ISOLATION AND SCREENING OF FRESHLY ISOLATED *BRADYRHIZOBIUM* AND *RHIZOBIUM* SPP. FOR MULTI-HOST PLANT GROWTH PROMOTION

By

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A dissertation submitted in fulfilment of the requirements for the degree of

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DISSERTATION SUMMARY

Biological nitrogen fixation (BNF) is an essential part of sustainable agriculture. Inorganic nitrogenous fertilizers are extensively used in modern, high input agriculture. However, they are expensive, and poorly absorbed by plants so much of the nitrogen that is applied is lost due to leaching, which results in soil acidification and ground water pollution. In contrast, rhizobia have the capacity to fix atmospheric nitrogen effectively, resulting in the uptake and assimilation of nitrogen into plants without the harmful environmental and related application costs. This study aimed to isolate, screen and identify freshly isolated *Bradyrhizobium* spp. and *Rhizobium* spp. for multi-host plant growth promotion.

Freshly isolated rhizobial isolates were obtained from the nodules on roots of soybean (*Glycine max* (L.) Merrill), cowpea (*Vigna unguiculata* (L.) Walp.), pigeonpea (*Cajanus cajan* (L.) Millsp.) and groundnut (*Arachis hypogaea* (L.) Kohler). Thirty-three isolates were isolated and stored in 40% glycerol solution at -80°C. Two commercial multi-host soybean strains, XS21 and WB74, to be used as controls, were obtained from the Agricultural Research Council, Mpumalanga, South Africa, in subsequent trials.

The development of visual growth scales of each of the test crops [soybean, cowpea, dolichos (*Lablab purpureus* (L.) Sweet) and dry bean (*Phaseolus vulgaris* (L.) var. Gadra)], were developed in order to study the dynamics of nitrogen fluxes that occurred in the growth cycles of each of the crops. The total chlorophyll and leaf nitrogen contents were determined, based on the chlorophyll and leaf nitrogen content at specific growth stages, for each crop, at multiple levels of nitrogen fertilization. A linear correlation was observed between the total chlorophyll content and that of the leaf nitrogen content of each test crop except for soybean. However, due to physiological crop damage due to red spider mite (*Tetranychus urticae* Koch.), powdery mildew (*Erysiphe polygoni* D.C.) and mealy bug (*Pseudococcus filamentosus* Guen.) infestations, that caused yellowing leaves, there was no linear correlation between chlorophyll content and leaf nitrogen content for soybean. There were significant differences in total chlorophyll content for dolichos and dry bean crops and for total leaf nitrogen content between the soybean, cowpea and dry bean crops.

The thirty-three freshly isolated rhizobial strains and the two commercial multi-host soybean strains (WB74 and XS21) were tested *in vitro* with for three secondary plant growth promoting traits: inorganic phosphate solubilization, siderophore production and indole-3-

acetic acid production. Eight freshly isolated rhizobial isolates tested positive for all three traits: Isolates CT1-6, Ukulinga I1-1, 93015 T1-1, Royes I3-3, Royes I1-2, CI1-G, 00040I2 and 87051 I2-1. Neither of the commercial controls performed well for these traits.

The 31 freshly isolated rhizobial strains and the two commercial strains were screened *in vivo*, compared with the two fertilizer controls (0% optimum nitrogen and 100% optimum nitrogen). Infection and nodulation were determined to occur at the V2 and V3 growth stages, depending on the rhizobial strain. Peak chlorophyll readings were noted at the V3 and R1 growth stages and thereafter, exponential declines in chlorophyll contents occurred at the R3, R6 and R7 growth stages. The best performing rhizobial isolates on soybean were: Ukulinga I1-1, Royes I1-2 and 93051 T1-1; on cowpea: Royes I1-2, 87091 C1-2, CCI3 and Ukulinga I1-1; on dolichos, CI2 and Royes I1-2; and dry bean, Isolate SCI performed nearly as well as the 100% Nitrogen Control for chlorophyll content, and XS21 performed nearly as well as the 100% Nitrogen Control for dry biomass.

The best 17 rhizobial isolates and the 2 commercial controls were screened with two fertilizer controls (0% optimum nitrogen and 100% optimum nitrogen) for nodulation, total nodule number and nodules containing leghaemoglobin. On soybean only four isolates produced nodules, and only one nodule contained leghaemoglobin. On cowpea 11 of the rhizobial isolates tested produced nodules, with nearly every nodule containing leghaemoglobin. On dolichos, 18 rhizobial isolates produced nodules, half of which contained leghaemoglobin. On dry bean, 9 rhizobial isolates produced nodules, and less than half of the nodules contained leghaemoglobin.

