the root angle among the cultivars were significant ($p<0.05$). The density of the roots for each cultivar was shown in Table 3.1, the largest root density was exhibited in PHB95Y 20R (46) and the lowest root density was in PAN 1521R (25). The vertical distribution (length) of roots varied among the cultivars. For instance, most roots were distributed in the top 15 cm of the main roots. Williams 82 appeared to have a shallow root system. In contrast, the root distributed in the top 15 cm were less in PAN 1521R and the root system were relatively deep in PHB94Y 80R and PHB95Y 20R. There is a positive relationship between growth root angle and root length $r = 0.47$ ($p<0.05$). The relationship was also positive between root density and root length $r = 0.54$ ($p<0.05$). This suggests that cultivars which show large root growth angle in the seedling stage have deep root system and density. The cultivar with the highest root angle means that the angle between the main root and the lateral roots was big so the root will in a more horizontal distance. However, a smaller root angle means the angle between the main and the lateral root is small thus, this means the root will grow in vertical direction (Oyanagi et al. 1993). A small root angle is of great advantage if the plants are growing in drought conditions because the roots will absorb water and nutrients deep in the soil. For this experiment a large root angle is preferred because the plants were grown under well watered conditions. Cultivars with high root density means the plants had a lot of roots when compared to the other cultivars. This is a good trait in soybeans because it means the roots will cover a large surface area, thus absorb more water and nutrients for better plant growth and development.

Table 3. 1: The root length, root angle and root density of soybean cultivars at germination

Cultivar	**Root length (cm)**	**Root angle (⁰)**	**Root density**
Williams 82	13.067 [c]	24.067 [d]	30.967 [c]
PAN 1521R	14.763 [b]	32.05 [b]	29.492 [c]
PHB 94Y 80R	17.128 [a]	35.592 [b]	38.783 [b]
PHB 95Y 20R	17.492 [a]	39.992 [a]	46.042 [a]
PHB 96T 06R	14.287 [b]	31.2 [c]	31 [c]
LSD (0.05)	**1.199***	**4.294***	**2.047***
Std Dev	**0.95**	**3.412**	**1.608**

[+] Values with the same lets are not significantly different according to Duncan's multiple range test at the 5% level
LSD-Least Significant Difference
*Significant at LSD (0.05) (P<0.0001).

3.2 Effect of inoculating soybeans with SMH1*2* and WB74-1

3.2.1 Plant Height

Inoculating with bacteria encouraged the growth of soybeans and cause an increase in plant height (Figure 3.3). Soybean cultivars inoculated with SMH12 exhibited an overall highest plant height but not significant, and the plants that were no inoculated (control) showed the lowest plant height. The plants inoculated with SMH12 were taller than those inoculated with WB74-1 within the same cultivar (Figure 3.4 a-c). The cultivar that exhibited the highest plant height when inoculated with either of the two strains was PAN 1521R (V3) (Figure 3.4c) and the cultivar that showed the lowest plant height was PHB94Y 80R (V1) (Figure 3.4a). There was a significant difference among the cultivars (Figure 3.3). This shows that PAN 1521 grew faster when compared to the other cultivars and the height increased faster when inoculated with SMH12 than when inoculated with WB74-1. There was a significant interaction between rhizobia and variety. These results agree with findings by García et al. (2004), who found a significant increase in the growth of soybean after inoculating soybean seeds with SMH12. Temprano-Vera *et al.* (2018) also found that inoculation with SMH12, increased plant growth by improving nitrogen-fixing nodules, which increase growth of plants.

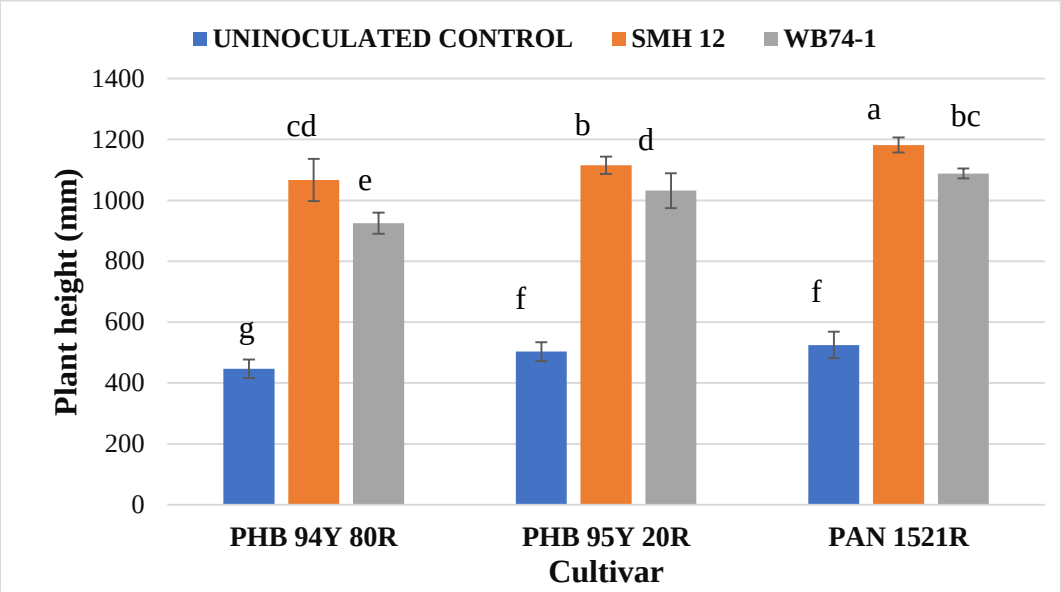

Figure 3. 3: The effect of SMH12 and WB74-1 on the plant height of soybean cultivars.

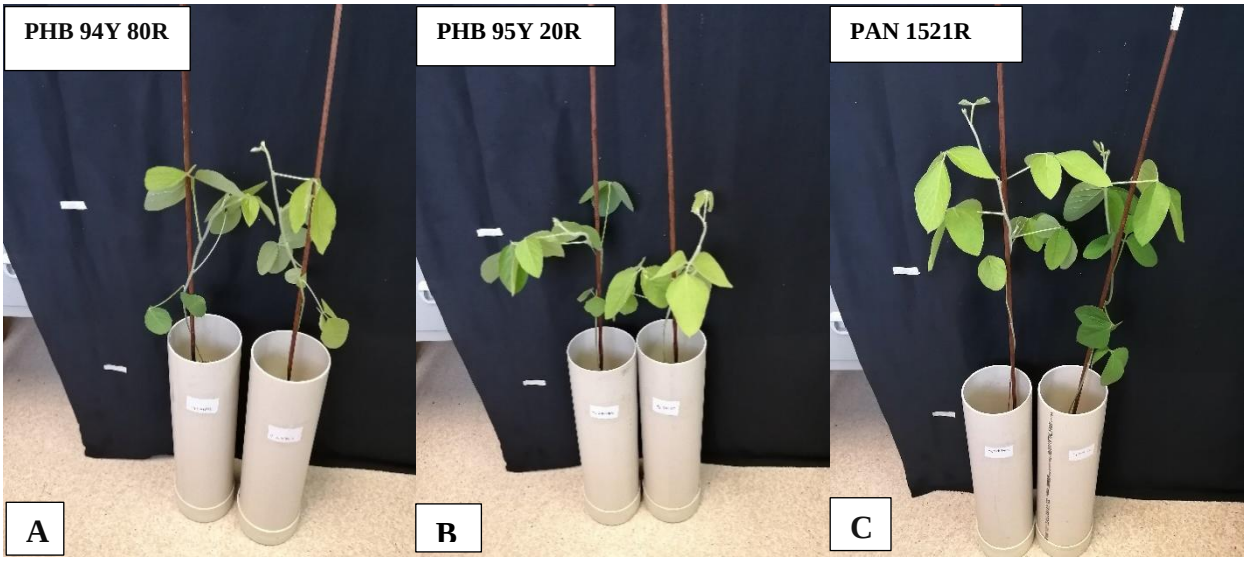

Figure 3. 4: The effect of SMH12 and WB74-1 on plant growth in different cultivars (a to c).

3.2.2 Root length

Inoculating soybean with rhizobium increased root growth of the plants (Figure 3.5). Cultivars inoculated with SMH12 had the highest root length and non-inoculated exhibited the lowest root length. Literature states that the increase in root length may be due to the nitrogen fixed by the plants through nodulation. The cultivar with the highest root length (640 mm) when inoculated with either SMH12 or WB74-1 is PAN 1521R and the cultivar with the lowest root length (436.67 mm) is PHB95Y 20R. There was a significant differences among the cultivars ($p<0.05$). This means that plants inoculated with SMH12 had longer root length in all the cultivars and this implies that they were better at absorbing water at deeper water table compared with plants inoculated with WB74-1. Fenta et al. (2014) suggested that cultivars with deeper and longer roots are better and may contribute to improved and better water uptake. There was no significant interaction between rhizobia and variety.

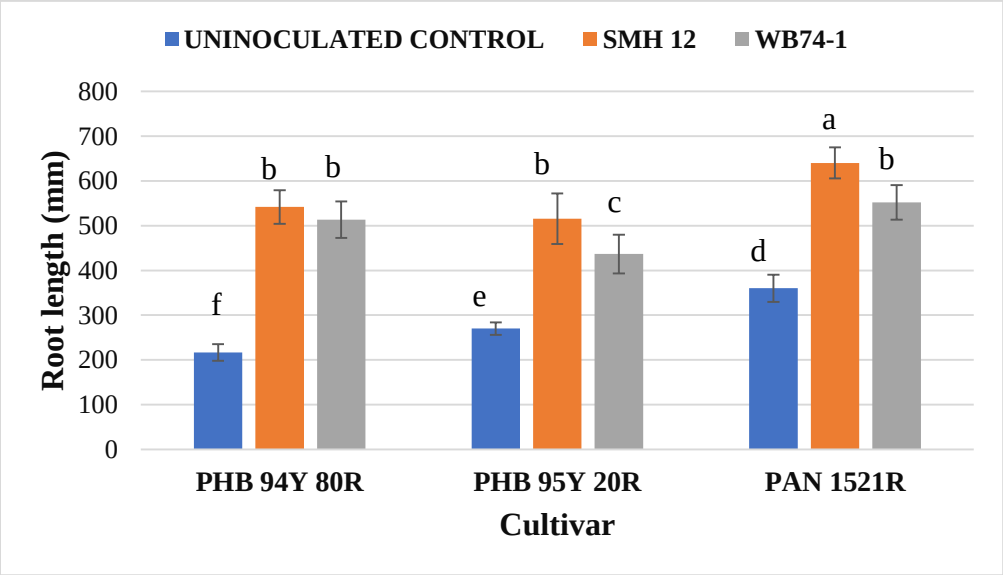

Figure 3. 5: The effect of SMH12 and WB74-1 on root length of soybean cultivars.

3.2.3 Root angle

Inoculating soybean plants with rhizobium resulted in plants with large root angles. Soybean plants inoculated with SMH12 had the highest root angle (Figure 3.6). The results show that the overall highest root angle was recorded in PHB 95Y 20R inoculated with SMH12 and the lowest root angle was recorded in cultivar PH B94Y 80R inoculated with WB74-1. This tells us that plants inoculated with SMH12 the plants grew more horizontal whereas plants inoculated with WB74-1 the angle tells us that roots grew more downwards than to the sides. Roots with a lower root angle have greater potential for absorbing water deep in the soil, when grown at drought conditions. There was a significant difference among the treatment at $p<0.05$ (Figure 3.6).

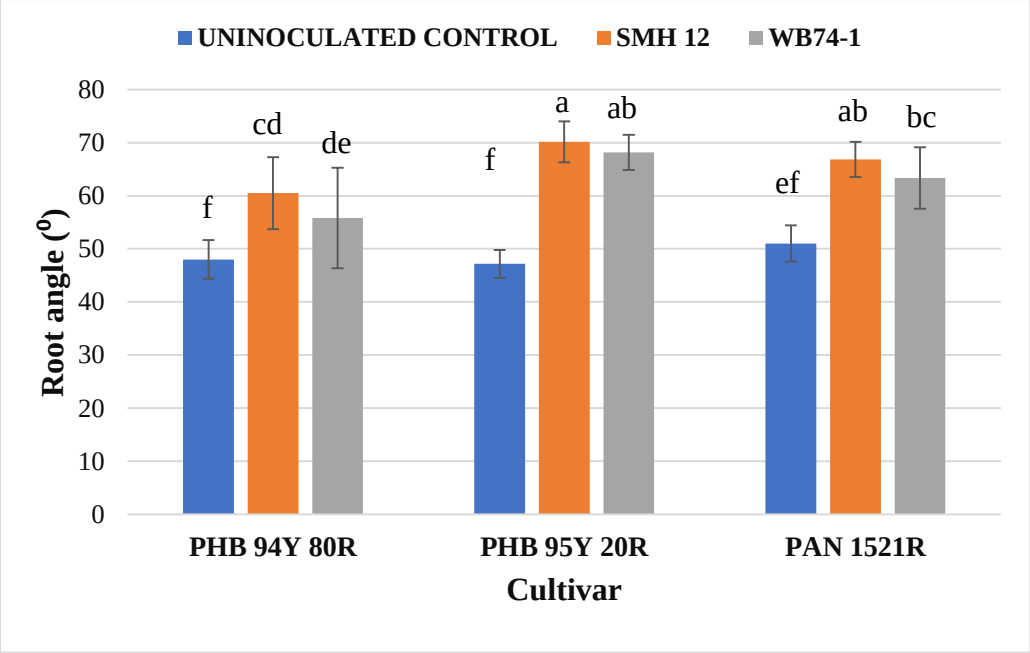

Figure 3. 6: The effect of SMH12 and WB74-1 on root angle of soybean cultivars.