Subsequently, the freshly isolated rhizobial strains that performed best were used in combination with potassium silicate (KSi), to determine their combined effects on chlorophyll content and dry biomass. A full factorial trial was not conducted due to lack of glasshouse space. The 17 rhizobial isolates, 2 commercial rhizobial strains and 4 fertilizer controls (0% optimum nitrogen, 100% optimum nitrogen, 0% optimum nitrogen + KSi and 100% optimum nitrogen + KSi) were tested. The total chlorophyll content and dry biomass was measured for each crop and analyzed using ANOVA, or Kruskal-Wallis analysis. The soybean plants treated with XS21 + KSi, and Royes I3-3 + KSi had higher chlorophyll contents than the 100% Nitrogen Control. Plants treated with CI2 + KSi, and Royes I3-3 + KSi had higher dry weights than the 100% Nitrogen Control. Twelve of the rhizobial isolates tested on cowpea plants produced more chlorophyll than the 100% Nitrogen Control. Nine of those rhizobial

isolates had higher chlorophyll contents than the plants treated with 100% Nitrogen + KSi Control. All of the rhizobial isolates tested on cowpea plants had higher dry weights than the plants treated with 0% nitrogen, 0% nitrogen + KSi and the 100% nitrogen. Sixteen of the nineteen rhizobial isolates tested on dolichos had higher chlorophyll contents than the plants treated with 0% N + KSi and 0% nitrogen. The plants treated with Isolates 87051 I3-1 + KSi had a higher chlorophyll content than the 100% Nitrogen Control. Eighteen of the rhizobial isolates tested on dry bean had higher dry weights than the 0% Nitrogen Control + KSi. Only Isolate CI2 + KSi on dolichos resulted in a dry weight comparable to both the 100% Nitrogen Control and 100% Nitrogen + KSi Control. None of the rhizobial isolates tested on dry bean plants caused chlorophyll contents comparable to both the 100% Nitrogen and 100% Nitrogen + KSi Controls.

Fourteen rhizobial isolates were identified using 16S DNA sequencing. Nine of these belonged to the genus *Bradyrhizobium* and the remaining five were identified as *Achromobacter xylosoxidans* Strain zc1, *Pseudomonas moraviensis* Strain IARI-HHS1-33 and *Pseudomonas* sp. LC182, *Pseudomonas chlororaphis* Strain UFB2 and *Bacillus acidiceler* Strain TSSAS2-2.

DECLARATION

I, Marylyn Matilda Christian, declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research

2. This dissertation has not been submitted for any degree examination at any other university

3. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted. Then:

    a. Their words have been re-written but the general information attributed to them has been referenced;

    b. Where their exact words have been used, then their writing has been placed inside quotation marks and referenced

4. This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the references sections

Signed ----------
Marylyn M. Christian (Candidate)

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Prof M.D. Laing (Supervisor)

Signed ----------
Dr K.S. Yobo (Co-supervisor)

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DEDICATION

To my amazing parents, Paul Jonathan Frederick Christian and Pamela Christian;

To my brothers, Adrian Ainsley Christian and Jerome Nathan Christian;

To my sister, Glenda L. Christian;

And to my nieces, Gabriella N.A. Christian and Elliana C.G. Christian;

I dedicate this dissertation to them

for being my pillars of strength, support, encouragement and inspiration.

Contents

DISSERTATION INTRODUCTION

Globally there has been a move to shift agriculture from its current paradigm of high input, high-output agriculture to lower inputs and greater sustainability. This includes the goal of reducing the levels of artificial chemical fertilizers, especially nitrogenous fertilisers, being applied to the soil. To maintain the high levels of agricultural productivity sustainably, much research has been directed at biological nitrogen fixation (BNF) (Peoples, et al., 1995). Nitrogen Use Efficiency (NUE) is critical in evaluating the role of nitrogen, and its availability, uptake, assimilation, utilization and remobilization during plant life cycles (Masclaux-Daubresse, et al., 2010). Nitrogen fixing rhizobia and their host crops play an integral part in the development of sustainable agricultural practices. Numerous benefits accrue from using symbiotic nitrogen fixing bacteria on leguminous hosts (O'Hara, 1998, Howieson, et al., 2000, O'Hara, et al., 2002). Soybean accounts for 50% of the global legume crop area planted, 68% of global production of plant protein legumes and generates almost 77% of the BNF fixed by legumes, an estimated 16.4 Tg of nitrogen (Herridge, et al., 2008). Some of the factors that hinder rhizobial inoculants in soil include: high levels of soil nitrogen that suppress rhizobial infection, nodulation and nitrogen fixation; high levels of native rhizobia that may outcompete commercial rhizobial inoculants, reducing infection and nitrogen fixation; poor quality of rhizobial product, especially low cell counts and low vigour; inappropriate storage conditions of inoculants may lead to short shelf lives; and finally, incorrect inoculation procedures followed by farmers may reduce nodulation (Bantilan and Johansen, 1995, Seneviratne, et al., 2000). Some strains of *Bradyrhizobium* spp. and *Rhizobium* spp. are considered multi-host, with the capacity for infection and effectively nodulating between 17 and 18 legume genera, respectively (Hernandez-Lucas, et al., 1995, Perret, et al., 2000).

The main aim of this research was to isolate and screen freshly isolated strains of *Bradyrhizobium* spp. and *Rhizobium* spp. *in vitro* and *in vivo* for multi-host plant growth promotion on four leguminous crops.

The specific objectives of this study were to undertake the following:

1. A literature review on *Rhizobium* spp., their sustainability, economic importance, mode of infection and the factors that affect nitrogen fixation.

2. The isolation and storage of freshly isolated rhizobia from within nodules containing leghaemoglobin of soybean, cowpea, pigeonpea and groundnut. Two commercial strains of *Bradyrhizobium*, WB74 and XS21, originally selected for their performance on soybean were obtained from the Agricultural Research Council and were used as controls in for subsequent trials.
3. Documentation of the growth stages of each of the test crops, soybean, cowpea, dolichos and dry bean.
4. Measurement of the chlorophyll content and leaf nitrogen content of the four crops at specific growth stages to determine the total chlorophyll content and leaf nitrogen content at various nitrogen fertilizer levels.
5. *In vitro* screening of the thirty one freshly isolated rhizobial isolates for growth stimulatory activities, including the solubilization of inorganic phosphate, and the production of siderophores and indole-3-acetic acid.
6. *In vivo* screening of the 31 freshly isolated rhizobia and commercial controls for multi-host plant growth promotion on soybean, cowpea, dry bean and dolichos, based on chlorophyll content and dry biomass.
7. *In vivo* screening of the best performing 17 freshly isolated rhizobia and 2 commercial controls for nodulation of soybean, cowpea, dolichos and dry bean.
8. *In vivo* screening of best performing 17 freshly isolated rhizobia and 2 commercial controls, when combined with silicon fertilization, based on chlorophyll content and dry biomass.

The dissertation has been written in the form of seven chapters, each chapter covering a specific aspect of the research conducted on the isolation and screening of freshly isolated rhizobia for multi-host plant growth promotion. With the exception of the literature review, each of the chapters has been written as an independent study, and prepared in the format of a scientific paper. This creates some redundancy in the introductory information, and the references. However, it is the standard dissertation model that has been adopted by University of KwaZulu-Natal.

This research was undertaken in the Discipline of Plant Pathology, at the University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Prof. M.D. Laing and Dr K.S. Yobo.

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Chapter 1

Literature review

1.1 Introduction

The family *Rhizobiaceae* consists of several genera, namely: the slow growing, *Bradyrhizobium*; the fast growing *Sinorhizobium*; *Allorhizobium*; *Mesorhizobium; Rhizobium*; the pathogenic *Agrobacterium*; and the stem nodulating *Azorhizobium* (Young and Haukka, 1996, de Lajudie, et al., 1998). *Rhizobium* spp. are characterised as small to medium rods that stain Gram negative and are classified as either fast or slow growing. Rhizobia are strict aerobes with certain strains of *Bradyrhizobium* able to grow autotrophically on atmospheric hydrogen (H_2) (Schlegel, 1993). Rhizobia exist in three types of environments: as free living bacteria in soil; in infected root nodules of leguminous plants; and in the rhizosphere of leguminous plants (Slattery, et al., 2001).

Rhizobia form symbiotic relationships with legumes. Symbiotic relationships are initiated by infection of legume root hairs by rhizobia, resulting in nodular growths (Malik, et al., 1984). Legumes infected with mutualistic rhizobial strains obtain extra nitrogen for plant growth and development (Brockwell, 1982). This, allows legumes to grow in nitrogen-poor environments (Martínez-Romero, 2009). Most *Rhizobium* strains are capable of infection of a restricted host range (Shantharam and Wong, 1982). However, some strains are capable of being "promiscuous" or multi-host, with an ability to inoculate a wide host range. Examples are *Rhizobium* sp. NGR234, *Rhizobium* sp. 127E15 and *Rhizobium fredii* USDA 257 (Pueppke and Broughton, 1999).

Correlations between plant colour and plant dry weight can be made with total nitrogen accumulated by plants (Wynne, et al., 1980). In a trial on 14 Cuban lines of groundnuts with several strains of *Rhizobium* spp. the best *Rhizobium* strains caused increases in crude oil, protein yield and seed weight per pod (Martinez-Viera, et al., 1988).