3.2.4 Root density

When soybean cultivars were inoculated with SMH12 and WB74-1 (Figure 3.7). The results indicate that plants inoculated with SMH12 had a significantly higher root density ($p<0.05$) and the lowest root density was recorded plants inoculated with WB74-1 (Figure 3.8b). Among the cultivars the highest root density (21.33 roots/plant) was recorded in PAN 1521R inoculated with SMH12 and the lowest (9.83 roots/plant) was recorded in PHB94Y 80R inoculated with WB74-1. This means that plants inoculated with SMH12 had more roots compared to those inoculated with WB74-1. More roots mean the roots cover a large soil surface in the field thus they will be able to absorb more water and nutrients compared to plants with lower root density. This also means there is more area to be occupied by nodules.

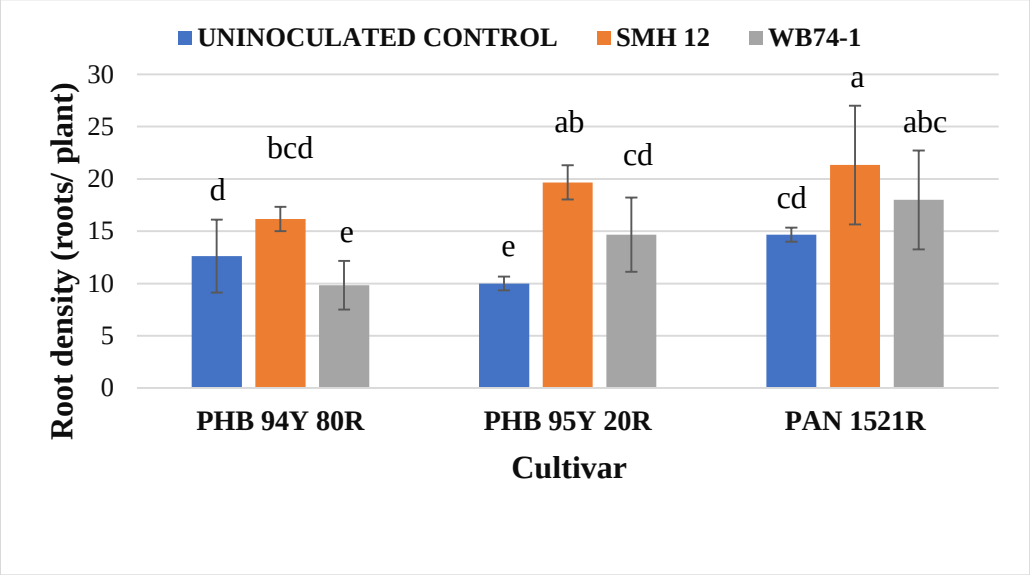

Figure 3. 7: The effect of SMH12 and WB74-1 on root density of soybean cultivars.

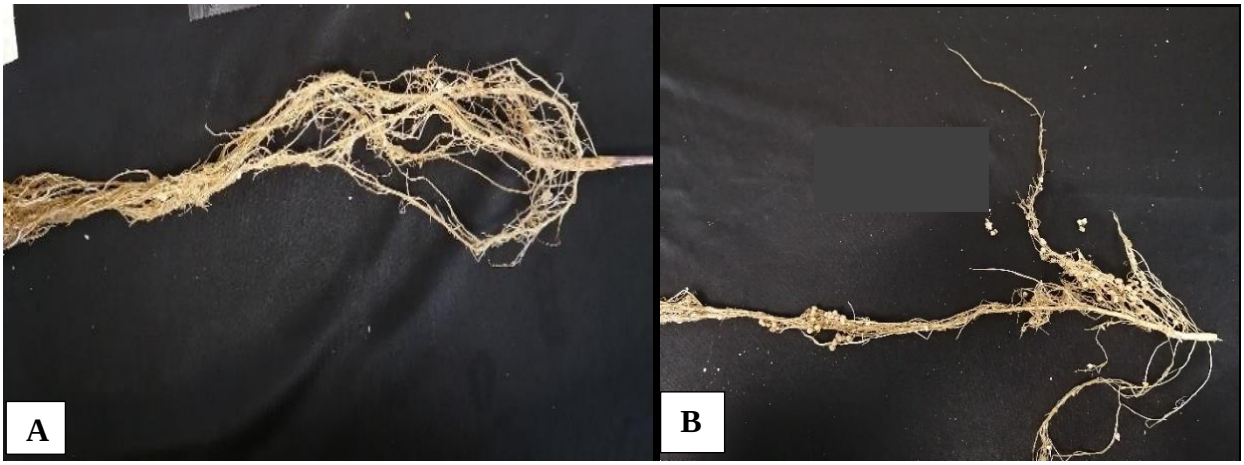

Figure 3. 8: The effect of SMH12 and WB74-1 on roots of cultivar PAN 1521R A. SMH12, B. WB74-1.

Table 3. 2: Standard deviation for SMH12 and WB74-1 on the plant height, root length root angle and root density of soybeans

Treatment	**Plant height (mm)**	**Root length (mm)**	**Root angle (°)**	**Root density (roots/ plant)**
V1 CONTROL	30.811	18.793	3.656	3.491
V2 CONTROL	30.798	14.043	2.639	0.666
V3 CONTROL	43.244	30.184	3.406	0.684
V1SMH12	69.186	37.639	6.775	1.169
V2SMH12	28.107	56.663	3.869	1.633
V3SMH12	24.833	34.641	3.312	5.680
V1WB74-1	34.496	40.825	9.453	9.453
V2WB74-1	57.067	43.205	3.312	3.312
V3WB74-1	16.669	38.687	5.785	5.785
LSD (0.05)	**30.82**	**10.039***	**6.903***	**4.217***

[+] Values with the same lets are not significantly different according to Duncan's multiple range test at the 5% level
LSD-Least Significant Difference
*Significant at LSD (0.05) ($P<0.0001$).

3.2.5 Shoot Mass

The results show soybean cultivars inoculated with SMH12 and WB74-1, there were differences in the dry mass of shoots (Figure 3.9). Inoculating soybean plants with SMH12 and WB74-1 increased the total dry mass of soybeans. There was a significant interaction between rhizobia and variety. The averall highest shoot dry mass was recorded in PAN 1521R inoculated with SMH12 and the lowest dry mass was recorded in PHB 94Y 80R. However, when the plants were inoculated with WB74-1 the highest dry mass was recorded in PHB94Y 80R and the lowest dry mass was recorded in PHB95Y 20R. This implies that plants inoculated with SMH12 had the highest shoot dry mass, which means they grow faster and were better in accumulation of nutrients thus the higher dry mass. There was not a significant difference per cultivar x strain but there were differences between the strains in how they responsded at $p<0.05$ (Figure 3.9).

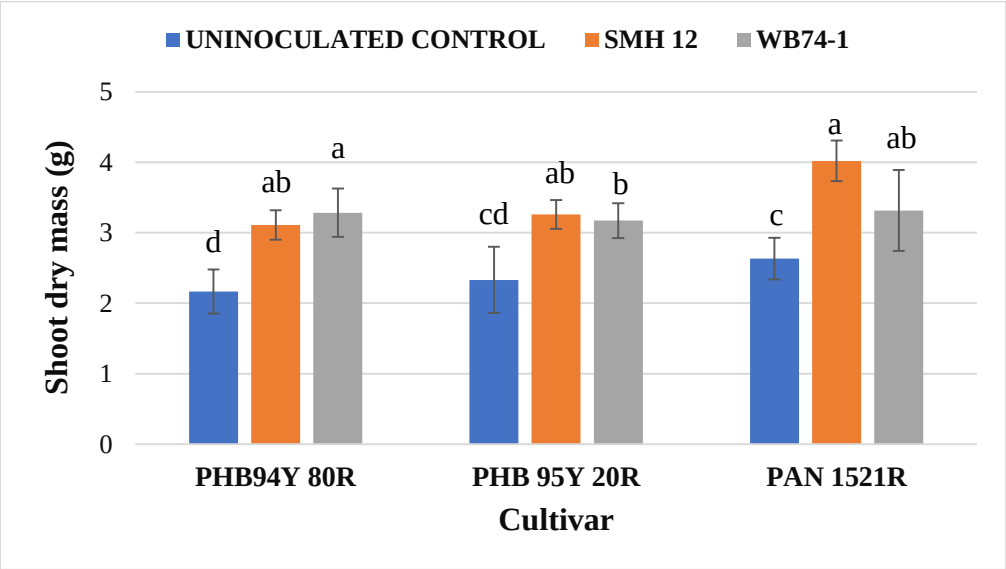

Figure 3. 9: The effect of SMH12 and WB74-1 on shoot mass of soybean cultivars.

3.2.6 Root Mass

The results show that soybean cultivars inoculated with SMH12 and WB74-1, had significant differences in the dry mass of roots (Figure 3.10). The highest dry mass (4.02g) was recorded in PAN 1521R when the plants were inoculated with SMH12 and the lowest dry mass (3.11g) was recorded in PHB 94Y 80R. However, when the plants were inoculated with WB74-1 the highest dry mass (3.317g) was recorded in PAN 1521R and the lowest dry mass (3.172g) was recorded in PHB 95Y 20R. This means that SMH12 was more effective at increasing root growth and development when compared to WB74-1. There was a significant interaction between rhizobia and variety.

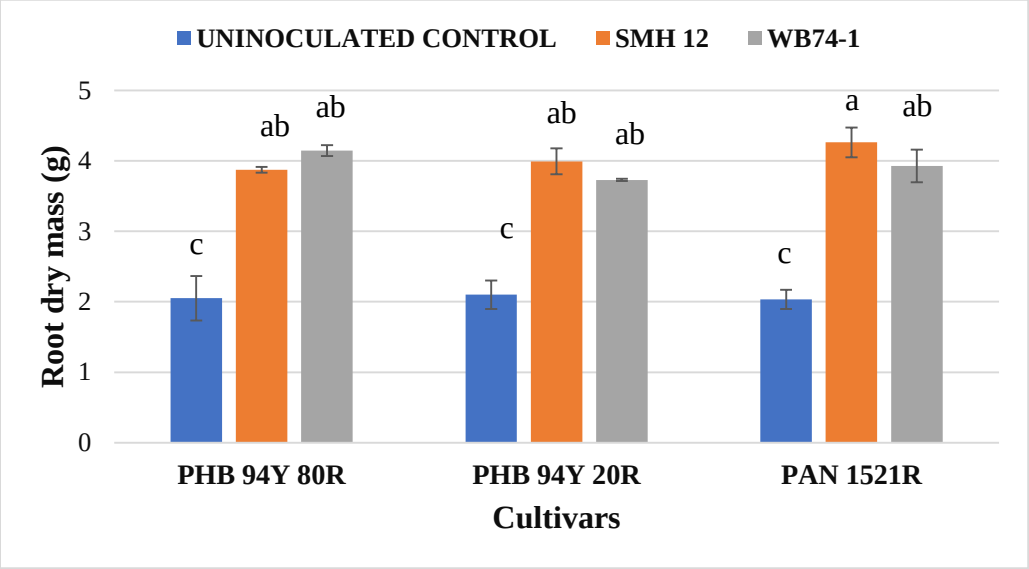

Figure 3. 10: The effect of SMH12 and WB74-1 on root mass of soybean cultivars.

3.2.7 Total mass

When soybean cultivars were inoculated with SMH12 and WB74-1, there was a difference in the total dry mass of the inoculate compared to uninoculated plants (Figure 3.11). For cultivar PHB 94Y 80R and PHB 59Y 20R there were no significant differences in the total mass when comparing to the two different inoculated partners. However, cultivar PAN 1521R showed a significant difference between all three treatments (uninoculated control, SMH12 and WB74-1) with the highest total mass obtained by plants inoculated with SMH12. The highest total dry mass in the cultivars inoculated with SMH12 was recorded in PAN1521R and the lowest total dry mass was recorded in PHB94Y 80R. The same results were observed when soybean cultivars are inoculated with WB74-1 however, the lowest total dry mass was recorded in PHB95Y 20R. These results mean that SMH12 was more effective at improving growth of the plants in the different cultivars. There was a significant interaction between rhizobia and variety.

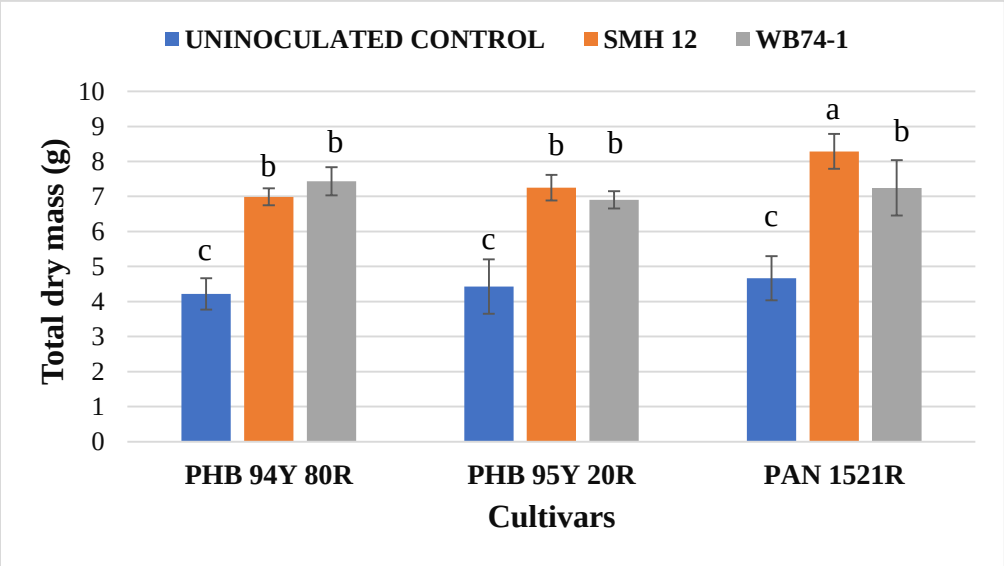

Figure 3. 11: The effect of SMH12 and WB74-1 on the total mass of soybean cultivars.