1.1.1 Impact and importance of nitrogen fixation

The family Leguminosae consist approximately of 750 genera and 20 000 species. Legumes are economically and agriculturally important crops. The symbiotic relationship between legumes and *Rhizobium* spp. results in the fixation of atmospheric nitrogen. This has an economic impact by reducing nitrogen fertilizer application as illustrated in Figure 1.1 It also contributes to more sustainable agriculture by generating nitrogen rich plants and seeds for food and fodder (Dixon and Wheeler, 1986). The rising cost of nitrogen fertilizers manufactured from fossil fuels thus requires the aid of biological nitrogen fixation to reduce agricultural costs (Wynne, et al., 1980). Biological nitrogen fixation is therefore an environmentally and economically feasible alternative to commercial nitrogen fertilizers.

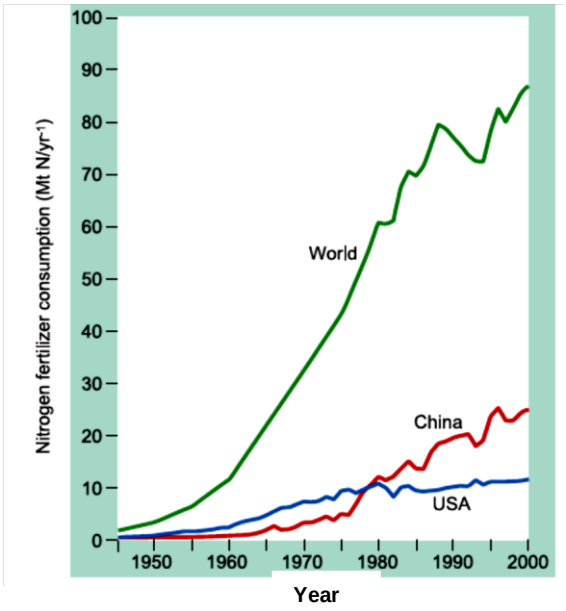

Figure 1.1: Nitrogen fertilizer consumption in the United States of America (USA), China and globally for the period of 1950 to 1999 (Smil, 2002)

Nitrogen is an integral part of human nutrition, required for the generation of amino acids, and is found in deoxyribose nucleic acids (DNA) and ribose nucleic acids (RNA). This element is required for protein and enzyme synthesis as well as maintenance of the body, with many by-products excreted by the body containing nitrogen (Pellett, 1990). The Haber-Bosch synthesis of ammonia industrialized nitrogen fixation (Figure 1.2), and today 40% of the world agriculture is dependent on this process (Smil, 1999). The human population is

expected to increase from around 6 billion to approximately 10 billion by the year 2050 (Byrnes and Bumb, 1998), which will require a substantial increase of world food production.

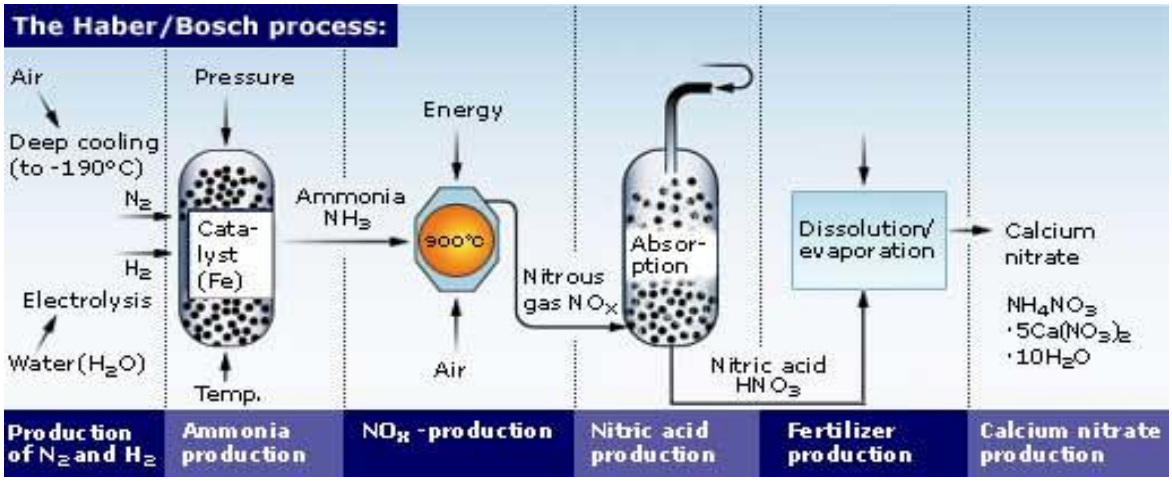

Figure 1.2: The Haber-Bosch process (Anonymous(a), 2007).

Intensified crop production due to the need for increased food production has therefore led to a greater reliance on nitrogen fertilizers (Loneragen, 1997). It is estimated that by 2020, world fertilizer requirements to satisfy world food needs is set to increase to 54 million tons (Galloway, et al., 1994). Nutrition depletion in soils is a growing concern, particularly in developing countries (Sanchez, 2002).