Table 3. 3: Standard deviation for SMH12 and WB74-1 on the shoot, root and total dry mass

Treatments	**Shoot Dry Mass (g)**	**Root Dry Mass (g)**	**Total Dry mass (g)**
V1 CONTROL	0.314	0.315	0.449
V2 CONTROL	0.469	0.203	0.774
V3 CONTROL	0.294	0.135	0.626
V1 SMH12	0.207	0.0408	0.242
V2 SMH 12	0.204	0.184	0.364
V3 SMH12	0.289	0.212	0.501
V1 WB74-1	0.345	0.0766	0.401
V2 WB74-1	0.249	0.0172	0.246
V3 WB74-1	0.575	0.231	0.789
LSD (0.05)	**0.397***	**0.345***	**0.546***

[+] Values with the same lets are not significantly different according to Duncan's multiple range test at the 5% level
LSD-Least Significant Difference
*Significant at LSD (0.05) ($P<0.0001$).

3.2.8 Number of Nodules

Soybean plants inoculated with SMH12 and WB74-1 exhibited differences in number of nodules per plant (Figure 3.12). The results show that averall SMH12 produced more nodules with an average of 93 nodules per plant for the three cultivars compared to WB74-1 with an average of 58 nodules per plant. The highest number of nodules were produced in cultivar PAN 1521R and the least recorded in cultivar PHB 94Y 80R. These results mean that SMH12 have the highest nodulation efficiency, because of the have the highest nodulation rate. The nodule when checked inside, by cutting horizontal they were pink inside this show they were fixing nitrogen. There was a significant interaction between rhizobia and variety. There was also a significant correlation ($r = 0.52$) (Appendix 18) between the root length and the number of nodules per plant. This suggest that there was increase in the number of nodules with an increase in root length and number. This is in agreement with results by (Delić et al. 2010) that inoculation with WB74-1 effectively increased number of nodules. There was no significant difference between cultivars inoculated with the same bacteria. However, there was a significant difference between cultivars inoculated with different strains of rhizobium at $p<0.05$.

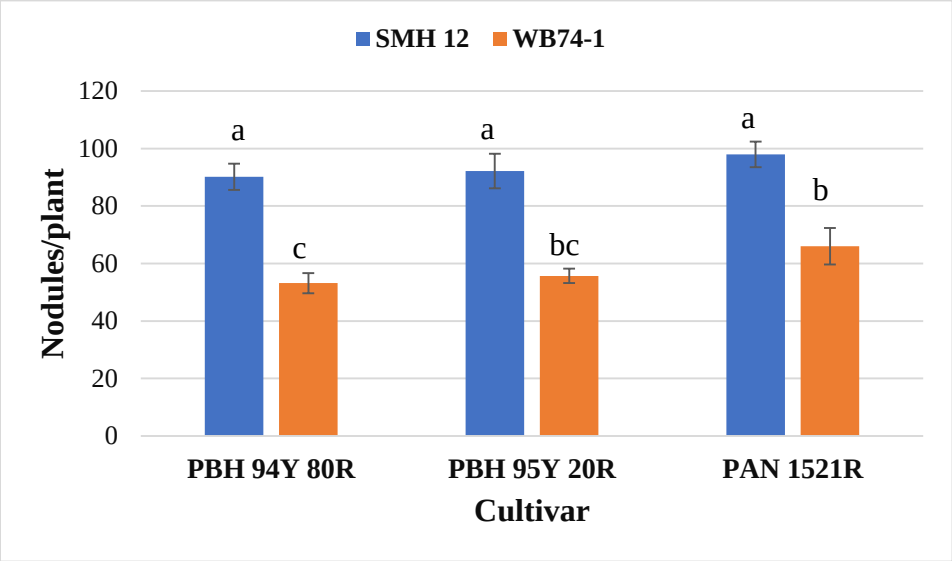

Figure 3. 12: The effect of SMH12 and WB74-1 on number of nodules of soybean cultivars.

3.2.9 Nodule size

When soybean was inoculated with the two rhizobium strains (Figure 3.13). The analysis shows that the largest nodules were produced by plants inoculated with SMH12. The average nodule size for plant inoculated with SMH12 was 3.11 mm. PAN 1521R produced the largest nodules. When comparing effect of the two strains on the same cultivars, those inoculated with SMH12 had large rnodules in PHB94Y 20R and PAN 1521R. The same observation was recorded in plants inoculated with WB74-1 at PAN1521R. Overall there was a significant difference between cultivar PHB 95Y 20R and PAN 1521R ($p<0.05$) in nodule size. However, there were no significant differences in nodule size of cultivar PHB 94Y 80R and PAN 1521R. Figure 3.14a shows that in SMH12 the nodules were mainly found in the upper 10 cm of the root zone, and these nodules were pink inside. When the nodules were cut horizontally, they were pink inside colour means they were fixing nitrogen. On the other side Figure 3.14b show the roots of soybean inoculated with WB74-1, the nodules are located in the upper root zone and also on the

lower root zone. However, when the nodules were cut horizontally, the nodules from the upper root zone were pink inside and the ones from the lower root zone were whitish inside.

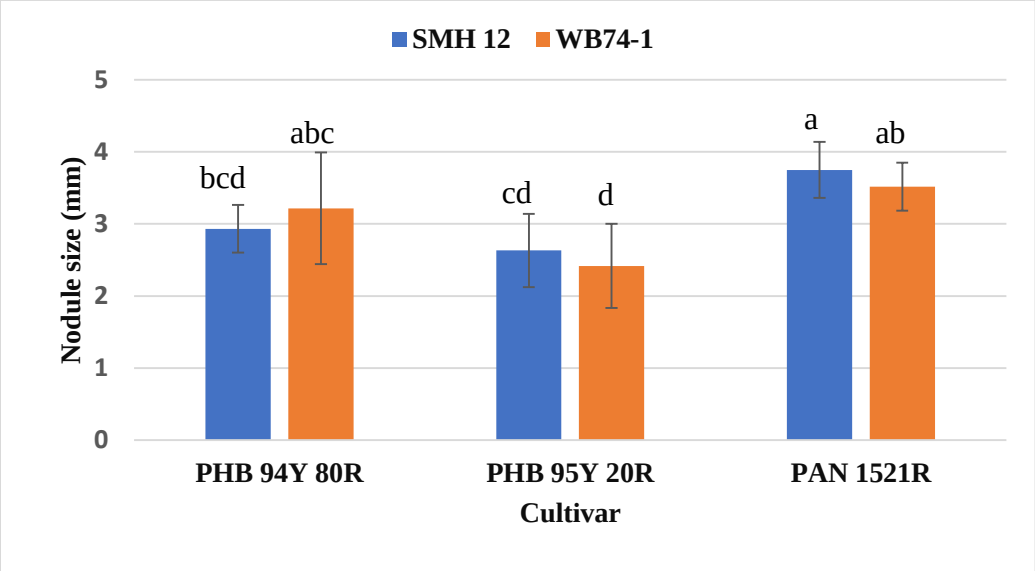

Figure 3. 13: The effect of SMH12 and WB74-1 on the nodule size of soybean cultivars.

Figure 3. 14: The nodules location on the roots of cultivar PAN 1521R inoculated with A. SMH12 and B. WB74-1.

3.2.10 Ureide content

The ureide content in the nodules of soybean plants inoculated varied within the different cultivars (Figure 3.15). Overall the ureide content was slightly higher in plants inoculated with SMH12 but only significantly higher in one cultivar PAN 1521R. This cultivar also gave the highest uride levels when inoculated with WB74-1 but it was not significantly higher compared to the other cultivars. There was a significant interaction between rhizobia and variety. The ureide content in the nodules tell us about the amount of nitrogen fixed by the plant. These results indicated more nitrogen in PAN 1521R inoculated with SMH12 when compared to the other cultivars. There was a positive correlation (r =0.5) (Appendix 18) between ureide content and the number of nodules per plant. The value of root dry mass significantly correlated (r =0.59)

with the with the total nitrogen content in the nodules as well as with plant height. The results also show that PAN 1521R is significantly different ($p<0.05$) from all the other cultivars.

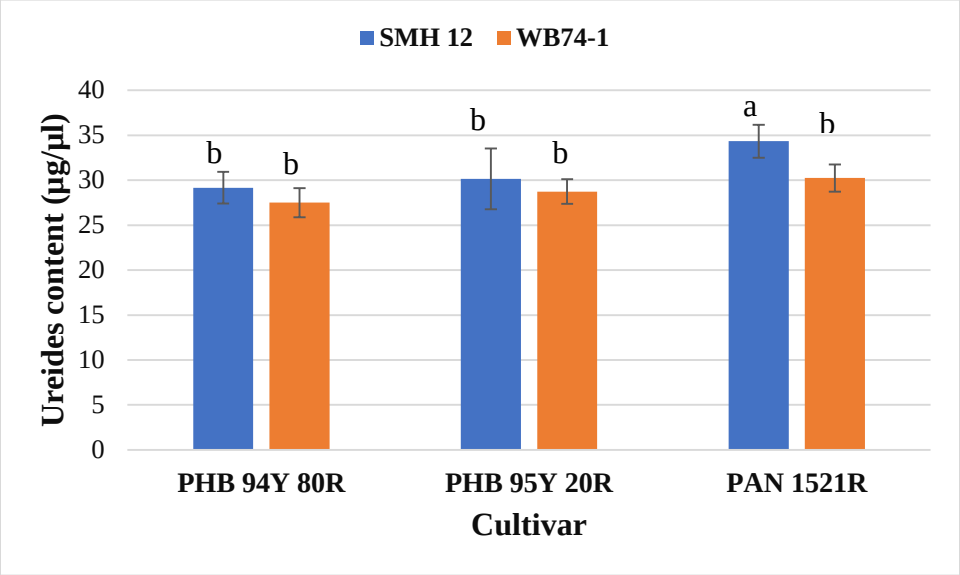

Figure 3. 3: The effect of SMH12 and WB74-1 on ureides content of soybean cultivars.

Table 3. 4: Standard deviation for SMH12 and WB74-1 on the nodules/plant, nodule size and ureide content

Treatments	Nodules/Plant	Nodule size (mm)	Ureide content (µg/µl)
V1 SHM12	4.590	0.333	1.764
V2 SMH12	5.981	0.509	3.369
V3 SMH12	4.457	0.389	1.821
V1 WB74-1	3.4881	0.776	1.6419
V2 WB74-1	2.503	0.585	1.369
V3 WB74-1	6.356	0.331	1.513
LSD (0.05)	**10.802***	**0.604***	**3.003***

[+] Values with the same lets are not significantly different according to Duncan's multiple range test at the 5% level
LSD-Least Significant Difference
*Significant at LSD (0.05) (P<0.0001).

3.3 Effect of Starter Nitrogen (KNO_3) on soybeans inoculated with SMH12 and WB74-1

3.3.1 Plant height and number of leaves

Starter nitrogen improved the growth and development of soybean (Figure 3.17). Application of nitrate signicicantly increased the plant height and number of leaves (Figure 3.18 a-c) of soybeans. The cultivar that was used for the starter Nitrogen experiment was PAN 1521R, the rationale to use this cultivar was its nodulation efficiency. Soybean plants grown in 15 mM Potassium nitrate (KNO_3) exhibited an overall higher plant height when inoculated with WB74-1 (Figure 3.17a) compared to the other nitrogen rates. There was a significant interaction between rhizobia and starter nitrogen. These results agree with results from Ohyama et al. (2017a), who found that a small amount of starter N supports vigorous vegetative growth. Huang et al. (2017) found starter N to show a positive effect in eliminating the N-deficiency period in *Pisum sativum* Linn., thus improving plant growth. There was also an increase in the number of leaves with the application of starter N. The highest number of leaves were produced in soybean grown in 15 mM KNO_3 inoculated with SMH12 and the lowest was recorded in soybean grown in 15 mM KNO_3 and inoculated with SMH12 (Figure 3.17b).

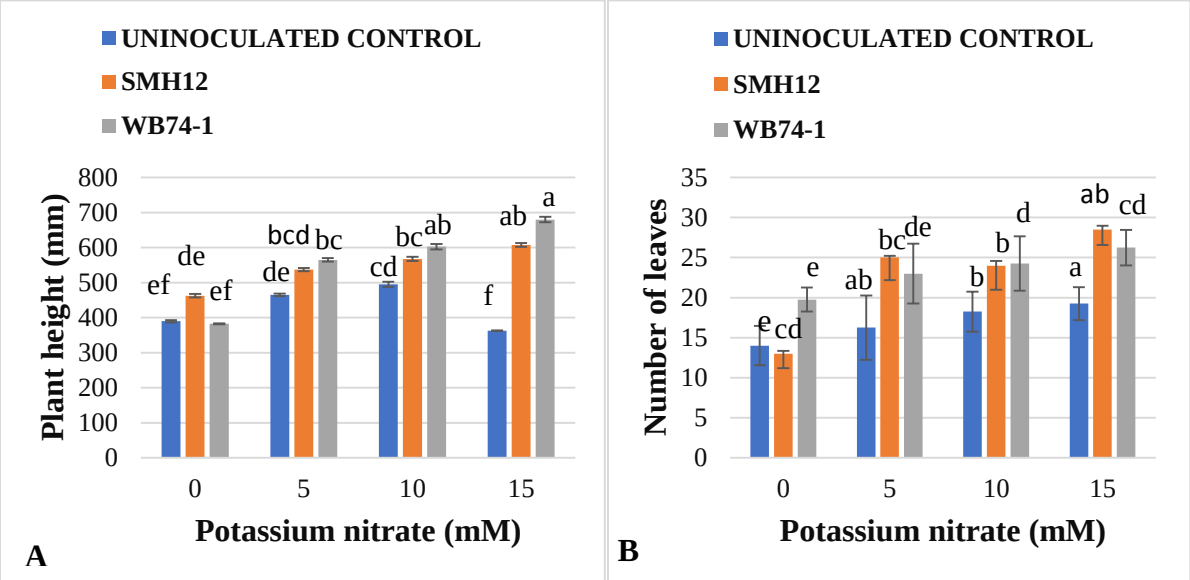

Figure 3. 4: The effect of different rates of starter N on A. Plant height B. Number of leaves.