1.2 Legumes

Globally, grain legumes are a major contributor to human nutrition; providing vital sources of proteins, vitamins and minerals. The major legume crops are: soybean (*Glycine max*); groundnut (*Arachis hypogea*); pigeonpea (*Cajanus cajan*); cowpea (*Vigna unguiculata*); lentil (*Lens culinaris*); mung bean (*Vigna radiata*) and chickpea (*Cicer arietinum*) (Singh and Singh, 1992). Legumes, working in symbiosis with *Rhizobium* spp., benefit agriculture with their ability to fix atmospheric nitrogen, introducing fresh nitrogen into the cropping system. Biological nitrogen fixation reduces the need for nitrogen fertilizers in agriculture (Power, 1987). Long term benefits of legumes on soils can outweigh the immediate monetary value of the crop, with enhancements of the organic matter content of soils and availability of nitrogen

for crops that are rotated with legumes (Hoyt and Hargrove, 1986). Two of the most commercially valuable legumes in South Africa are soybean and groundnut.

1.2.1 Soybeans

Soybeans are an expanding commercial crop in Southern Africa, benefitting small scale farmers more than crops such as groundnuts and cowpeas (Mpepereki, et al., 1996). This is visible in Figure 1.3, illustrating the increases in soybean production and maintenance of yield averages amongst African countries (FAO, 2015). Soybean production has increased steadily on the global market, beginning in Asia, predominately in China and Japan and expanding to areas of Europe and the United Kingdom, and eventually America (Probst and Judd, 1973). In India, soybeans are a lucrative oilseed crop (Gupta and Rajput, 2001). Soybean is processed for oil, flour, meal and animal feed products. Processing of soybean is different to that of grains, in order to obtain the optimum food and feed products. Moist heat treatments causes the inactivation of anti-nutritional proteins, making soya flour and meal suitable for consumption by humans and animal feed (Cowan, 1973). According to Black and Mattel (1951), crude soybean oil is refined for production of mayonnaise, salad dressings, margarine, salad oils and even sandwich spreads, with the lecithin fraction being used in stabilizing agents, emulsifiers and anti-spattering agents. Industrial products such as paint, ink, soap and cosmetics are also produced from soybean oil (Bradley, 1951).

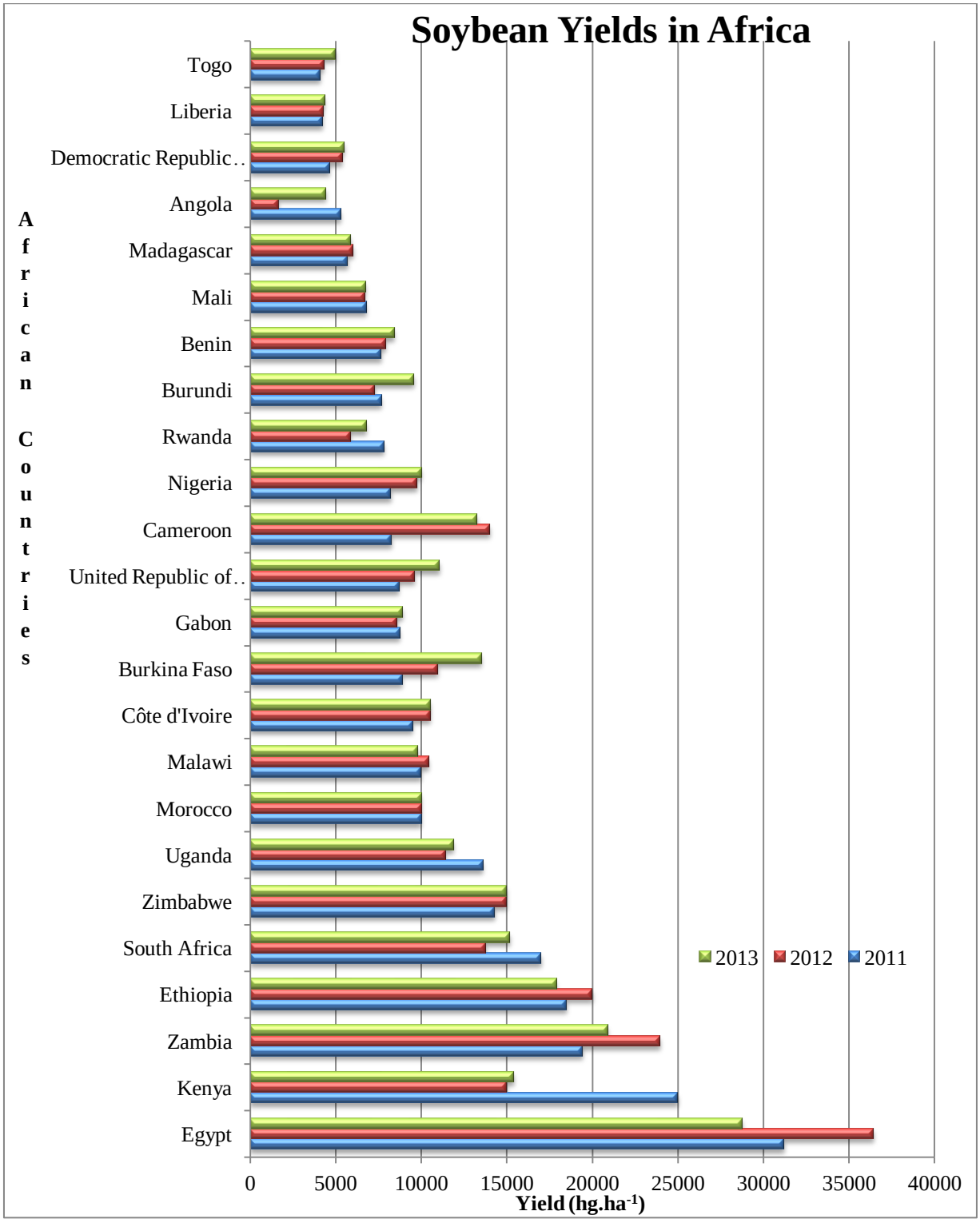

Figure 1.3: Yield per hectare for African countries for the periods 2011, 2012 and 2013 (FAO, 2015).

In Zimbabwe, over the period 2011-2013 the mean soybean production was 14762 $hg.ha^{-1}$, whilst South Africa had a soybean production of 15313 $hg.ha^{-1}$ for the same period (Figure 1.3) (FAO, 2015). Soybean can increase the nitrogen content of nitrogen-poor soils

due to its ability to form symbiotic relationships with *Rhizobium* spp. which aids in sustainable cropping systems. The soybean varieties are either susceptible or not susceptible to *Rhizobium* spp. infections. Promiscuous or multi-host varieties of soybeans can form nodules with many strains and are capable of nitrogen fixation, even on farms with no history of *Rhizobium* inoculation, particularly in Zimbabwe, Zambia and Malawi (Mpepereki, et al., 2000). Soybean is considered to be the most inexpensive source of protein based on the production costs, per kilogram of protein (Judd, 1970).

1.2.2 Groundnuts

Groundnut (*Arachis hypogaea*) is also known as the earthnut, monkeynut, Malinanut or peanut and is regarded as the world's fourth largest crop for edible vegetable oil and vegetable protein (Lusas, 1979). Consumption of whole groundnut kernels has increased whilst there is a decreased usage of groundnut oil, in comparison to soybean oil (Revoredo and Fletcher, 2002). The largest producers of groundnut are India, China, Brazil, America, and West and Southern Africa, with temperatures of between 25-28 °C and rainfall greater than 500 mm (Maiti, 2002). The world utilization of groundnut during the period 1996-2000 was 29 million metric tons (Revoredo and Fletcher, 2002). High yields of groundnut in African countries, as shown in Figure 1.4, reflect the importance of groundnut in this region (FAO, 2015).

One of the major factors that affect groundnut growth is its ability to fix nitrogen (Maiti, et al., 2002). Depending on the cultivar of groundnut used, nitrogen fixation has been noted to peak at around 84 days post planting and seems to decrease after this. Nodulation increases until 84 days after planting but can be as late as 98 days post planting (Wynne, et al., 1980). Symbiotic interactions between *Rhizobium* spp. and groundnuts are well documented.

The quality of groundnut is important for both human and animal consumption. Factors that may affect groundnut quality are insect damage, fungal infections, climatic factors and harvesting methods (Maiti and Wesche-Ebeling, 2002). Nitrogen deficiency will limit groundnut productivity. Nitrogen fixation by *Rhizobium* strains improves crop quality and productivity (Maiti, et al., 2002). Higher yields have been noted in groundnut inoculated with *Rhizobium* spp. in comparison with uninoculated groundnut (Dongarwar and Thosar, 1991).