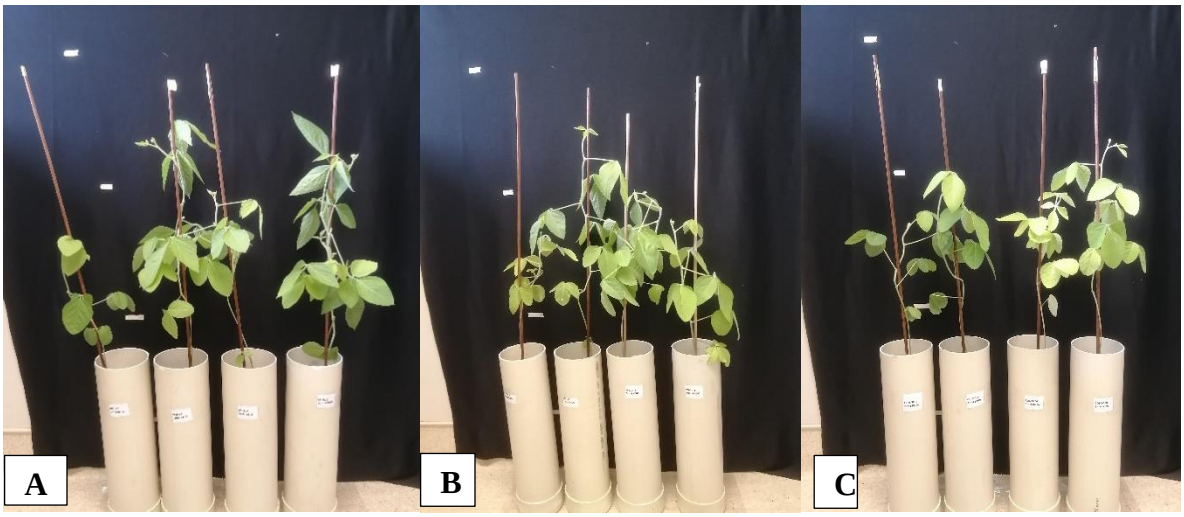

Figure 3. 5: The effect of different rates of starter N on plant height plants with A. SMH12 B. WB74-1 and C. Uninoculated Control.

3.3.2 Root length

The root length of soybeans was highly improved by the application of starter nitrogen (Figure 3.19). There was an increase in the root length of the plants with an increase in the rate of potassium nitrate until 10 mM KNO_3. Soybean plants grown in 10 mM KNO_3 exhibited a significantly higher root length when inoculated with SMH12 and the plants that received 0 mM KNO_3 exhibited the lowest root length. These results show that nitrate improves the growth of roots by providing the required nutrients for growth and development. These results agree with results by Razaq et al. (2017) who found that optimum N fertilizer at planting has a favorable impact on the root growth of *Larix gmelinii*. However, 15 mM KNO_3 suppressed the growth of the primary root, it produced shorter roots.

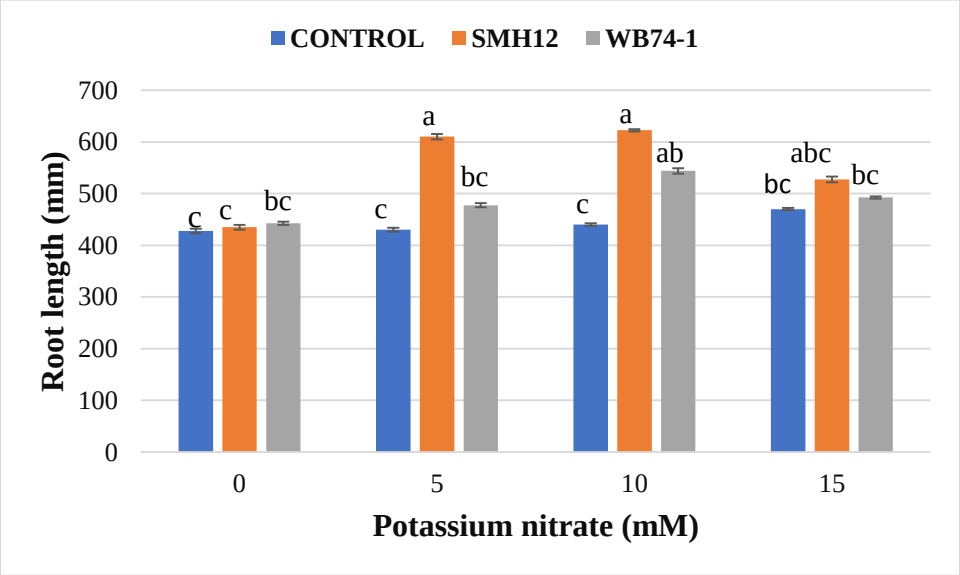

Figure 3. 6: The effect of different rates of starter N on root length.

3.3.3 Root angle

Potassium nitrate improved the root angle in soybean (Figure 3.20). There was an increase in the root angle with an increase in KNO_3concentration. The highest root angle (45°) was recorded in 15 mM KNO_3 and the lowest (35.5°) was recorded in 0 mM KNO_3. The non-inoculated control had significantly higher root angle from 0 mM KNO_3 up to 10 mM KNO_3, followed by SMH12 thereafter it had moreless the same as those inoculated with rhizobia (Figure 3.20). Thereafter WB74-1 had a slightly higher root angle when compare to plants inoculated with SMH12. However, the difference was not significant between cultivars. The root angle is controlled by generic factors. The presence of KNO_3 in the rhizosphere influenced the roots to grow in horizontal orientation. The nutrients were readily available for uptake instead of searching deeper in the soil. Plants with steep root angles are at an advantage of to capture mobile nutrients and water deep in soil the surface (Schneider et al.2021). There was a significant difference between plants of the same cultivar, when different rates of KNO_3 were applied at $p \leq 0.05$.

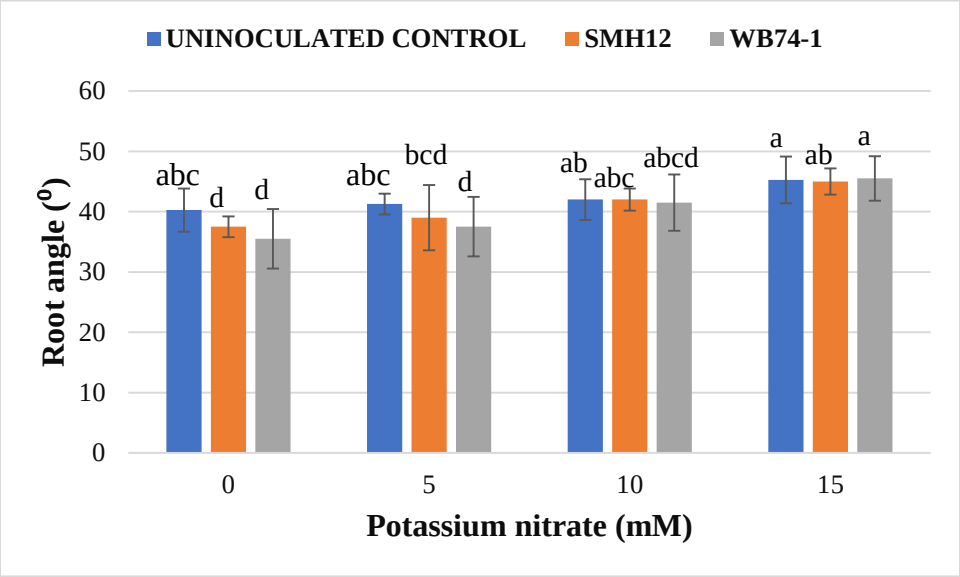

Figure 3. 7: The effect of different rates of starter N on Root angle.

3.3.4 Root density

Soybean plants grown in different potassium nitrate concentrations exhibited a significant increase in root density with an increase in potassium nitrate concentration (Figure 3.21). These results show that the highest root density was recorded in plants grown in 15 mM KNO_3 (Figure 3.22) and the lowest root density was recorded in plants where 0 mM KNO_3 was applied. The plants inoculated with SMH12 had significantly higher root density when compared to those inoculated with WB74-1, the control (non-inoculated) had the lowest root density at rates of KNO_3 (Figure 3.21). There was a significant interaction between rhizobia and starter nitrogen. This means that plants grown in 15 mM KNO_3 have a large number of roots and they are most likely to more effective in nitrogen fixation. This is because there will be more space for the formation of nodules and increased nitrogen fixation.

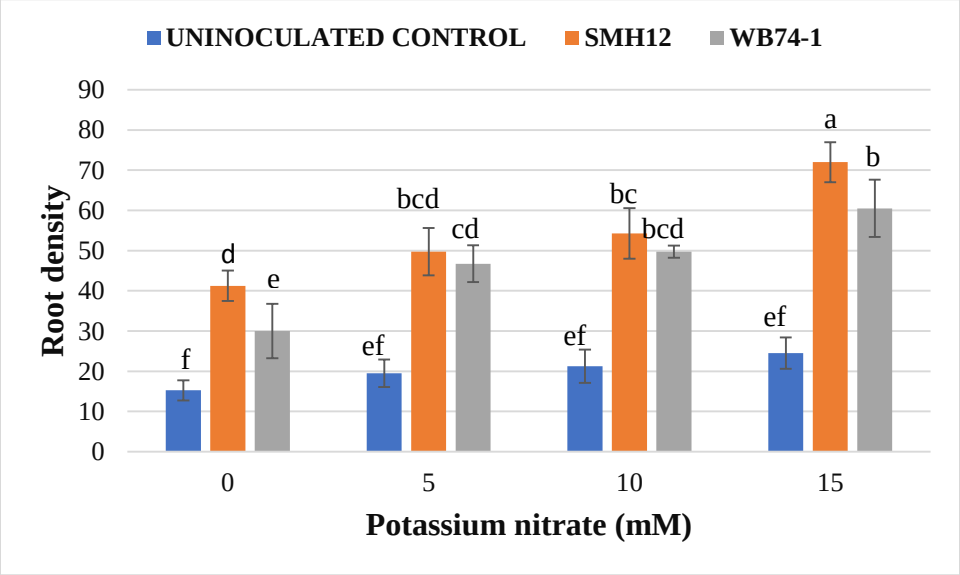

Figure 3. 8: The effect of different rates of starter N on root density.

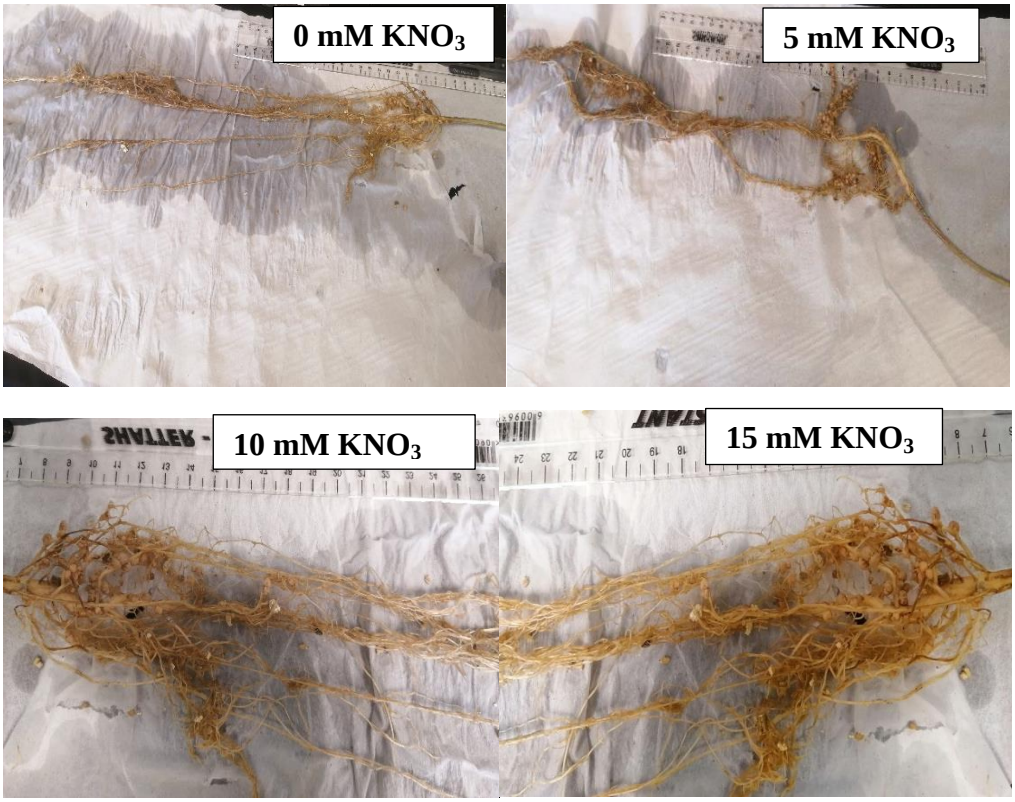

Figure 3. 9: The effect of different levels of starter N on root density.

Table 3. 5: Standard deviation for the plant height, root length angle and density

Treatment	Plant height (mm)	Root length (mm)	Root angle (°)	Root density
0 mM CONTROL	3.162	4.425	3.594	2.5
5 mM CONTROL	3.873	3.652	1.708	3.416
10 mM CONTROL	7.550	2.708	3.367	4.113
15 mM CONTROL	0.957	2.160	3.862	3.873
0 mM SMH12	5.252	4.655	1.732	3.775
5 mM SMH12	4.573	5.123	5.416	5.909
10 mM SMH12	5.852	2.160	1.826	6.292
15 mM SMH12	5.123	5.679	2.160	4.967
0 mM WB74-1	1.5	3.304	4.933	6.782
5 mM WB74-1	4.796	3.948	4.933	4.573
10 mM WB74-1	7.366	5.560	4.655	1.5
15 mM WB74-1	7.703	2.287	3.697	7.141
LSD (0.05)	**9.826****	**10.127***	**6.090***	**10.954****
Std Dev	**11.419**	**9.079**	**4.866**	**18.753**

[+] Values with the same lets are not significantly different according to Duncan's multiple range test at the 5% level
LSD-Least Significant Difference
*Significant at LSD (0.05) (P<0.0001).