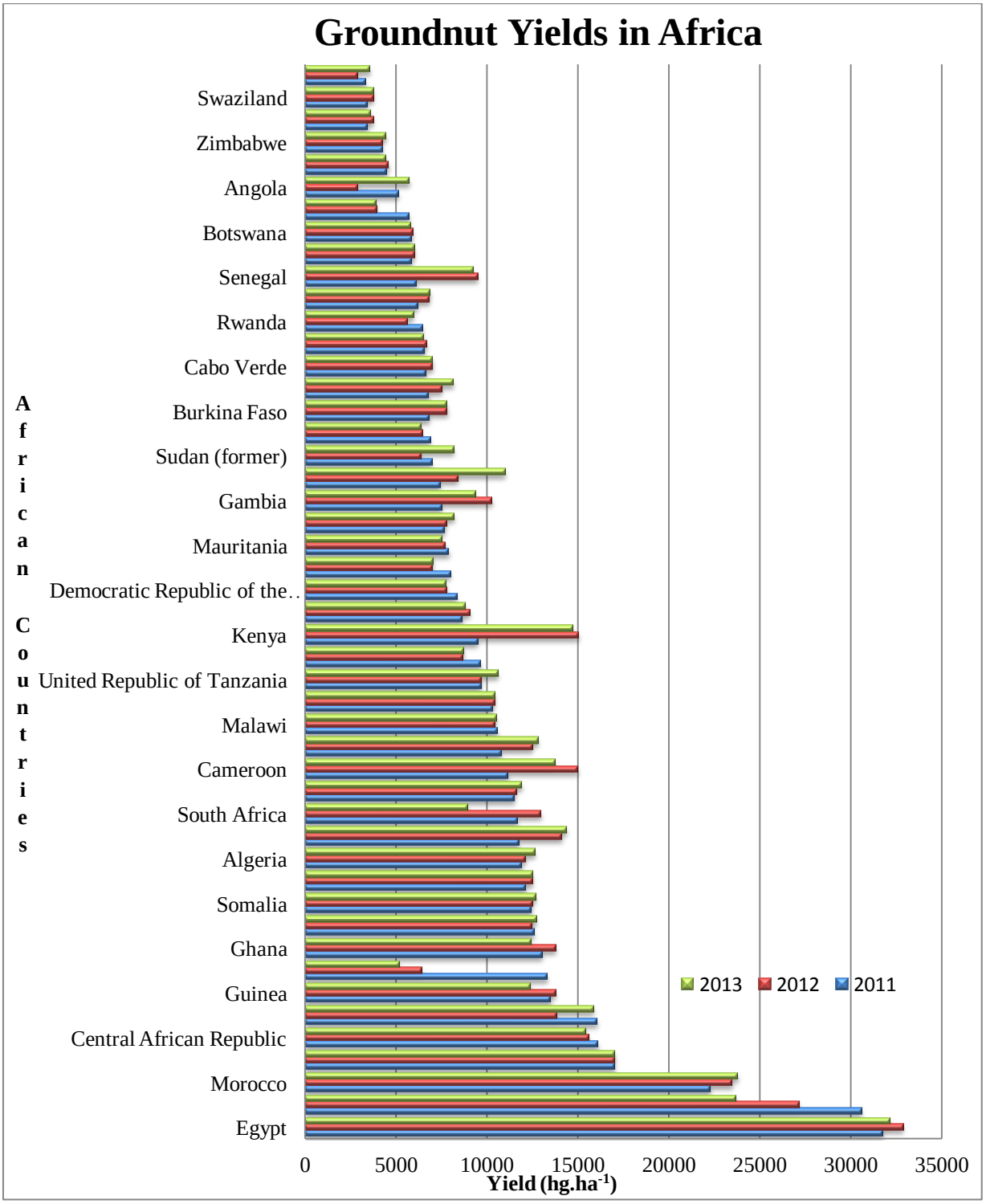

Figure 1.4: Yield per hectare of groundnuts in African countries for the periods 2011, 2012 and 2013 (FAO, 2015).

There has been a steady increase in the yield of groundnuts in African countries, between the years of 2011 and 2013, particularly in Egypt, which overshadows the rest of the African countries in production, as shown in Figure 1.4 (FAO, 2015). Egypt also increased production

of groundnuts between 1972 and 2000. Groundnut production in the Asian and African continents showed increases during this period, whilst production in North America and other developed countries remained fairly stagnant, with little increase between 1972 and 2000 (Revoredo and Fletcher, 2002).

1.2.3 Cowpea

Cowpea (*Vigna unguiculata* (L.) Walp.) is a staple food in many developing countries, eaten as dried seeds, green pods, green seeds, and even the tender green leaves can be consumed. Other uses of cowpea include animal fodder and as an effective ground cover. The nitrogen fixing capability of cowpeas with rhizobia is up to 240 kg of nitrogen per hectare, which leaves substantial amount of nitrogen in soils for succeeding crops (Rachie, 1985). Cowpea is a major crop in sub-Saharan Africa, particularly in the dry regions of West Africa. The crop provides essential nutrients and plant proteins for humans and animals (Dugje, et al., 2009).