3.3.5 Shoot dry mass

Potassium nitrate improved the shoot dry mass of soybeans (Figure 3.23). The increase in the rate of Potassium nitrate applied increased the dry mass of soybeans. The overall shoot dry mass was significantly higher in plants grown in 15 mM KNO_3 and the lowest shoot dry mass was produced in plants grown in 0 mM KNO_3. These results are agreement with findings of Xia et al. (2017) showed a significant increase in the dry mass of shoots with an increase in NO_3^- concentration in soybeans. The shoot dry mass of plants inoculated with SMH12 was higher than of those inoculated with WB74-1, plant not inoculated with rhizobium had the lowest shoot dry mass at all levels of KNO_3.

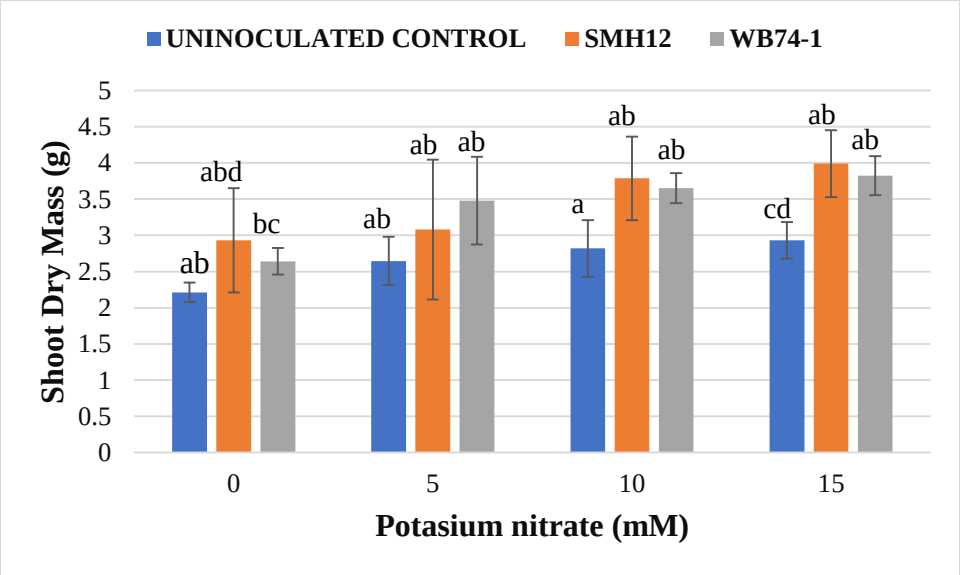

Figure 3. 10: The effect of different levels of starter N on shoot dry mass.

3.3.6 Root dry mass

As shown in Figure 3.24, starter nitrogen improved the growth of soybeans and caused a significant increase in root dry mass. The root dry mass increased with an increase of nitrogen concentration, 10 mM KNO_3 concentration reached maximum then it decreased, and 0mM KNO_3 concentration was the minimum. Soybean root mass for 0 Mm KNO_3 was significantly lower compared to the rest of the nitrogen rates, more especially the highest nitrogen rate (15 mM KNO_3). The root dry mass of plants inoculated with WB74-1 was significantly ($p \leq 0.05$) higher compared to the other inoculant. These results are in line with findings of Xia et al. (2017) he reported an increase in root dry mass of soybean when grown in increasing concentration of NO_3^-. Zhang et al. (2017) also highlighted that nitrogen and water enhance plant growth and the distribution of roots in the layer in which it is applied, this result in the increase in nutrient absorption and photosynthetic capacity of soybean.

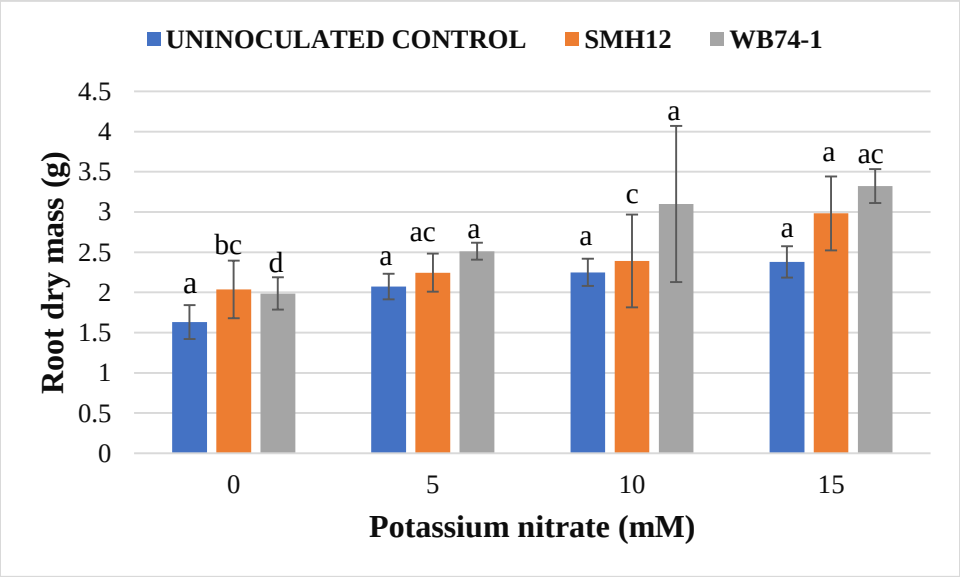

Figure 3. 11: The effect of different levels of starter N on root dry mass.

Table 3. 6: Standard deviation for shoot dry mass, root dry mass and number of leaves

Treatment	Shoot dry weight (g)	Root dry mass (g)	Number of leaves
0 mM CONTROL	0.135	0.211	2.449
5 mM CONTROL	0.334	0.160	4.031
10 mM CONTROL	0.392	0.168	2.5
15 mM CONTROL	0.251	0.194	2.062
0 mM SMH12	0.721	0.358	1.826
5 mM SMH12	0.967	0.237	2.837
10 mM SMH12	0.576	0.576	3
15 mM SMH12	0.463	0.45	1.915
0 mM WB74-1	0.184	0.201	1.5
5 mM WB74-1	0.606	0.106	3.742
10 mM WB74-1	0.0847	0.970	3.403
15 mM WB74-1	0.0191	0.209	2.217
LSD (0.05)	**0.337**	**0.251**	**3.917***

[+] Values with the same lets are not significantly different according to Duncan's multiple range test at the 5% level
LSD-Least Significant Difference
*Significant at LSD (0.05) (P<0.0001).

3.3.7 Number of Nodules

Starter nitrogen significantly increased the number of nodules of soybean (Table 3.7). The number of nodules increased significantly with an increase in nitrogen application up to a certain KNO_3 rate (Figure 3.25). In plants grown under SMH12, the number of nodules increased until 10 Mm KNO_3 and thereafter the number of nodules decreased. On the other hand, in plants inoculated with WB74-1, a decrease in number of nodules was observed when the nitrogen concentration was above 5 mM. This means the highest number of nodules in plants inoculated with WB74-1 was recorded at 5 mM KNO_3. Plants grown in soil that was not inoculated with either of the rhizobium strain did not have any nodules in the roots, this indicated that the soil was properly sterilized before planting. There was asignificant interaction between rhizobia and starter nitrogen.

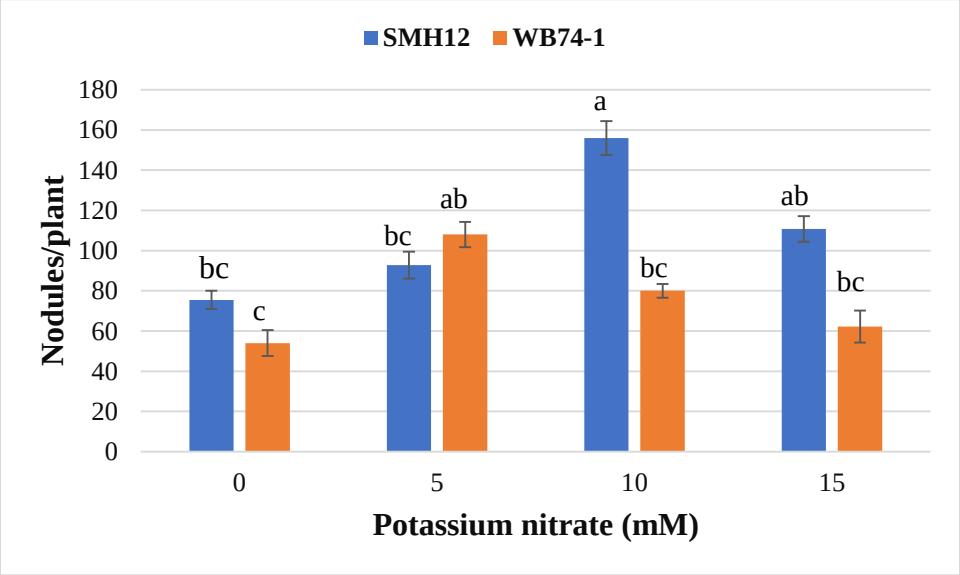

Figure 3. 12: The effect different rates of starter N on number of nodules.

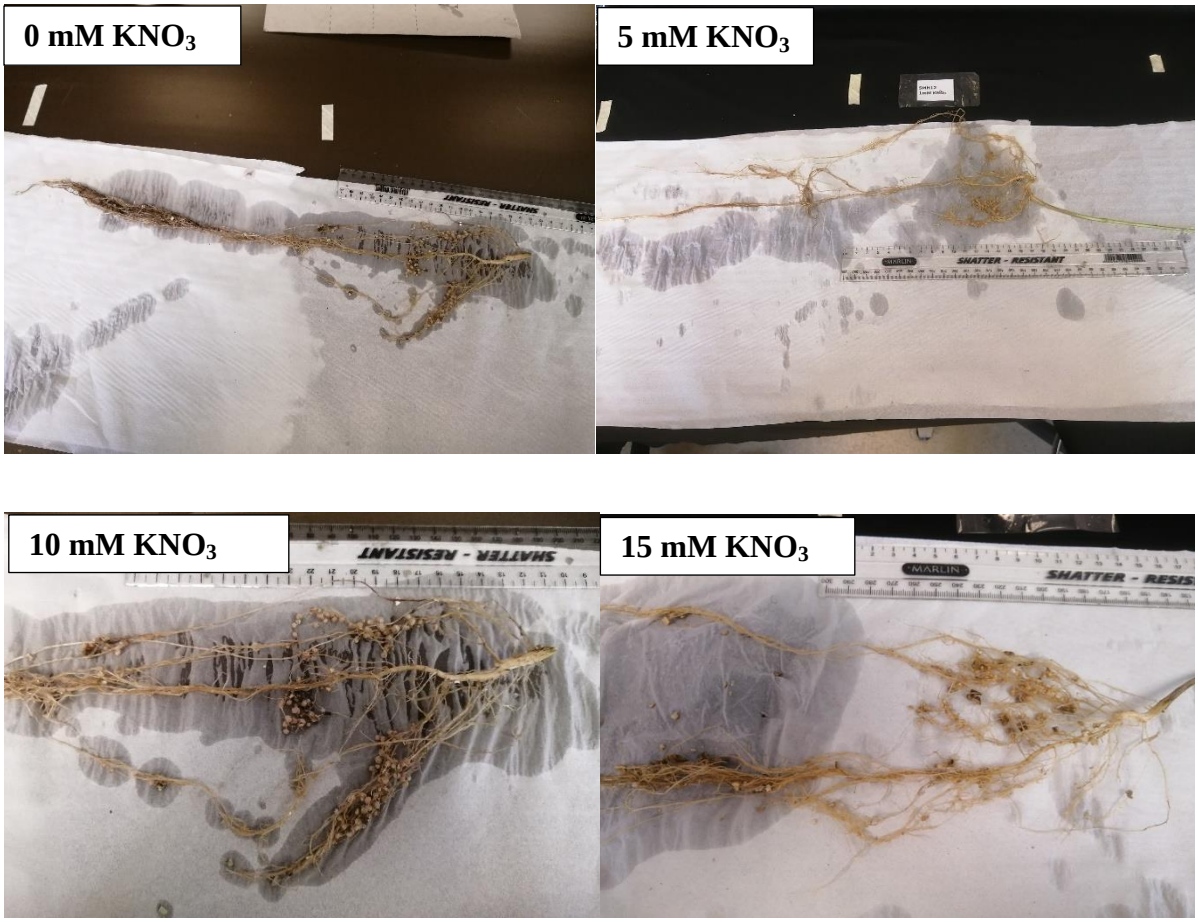

Figure 3. 13: The effects of different rate of starter N on nodulation in plants inoculated with SMH12.

3.3.8 Nodule size

The nodule size of soybeans was affected by starter nitrogen (Figure 3.27). The nodule size increased with an increase in nitrogen concentration up until 5 mM KNO_3, thereafter a decrease was observed. SMH12 recorded nodules with the biggest size (diameter) compared to WB74-1 (Figure 3.28), however, the difference was not significant. The highest nodule size was recorded when the KNO_3 was 5 mM and the lowest was observed when the KNO_3 was 15 mM in SMH12 and 0 mM in WB74-1. There was a significant interaction between rhizobia and starter nitrogen. These results show that increasing starter nitrogen concentration results in nodules with a small size. These nodules will not be efficient in fixing nitrogen. These results are in close conformity with findings of Saito et al. (2014) who showed that under nitrate nodule growth was inhibited due to changes in the photoassimilates supply from the nodules to the roots.

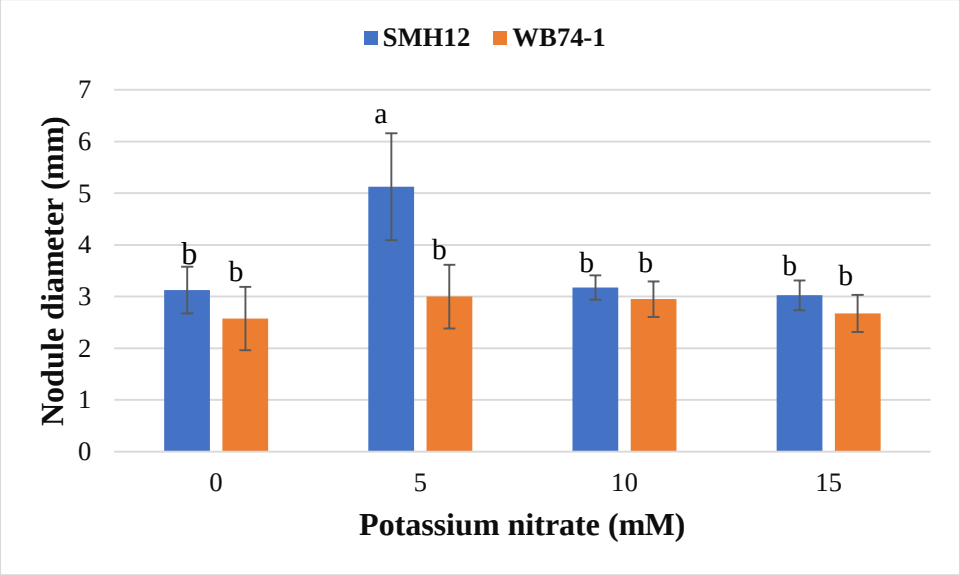

Figure 3. 14: The effect of different rates of starter N on nodule size.

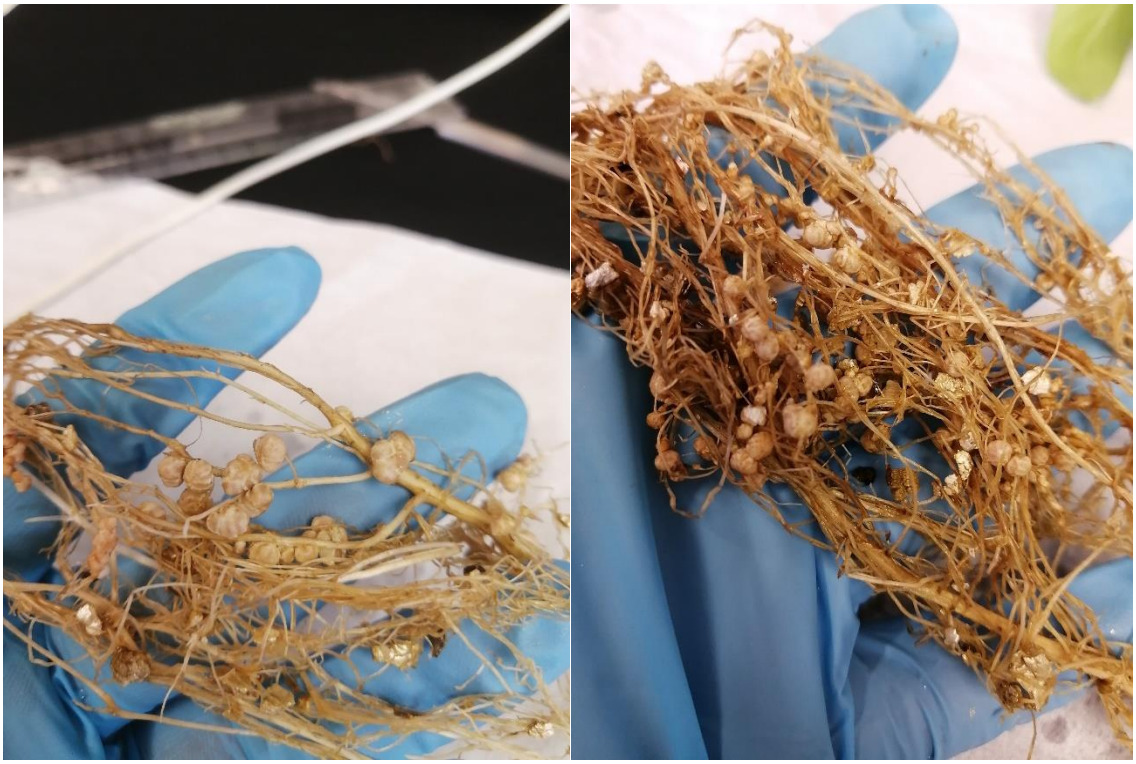

Figure 3. 15: The size of nodules from plants with starter N.

3.3.9 Nodule weight

The nodule weight of soybean was significantly affected by starter nitrogen. The nodule dry weight was increased with an increase up until a certain rate of starter nitrogen was applied (Figure 3.29). The results indicated that SMH12 and WB74-1 significantly ($p \leq 0.05$) increase the nodule dry weight from 0 t0 10 mM KNO3 and a further increase in the rate of starter N resulted in a decreased. The highest nodule weight was recorded at a concentration of 10 mM KNO_3 and the lowest was recorded in 0 mM KNO_3. When the plants were inoculated with WB74-1 the highest nodule dry weight was observed at 10 mM KNO_3 and the lowest was recorded in 0 mM KNO_3. The nodules were pinkish inside, this shows they were fixing nitrogen. There was a reduction in nodule weight when the nitrogen concentration was 15 mM KNO_3. These results are in agreement with results by Huang et al. (2017) who found that overapplication of starter N has detrimental effects on nodule growth and yield in legumes.

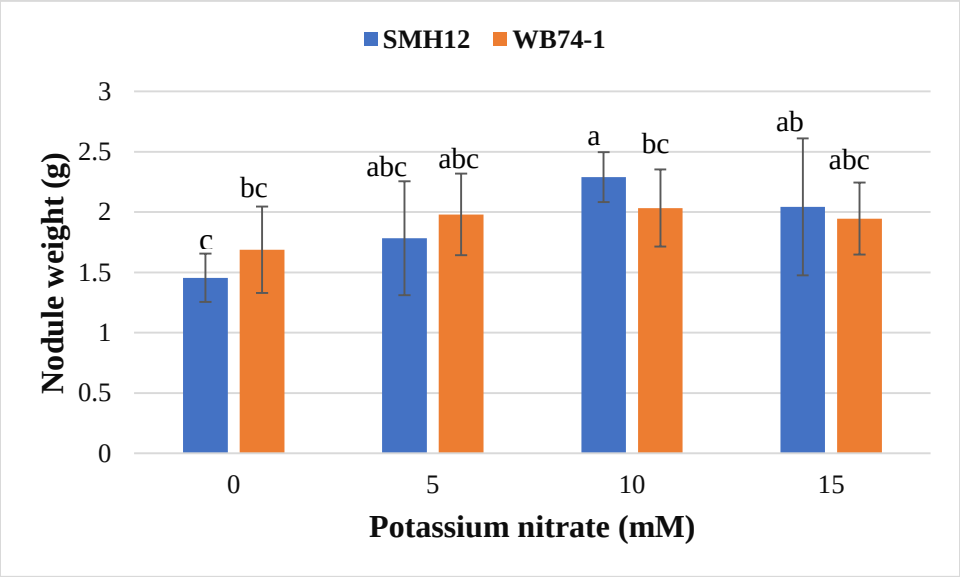

Figure 3. 16: The effect of different rates of starter N on nodule weight.

Table 3. 7: Standard deviation for nodules/plant, nodule size and nodule weight

Treatment	**Nodules/plant**	**Nodule size (mm)**	**Nodule weight (g)**
0 mM SMH12	4.509	0.45	0.202
5 mM SMH12	6.702	1.031	0.472
10 mM SMH12	8.406	0.236	0.207
15 mM SMH12	6.397	0.287	0.568
0 mM WB74-1	6.481	0.613	0.358
5 mM WB74-1	6.218	0.616	0.338
10 mM WB74-1	3.464	0.342	0.321
15 mM WB74-1	7.932	0.359	0.298
LSD (0.05)	**50.334***	**0.904**	**0.532***

[+] Values with the same lets are not significantly different according to Duncan's multiple range test at the 5% level
LSD-Least Significant Difference
*Significant at LSD (0.05) (P<0.0001).

3.3.10 Ureides Content

We measured the ureide content, as allantoin formation. This was measured in both leaves and nodules (Figure 3.30A and B). Ureide content was determined as the nitrogen fixed in SMH12 and WB74-1 in inoculated plants. Different patterns were observed in the Ureide content of nodules and leaves. Nodules from SMH12 showed an increase in the nitrogen fixed (ureide content) until 10 mM KNO_3 (Figure 3.3A), however the increase was not significant (Table 3.8). A further increase (15 mM KNO_3) in starter N resulted in a decrease in the amount of nitrogen fixed. The highest ureides content was recorded in 10 mM KNO_3 and the lowest was recorded in 15 mM KNO_3. On the other hand, it was observed that when starter N was applied to plants inoculated with WB74-1, the highest ureides content was recorded at 5 mM KNO_3 and the lowest was when 15 mM KNO_3 was applied. There was a significant interaction between rhizobia and starter nitrogen. There was a significant difference in the ureide content, in plants inoculated with WB74-1. The ureide content of the leave was also measured, leaves from WB74-1 inoculated plants had the highest average ureide content compared to leaves from SMH12 inoculated plants at 0, 5 and 15 mM KNO_3, however at 10 mM KNO_3 SMH12 recorded the highest ureide content (Figure 3.30B). There was a significant difference ($p \leq 0.05$) between the ureide content of soybean plants treated with different rates starter N.

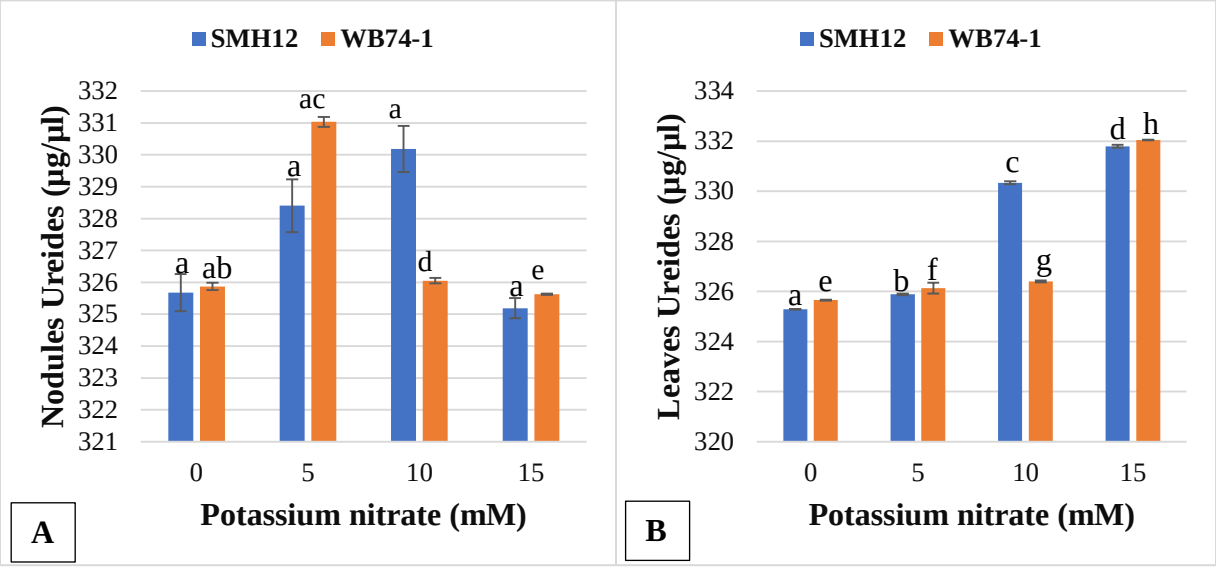

Figure 3. 17: The effect of different rates of starter N ureide content on A. Nodules B. Leaves.

Table 3. 8: Standard deviation for ureide content in nodules and leaves

Treatment	**Ureide content (µg/g)** **Nodules**	**Ureide content (µg/g)** **Leaves**
0 mM SMH12	0.580	0.0165
5 mM SMH12	0.829	0.033
10 mM SMH12	0.726	0.0595
15 mM SMH12	0.315	0.0625
0 mM WB74-1	0.115	0.0165
5 mM WB74-1	0.158	0.215
10 mM WB74-1	0.0847	0.0381
15 mM WB74-1	0.0191	0.0952
LSD (0.05)	**0.971**	**0.654**

[+] Values with the same lets are not significantly different according to Duncan's multiple range test at the 5% level
LSD-Least Significant Difference
*Significant at LSD (0.05) ($P<0.0001$).

CHAPTER 4

4.0 DISCUSSION

4.1 Effect of inoculating soybeans with SMH12 and WB74-1

Soybean is one of the most importance agricultural crops in South Africa. Soybean is mainly grown as a excellent source of vegetable oil and protein in animal feed. Farmer grow soybeans in large scale and there has been a sharp increase in production over the few years. The demand for soybean has also increased. This is attributed to the increased demand in the meat industry. The use of inorganic nitrogen fertilizers in growing may lead to environmental pollution, farmers have opted to biological nitrogen fixation to achieve optimum yields. Farmers have always been using WB74-1 as an inoculant to increase symbiotic nitrogen fixation. In recent years, there have been an introduction of a new rhizobium strain from Spain. It is called SMH12, this strain has raised hope among farmers. This is because it is fast growing compared to the rhizobium strain that have always been used. This experiment is aimed at finding the cultivar that will be more compatible with the rhizobium strain with high nodulation efficiency.

This study was conducted under greenhouse conditions. This is because SMH12 is a new rhizobium strain, not yet registered in SA under investigation, we could not use it under field conditions. The objectives and aim of this study were to look at the interaction between different cultivars (cultivars chosen based on different root phenotypes) and two rhizobium strains. We also determined the nodulation efficiency in of different soybean cultivars inoculated with different rhizobium strains. Lastly, we found the effect of different rates of starter nitrogen application in nodulation of soybean. The use of rhizobium to facilitate biological nitrogen

fixation in legumes and improve plant growth as well as soil fertility is an essential alternative to synthetic N in sustainable agriculture. SMH12 and WB74-1 are well-known strains which when inoculated into the soil facilitate nodulation and improve plant growth. In the present work, the results showed nitrogen fixation improved growth, when inoculated with the two rhizobium strains. Shoot dry mass was the best indicator nodulation efficiency.

There were phenotypic differences in the root pattern and growth of different soybean cultivars. The germination test showed that cultivar PHB 96T 06R had the highest germination percentage 1, 3 and 10 after planting. It was noted that the plants were stronger and greener in color as compared to plants from other cultivars. Plants from PHB 96T 06R germinated earlier than plants from other cultivars, this tells us that seeds from this cultivar have high seed vigor. This means they most likely grew into stronger plants. When measuring root length and density it was noted that PHB 95Y 20R had the highest. This means that this cultivar has a deeper root system and has more roots which make it to be at an advantage in times of water shortage. PHB 95T 06R had lower root angle and can extract water from deep surface areas of the soil. The three cultivars PHB 94Y 80R, PBH 95Y 20R and PAN 1521R proved to have superior root characteristics, these cultivars were then used in phase two.

Three soybean cultivars were grown in phase two of the experiment, to determine nodulation efficiency when inoculated with two strains of rhizobium. Rhizobium encouraged the growth of soybeans. The plant height of soybean was increased. It is noted that plants inoculated with SMH12 the highest plant height within all the cultivars when compared inoculation with to WB74-1. These results are in line with findings by (Peoples et al. 2009), who found that biological nitrogen fixation improved plant growth, because the plants gain extra nitrogen. Cultivar PAN 1521R exhibited the highest plant height compared with the other two cultivars.

Samudin and Kuswantoro (2018) reported that different soybean genotypes might generate different heritability causing different response to the environment such as rhizobium inoculation. It is evident that cultivar PAN 1521R had significantly the highest root length when compared with the other cultivars. Even with this parameter plants inoculated with SMH12 had the highest average root length. The increased root length made the plant to be able to grow deep in soil to absorb more nutrient and water for improved plant growth (height). This also led to an increase in nodulation.

Root angle is an important parameter in root phenotyping, it determines whether the roots will grow more vertically or horizontally. Ambient stress is related to the deepness of the root, more especially in plants grown in soils experiencing nutrients and water shortage (An et al. 2017). The growth of the roots down the soil profile is mainly determined by root elongation which is influenced by root growth angle. These findings show that cultivar PHB 95Y 20R had significantly the highest root angle when inoculated with SMH12 compared to when inoculated with WB74-1.

These results are in line with An et al. (2017) who found hybrids to have extreme phenotypes, that have numerous genes determining the average root length and growth angle. Oyanagi et al. (1993) also found that aside from genetic background, RGA has shown to be also influenced by multiple environmental conditions, such as gravity, light and water or nutrient potential. Graravitropism the force due to gravity also controls the root angle. The gravitational signals, auxin at the root tip which are always transported by auxin effluent carrier PIN- FORMED 2 (PIN2) to the zone of root elongation control the bending of roots. The amount of AtPIN2 or reduced function will have shorter roots (Roychoudhry and Kepinski 2015).

A larger root angle means the plant will cover a larger soil surface for the absorption of water and nutrients. However, a smaller root angle is an advantage in soils affected by drought because roots grow in a vertical direction, thus are more capable of absorbing water deep in the soil profile. Differences in root structure related to varieties such as thickness and root length also determine the vertical root distribution. Plants with thick roots tend to allocate more assimilates to deep roots, and consequently develop greater deep root length.

García et al. (2004) conducted a study on the soybean inoculated with three and PGPRs and concluded that strains produce growth regulator and change the growth pattern. These growth regulators included gibberellins and auxins. The auxins are important as they increase contact between the rhizosphere and rootssurface, consequently enhancing nutrient uptake which results to improved plant growth. This also improved root metabolic activity and plant nutrition. SMH12 also caused an increase in aerial growth.

The results show that cultivar PAN 1512R had the highest root density, inoculated with SMH12. This means plants inoculated with SMH12 from cultivar PAN 1521R had many roots compared to the other treatments. A higher root density means there will be more roots for water and nutrients absorption. There were also more sites for the development of root nodules. Fried et al. 2018 found nutrient and water absorption to be proportional to the contact area between root and soil.

The highest shoot and root dry mass were recorded in cultivar PAN 1521R inoculated with SMH12. These results are in conformity with Kibido et al. (2020) who reported that SMH12 encouraged root growth and consequently the growth of the above-ground parts (shoots). There was a positive correlation between shoot dry mass and number of nodules. Results by Stajkovic

et al. (2011), showed that the production of IAA by rhizobium was often directly associated with the potential to stimulate plant growth. The increase in nodulation is also connected with plant growth.

Plants inoculated with SMH12 had the highest nodulation rate in all the cultivars when compared with plants inoculated with WB74-1. These results conform with García et al. (2004) who found that SMH12 is a fast-growing strain, it therefore better in colonized the rhizosphere and fixing nitrogen. The cultivar that had the highest nodulation rate was PAN 1521R. These nodules were more efficient at fixing nitrogen when the nodules were cut into half, they were pink inside which signals nitrogen fixation. These findings are interesting. The nitrogen concentration was higher when inoculated with SMH12 compared with WB74-1. Nodules were located along the upper section of the taproot in SMH12. However, in WB74-1 the nodules were in the upper lateral roots, there were also some nodules located in the lower section of the roots. It was observed that nodules from the lower root section when cut horizontal, they were not pink inside and very small, compared to nodules from the top and mid-section that were bigger in size and pink inside.

When comparing the nodule sizes for the two-rhizobium strain, it was observed that nodules from SMH12 were bigger compared to those from WB74-1. García et al. (2004) found that SMH12 did not only produce physiological changes, which lead to an improvement in nitrogen uptake, but it also stimulated physiological changes which resulted to the improvement of the Biological Nitrogen Fixation (BNF). SMH12 is Gram-negative, and it produces siderophores. Plant root flavonoids are the inducers of the nodulation gene (nod genes) expressed in rhizobium. The increase in nodulation may also be due to the action mechanism that could be related to the availability of adequate sites for rhizobium infection. The hormonal effect also increased the root

surface thus increase nodulation. In this experiment SMH12 was more effective in nodulation compared to WB74-1.

The nodulation efficiency was measured based on shoot dry mass. Andrews and Andrews (2017) found that the effectiveness of rhizobium strain is measured or visually assessed by the evidence of red/pink nodules (sign of leghemoglobin therefore, nodule active), increased total plant or shoot dry mass or N content, noticeably greener (increased chlorophyll) and an improved plant vigor. However, in some scenariors, increased growth, vigor and/ or greenness may be due to by plant hormone secreted by the bacterium. As it was noted that plants grown in vermiculite inoculated with SMH12 had nodules bigger in size compared to those inoculated with WB74-1. There ureide content was higher in plants inoculated with SMH12 compared to in WB74-1 in cultivar PAN 1521R.

There was a strong positive correlation between plant height and root length ($r=0.91$, $p<0.01$) (Appendix 19), this relationship is positive between plant height and root length, meaning that greater plant height is associated with greater root length. There was also a strong positive correlation between root length and shoot dry mass ($r=0.84$, $p<0.01$) (Appendix 19), which is used as the measure for plant growth. This relationship means that an increase in root length increase the above ground plant growth. An increase in root length means plant have high ability to absorb water and nutrients deep in the soil even in periods of water shortage thus improving plant growth. There was a positive correlation between plant height and nodulation rate ($r=0.71$, $p<0.01$), this means an increase in nodulation rate and the number of nodules increase plant growth in soybeans. The positive correlation between nodulation rate and ureide content ($r=0.51$, $p< 0.01$) (Appendix 19) in the roots, this relationship shows an increase in the ureide content

with an increase in the number of nodules. An enhanced root system and nitrogen-fixing nodules increase soil fertility resulting in improvememt of crop yields.

4.2 Effect of starter N on nodulation

Some soybean producers depend on exclusively on rhizobia inoculation without the application of N fertilizer, some farmers use starter N fertilizers to supplement rhizobia inoculation. Starter nitrogen is important in the growth and development of legumes during the early stages of development. Research shows that the establishment of an effective plant-bacteria symbiosis in legume root system requires time. The N hunger period can adversely affect yield in grain legumes. Starter N eliminate deficiency period, especially when soil initial N is low (Van Kessel and Hartley 2000). Therefore, additional studies on and rhizobium inoculant for soybeans are necessary to improve yield and quality of the produce, enhance N use efficiency, minimize N losses, and maximize economic returns.

Our study shows that application of N fertilizer before sowing had a positive effect on the growth of soybeans. The plant height of soybean was increased when the starter N was applied. However, when the starter nitrogen was high plant height of soybean was reduced. The rate of N that produced the best results in terms of plant growth was 10 mM KNO_3. Further, we found detrimental effect of N exceeding 10 mM KNO_3 on seedling germination and vigor. These results are in line with finding of (Huang et al. 2017) in peas, application of high rate of starter N did not have a desired effects. The author emphasized that the low yields were primarily due to moisture availability and not nutrient availability.

The root length, angle, density, and the number of leaves increased with an increase in starter N. Nitrate improved the growth of roots, however, the root length increased up until 10 mM KNO3,

thereafter there was a decrease. Our results are in agreement with Gai, Zhang and Li (2017), who pointed out that excessive or insufficient ntitrogen fertilizer negatively affect grain yield of soybean. The assimilation of N in the rhizosphere might consume carbohydrates, resulting in the reduction in the amount of carbohydrates supplied to nodules.

Ohyama et al. 2011 reported an inhibitory effect of nitrate on nodulation at a concentration as low as 3 mM nitrate. The nitrate inhibition of nodulation is proposed to be caused by carbohydrate deprivation, feeedback inhibition by the products of nitrate metabolism such as glutamine and asparagine and the reduction in O_2 diffusion into nodules which decrease respiration of bacteroids. (Saito et al. 2014) also suggested that the supply of nitrate has complex and multiple effects on nodule growth and nitrogen fixation, these include a decrease in the supply of photoassimilates to the nodules. Plants inoculated with SMH12 exhibited better growth rate compared to WB74-1, however, the difference between the two strains was no significant.

The shoot and root mass of soybean was significantly improved by the application of starter N. The highest shoot mass was recorded in plants inoculated with SMH12 and the lowest was recorded in the control (non-inoculated). The shoot mass of plants inoculated with WB74-1 was higher only at 5 mM KNO_{3} when compared to those inoculated with SMH12. At 10 and 15 mM KNO_3 plants inoculated with SMH12 had the highest shoot mass.This implies that at this rate plants inoculated with SMH12 was more tolerant of nitrate. The highest root mass was recorded in plants inoculated with WB74-1. The treatment of soybean plants 10 mM KNO_3 (nitrate) had an inhibitory effect on the root growth of soybeans. Paradiso et al. (2015) reported that an increase of nitrate concentration had a stronger inhibition of the root growth and dry matter accumulation. Based on these research impedement of soybean root and nodule growth, contributed to the aboveground portion by reducing the rate of photosynthesis.

The nodulation rate was higher in plants inoculated with SMH12 when compared to WB74-1. In plants inoculated with SMH12 the highest number of nodules per plant were recorded in 10 mM of KNO_{3_3} and there was a decrease thereafter. This implies that 10 mM KNO_3 starter N was more efficient at increasing nodulation rate when plants were inoculated with SMH12. The results also indicate that 15 mM KNO_3 inhibited nodulation. SMH12 is more compatible with PAN 1521R and tolerant with increased levels of N (Huang et al. 2017) it has better legume-rhizobia interaction and it initiates production of the Nod Factor earlier, triggering a signal transduction pathway in the root cells, activating bacterial infection and nodule organogenesis faster compare to WB74-1 (Clúi et al 2018). On the other hand, plants when inoculated with WB74-1, the highest number of nodules per plant were recorded in 5 mM KNO_3 when the rate of KNO_3 was further increased, the number of nodules per plant decreased. This reveals that 10 mM KNO_3 inhibits nodulation in soybeans inoculated with WB74-1. Plants inoculated with WB74-1 were more sensitive to starter N compared to those inoculated with SMH12.

The nodules with the biggest size were recorded in plants inoculated with SMH12. When the plants were inoculated with SMH12 the largest nodules were observed when 5 mM KNO_3 was applied and the smallest nodules were in 15 mM KNO_3. The rate of 10 mM KNO_3, which had a larger number of nodules also had a small size of nodules. There was no significant difference in the size of nodules in plants inoculated with WB74-1. The size of the nodules was large in 5 mM KNO_3, and the lowest size was recorded in plants where 0 mM KNO_3 was applied (no starter nitrogen). The application of starter N had an inhibitory effect on root nodulation and size. These results are in conformity with Paradiso et al. (2015) who reported a decrease in nodulation and nodulation dry mass accumulation with an increase as the nitrate concentration increases.

The highest average nodule weight was recorded in plants inoculated with SMH12 when compared with WB74-1. In plants inoculated with SMH12 the highest average weight of nodules was recorded in 10 mM KNO_3 and when 15 mM KNO_3 was applied the average nodule weight decreased. The control (0 mM KNO_3) recorded the lowest average nodule weight. In plants inoculated with WB74-1 the nodule weight increased up to 10 mM KNO_3 and then decreased. The nodules weight where 0 mM KNO_3 was applied had the lowest nodule weight. From these results, we can infer that starter N increases the average weight of nodules up to 10 mM KNO_3, when the rate is increased further than that it decreased the weight of nodules.

Previous research proved that ureides accumulation into soybean nodules increase with an increase in starter N applied up until 12mM (Paradiso et al. 2015). This study of the ureide concentration in plants subjected to starter N, confirmed that nodule ureide levels increased after exposure to starter N. The highest ureide content was in plants exposed to SMH12 when compared to WB74-1. In plants exposed to SMH12 the ureide levels increased up until 10 mM KNO_3 and thereafter it decreased. In WB74-1, the ureide levels increased up until 5 mM KNO_3. When the KNO_3 applied was greater than 5 mM KNO_3 the ureide concentration in the nodules decreased. Tan et al. (2000) reported similar results, where there was a high concentration of ureides in root nodules than in leaves. This was due to poor absorption, limited translocation, and slow assimilation of N from the root nodules to plant. This was the main cause of a reduction in plant growth. Supplying plants with nitrate increased the ureide content in leaves, this means the plants were able to translocate ureides from the roots to rest of the plant, the nitrogen was used in carrying-out functions of the plant. SMH12 when compared to WB74-1 is nitrate-tolarent and therefore can nodulate and fix high levels of nitrogen in the presence of nitrate.

CHAPTER 5

5.0 CONCLUSION

In conclusion, the germination test used for quantifying phenotypic differences on root pattern among different soyben cultivars proved that PHB 94Y 80R, PHB 95Y 20R AND PAN 1521R have superior root characteristics. The selection of a cultivar that is more effective in nodulation with a specific rhizobium strain SMH12 is of great importance. Farmers need to know if SMH12 is more effective in nodulation compared to WB74-1. In the past, slow growing rhizobia, like WB74-1, have been regarded as poor N_2-fixers in South Africa with limited value for a commercial inoculant. Our study was testing the effect of WB74-1 and SMH12 on different cultivars. It is concluded that SMH12 is more effective in nodulation compared to WB74-1. It would be ideal for use in promoting soybean production in South Africa. PAN 1521R was found to be the best performing cultivar, it had high affinity to SMH12 compared to the other cultivars. The study of different rates of starter N in nodulation were also investigated. The different rates were tested on PAN 1512R inoculated with SMH12 and WB74-1. The study on starter nitrogen showed that strain SMH12 is nitrate-and tolerant therefore can nodulate soybean and fix high levels of nitrogen in the presence of high soil nitrogen. The recommended N rates to be used with SMH12 is 10 mM KNO_3 and WB74-1 is 5 mM KNO_3.

FUTURE PROSPECT

The research should be done in field conditions to find out if other environmental conditions will have an effect on nodulation efficiency. This will particularly allow more inoculation trials and the use of different sources of N.

CHAPTER 6

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CHAPTER 7

7.0 APPENDIX

Effect of inoculating soybeans with SMH12 and WB74-1

Appendix 1: ANOVA for plant height

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	8	4230181.050	528772.631	324.05	<.0001
Treament	8	4230181.050	528772.631	324.05	<.0001
Error	45	73428.733	1631.750		
Corrected Total	53	4303609.784			
R-Square	**Coeff Var**	**Root MSE**	**Height Mean**		
0.982938	4.611682	40.39492	875.9259		

Appendix 2: ANOVA for root length

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	8	4230181.050	528772.631	324.05	<.0001
Treament	8	4230181.050	528772.631	324.05	<.0001
Error	45	73428.733	1631.750		
Corrected Total	53	4303609.784			
R-Square	**Coeff Var**	**Root MSE**	**Root length Mean**		
0.939963	8.190405	36.99667	451.7074		

Appendix 3: ANOVA for root angle

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	8	3779.148148	472.393519	17.90	<.0001
Treament	8	3779.148148	472.393519	17.90	<.0001
Error	45	1187.833333	26.396296		
Corrected Total	53	4966.981481			
R-Square	**Coeff Var**	**Root MSE**	**Root angle Mean**		
0.760854	8.710755	5.137733	58.98148		

Appendix 4: ANOVA for root density

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	8	742.250370	92.781296	9.30	<.0001
Treament	8	742.2503704	92.7812963	9.30	<.0001
Error	45	448.708333	9.971296		
Corrected Total	53	1190.958704			
R-Square	**Coeff Var**	**Root MSE**	**Root density Mean**		
0.623238	20.53688	3.157736	15.37593		

Appendix 5: ANOVA for shoot mass

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	8	31.63418148	3.95427269	32.82	<.0001
Treament	8	31.63418148	3.95427269	32.82	<.0001
Error	45	5.42125	0.12047222		
Corrected Total	53	37.05543148			
R-Square	**Coeff Var**	**Root MSE**	**Shoot dry mass Mean**		
0.853699	10.02134	0.347091	3.463519		

Appendix 6: ANOVA for total dry mass

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	8	109.9776667	13.7472083	49.90	<.0001
Treament	8	109.9776667	13.7472083	49.90	<.0001
Error	45	12.3960667	0.2754681		
Corrected Total	53	122.3737333			
R-Square	**Coeff Var**	**Root MSE**	**Total dry mass Mean**		
0.898703	8.227931	0.524851	6.378889		

Appendix 7: ANOVA for Nodulation rate

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	5	11484.88889	2296.97778	101.69	<.0001
Treament	5	11484.88889	2296.97778	101.69	<.0001
Error	30	677.66667	22.58889		
Corrected Total	35	12162.55556			
R-Square	**Coeff Var**	**Root MSE**	**Nodule/plant Mean**		
0.944283	6.285818	4.752777	75.61111		

Appendix 8: ANOVA for nodule size

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	5	7.91555556	1.58311111	6.04	<.0006
Treament	5	7.91555556	1.58311111	6.04	<.0006
Error	30	7.8666667	0.26222222		
Corrected Total	35	15.78222222			
R-Square	**Coeff Var**	**Root MSE**	**Nodule size Mean**		
0.501549	16.63786	0.512076	3.077778		

Appendix 9: ANOVA for ureides

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	5	109.7613708	21.9522742	5.37	<.0034
Treament	5	109.7613708	21.9522742	5.37	<.0034
Error	18	73.5391250	4.0855069		
Corrected Total	21	183.3004958			
R-Square	**Coeff Var**	**Root MSE**	**Ureides Mean**		
0.598806	6.7344646	2.021264	30.01292		

Effect of Starter Nitrogen (KNO_3) on soybeans inoculated with SMH12 and WB74-1

Appendix 10: ANOVA for plant height

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	11	4438.729167	403.520833	8.59	<.0001
Treatment	11	4438.729167	403.520833	8.59	<.0001
Error	36	1690.25	46.951389		
Corrected Total	47	6128.979167			
R-Square	**Coeff Var**	**Root MSE**	**Plant height Mean**		
0.724220	13.44100	6.852108	50.97917		

Appendix 11: ANOVA for root length

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	11	2079.057292	189.005208	3.79	<.00012
Treament	11	2079.057292	189.005208	3.79	<.00012
Error	36	1795.187500	49.866319		
Corrected Total	47	3874.244792			
R-Square	**Coeff Var**	**Root MSE**	**Root length Mean**		
0.536636	14.31710	7.061609	49.32292		

Appendix 12: ANOVA for root angle

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	11	463.7291667	42.1571970	2.34	0.0272
Treament	11	463.7291667	42.1571970	2.34	0.0272
Error	36	649.25	18.034722		
Corrected Total	47	1112.979167			
R-Square	**Coeff Var**	**Root MSE**	**Root angle Mean**		
0.416656	10.35262	4.246731	41.02083		

Appendix 13: ANOVA for root density

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	11	14429.22917	1311.74811	22.48	<.0001
Treament	11	14429.22917	1311.74811	22.48	<.0001
Error	36	2100.25	58.34028		
Corrected Total	47	16529.47917			
R-Square	**Coeff Var**	**Root MSE**	**Root density Mean**		
0.872939	18.90809	7.63081	40.39583		

Appendix 14: ANOVA for number of leaves

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	11	1097.5	99.772727	13.38	<.0001
Treament	11	1097.5	99.772727	13.38	<.0001
Error	36	268.5	7.45833		
Corrected Total	47	1366			
R-Square	**Coeff Var**	**Root MSE**	**Number of leaves Mean**		
0.803441	13.00474	2.730995	21		

Appendix 15: ANOVA for nodulation rate

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	7	29660.71875	4237.24554	3.56	00091
Treament	7	29660.71875	4237.24554	3.56	0.0091
Error	24	28549.25	1189.55208		
Corrected Total	31	58209.96875			
R-Square	**Coeff Var**	**Root MSE**	**Nodule/plant Mean**		
0.509547	37.27377	34.48988	92.53125		

Appendix 16: ANOVA for nodule size

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	7	18.04375	2.57767857	6.71	0.0002
Treament	7	18.04375	2.57767857	6.71	0.0002
Error	24	9.215	0.38395833		
Corrected Total	31	27.25875			
R-Square	**Coeff Var**	**Root MSE**	**Nodule size Mean**		
0.661943	19.32612	0.619644	3.206250		

Appendix 17: ANOVA for nodule weight

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	5	1.82138750	0.26019821	1.96	0.1036
Treament	5	1.82138750	0.26019821	1.96	0.1036
Error	18	3.1855	0.13272917		
Corrected Total	21	5.0068875			
R-Square	**Coeff Var**	**Root MSE**	**Nodule weight Mean**		
0.363776	19.15584	0.364320	1.901875		

Pearson correlation analysis

Appendix 18: Correlation analysis for phase 2

	Plant height (cm)	Root length (cm)	Root angle (^{0})	Root density	Shoot D.M	Roots D.M	Nodules/plant	nodule size (mm)	Ureide content (µg/µl)
Plant height (cm)	1								
Root length (cm)	0.373	1							
Root angle (^{0})	0.501	0.832	1						
Root density	0.263	0.347	0.429	1					
Shoot D.M	0.905	0.656	0.366	0.957	1				
Roots D.M	0.248	0.367	0.461	0.356	0.626	1			
Nodules/plant	0.345	0.522	0.232	0.523	0.502	0.371	1		
nodule size (mm)	0.219	0.475	0.285	0.527	0.271	0.488	0.477	1	
Ureide content (µg/µl)	0.663	0.438	0.42	0.579	0.234	0.589	0.474	0.269	1

Appendix 19: Correlation analysis phase 3

	Plant height (cm)	Root length (cm)	Root angle (^{0})	Root density	Shoot D.M	Roots D.M	Leaf number	Nodules/plant	nodule size (mm)	Nodule weight (g)	Ureide content (µg/µl)
Plant height (cm)	1.000										
Root length (cm)	0.911	1.000									
Root angle (^{0})	0.068	0.195	1.000								
Root density	0.707	0.534	0.196	1.00							
Shoot D.M	0.755	0.841	0.097	0.71	1.0						
Roots D.M	0.591	0.423	0.205	0.60	0.6	1.0					
Leaf number	0.519	0.554	0.348	0.71	0.6	0.6	1.00				
Nodules/ plant	0.714	0.521	0.212	0.13	0.3	0.8	0.9	1.000			
nodule size (mm)	0.156	0.168	0.293	0.02	0.1	0.1	0.05	0.190	1.00		
Nodule weight (g)	0.512	0.322	0.202	0.23	0.6	0.1	0.37	0.645	0.161	1.000	
Ureide content (µg/µl)	0.591	0.063	0.144	0.10	0.3	0.59	0.19	0.511	0.120	0.042	1

Appendix 20: Correlation Analysis for SMH12

	Plant height (cm)	Root length (cm)	Root angle (^{0})	Root density	Shoot D.M	Roots D.M	Leaf number	Nodules/plant	nodule size (mm)	Nodule weight (g)
Plant height (cm)	1.000									
Root length (cm)	0.422	1.000								
Root angle (^{0})	0.100	0.199	1.000							
Root density	0.629	0.448	0.455	1.000						
Shoot D.M	0.819	0.370	0.013	0.470	1.00					
Roots D.M	0.596	0.691	0.300	0.680	0.63	1.00				
Leaf number	0.555	0.575	0.578	0.683	0.41	0.65	1.000			
Nodules/plant	0.363	0.367	0.367	0.001	0.28	0.08	0.262	1.000		
nodule size (mm)	0.187	0.036	-0.482	0.244	0.08	0.02	0.084	-0.059	1.000	
Nodule weight (g)	0.606	0.342	0.308	0.269	0.63	0.23	0.495	0.807	0.069	1.000
Ureide content (µg/µl)	0.157	0.233	0.076	0.032	0.02	0.07	0.272	0.082	0.154	0.197

Appendix 21: Correlation Analysis for WB74-1

	Plant height (cm)	Root length (cm)	Root angle (^{0})	Root density	Shoot D.M	Roots D.M	Leaf number	Nodules/plant	nodule size (mm)	Nodule weight (g)
Plant height (cm)	1.000									
Root length (cm)	0.416	1.000								
Root angle (^{0})	0.396	0.446	1.000							
Root density	0.733	0.229	0.620	1.000						
Shoot D.M	0.898	0.467	0.397	0.774	1.00					
Roots D.M	0.617	0.240	0.303	0.578	0.53	1.00				
Leaf number	0.448	0.385	0.717	0.549	0.40	0.35	1.000			
Nodules/plant	0.252	-0.087	-0.094	0.104	0.28	0.05	0.112	1.000		
nodule size (mm)	0.341	0.033	-0.239	0.004	0.32	0.12	0.101	0.442	1.000	
Nodule weight (g)	0.532	0.360	0.081	0.211	0.56	0.02	0.039	0.440	0.619	1.000
Ureide content (µg/µl)	-0.112	-0.247	-0.349	0.277	0.11	0.04	0.091	0.385	0.043	-0.178