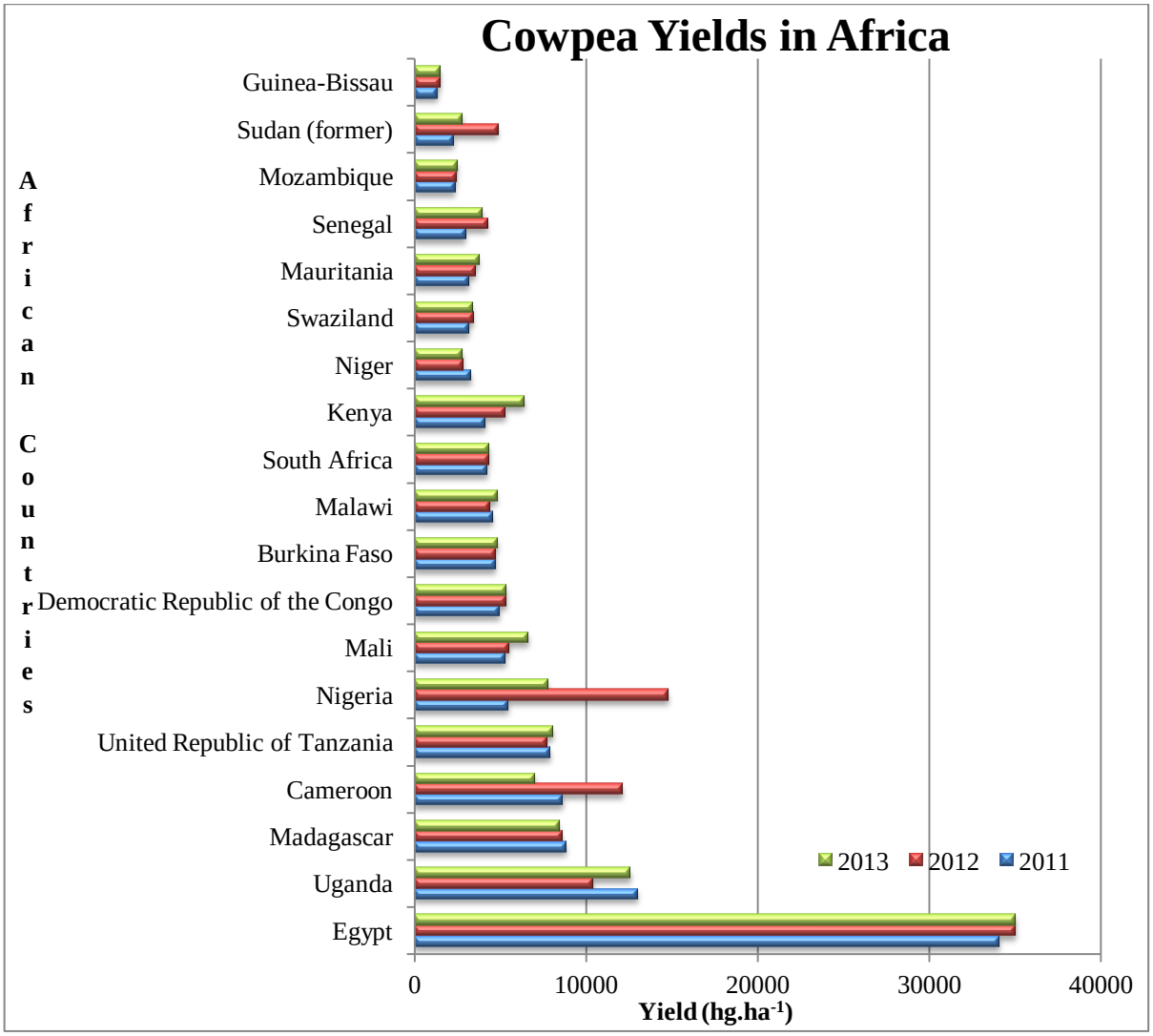

Figure 1.5: Cowpea yield per hectare for African countries for periods 2011, 2012 and 2013 (FAO, 2015).

Nigeria had a mean yield for the period 2011 to 2013 of 9329 $hg.ha^{-1}$ and Niger an average of 2953 $hg.ha^{-1}$ (Figure 1.5) (FAO, 2015). Ortiz (1998) observed that Nigeria and Niger together produced approximately 87% of the worlds' cowpea crop.

1.2.4 Pigeonpea

Pigeonpea (*Cajanus cajan* (L.) is a major grain crop in the tropics and subtropics. It is known also by another name, "dhal", which is dry, dehulled and split pigeonpea, used for cooking. The tender green pods are used as a vegetable, the green leaves are used as fodder, dry seeds as an animal feed, and the stems are used for fuel for fires, or to make huts and baskets (Nene and Sheila, 1990). Pigeonpea is capable of nitrogen fixation with the aid of rhizobia. Mature plants drop their leaves, ensuring the added nitrogen is returned to soil and organic matter (Kumar Rao, et al., 1981). Pigeonpea is infected and forms nodules, with rhizobia belonging to the "cowpea-miscellany" group that has the ability to nodulate in tropical and sub-tropical legumes (Jadhav and Moniz, 1972).

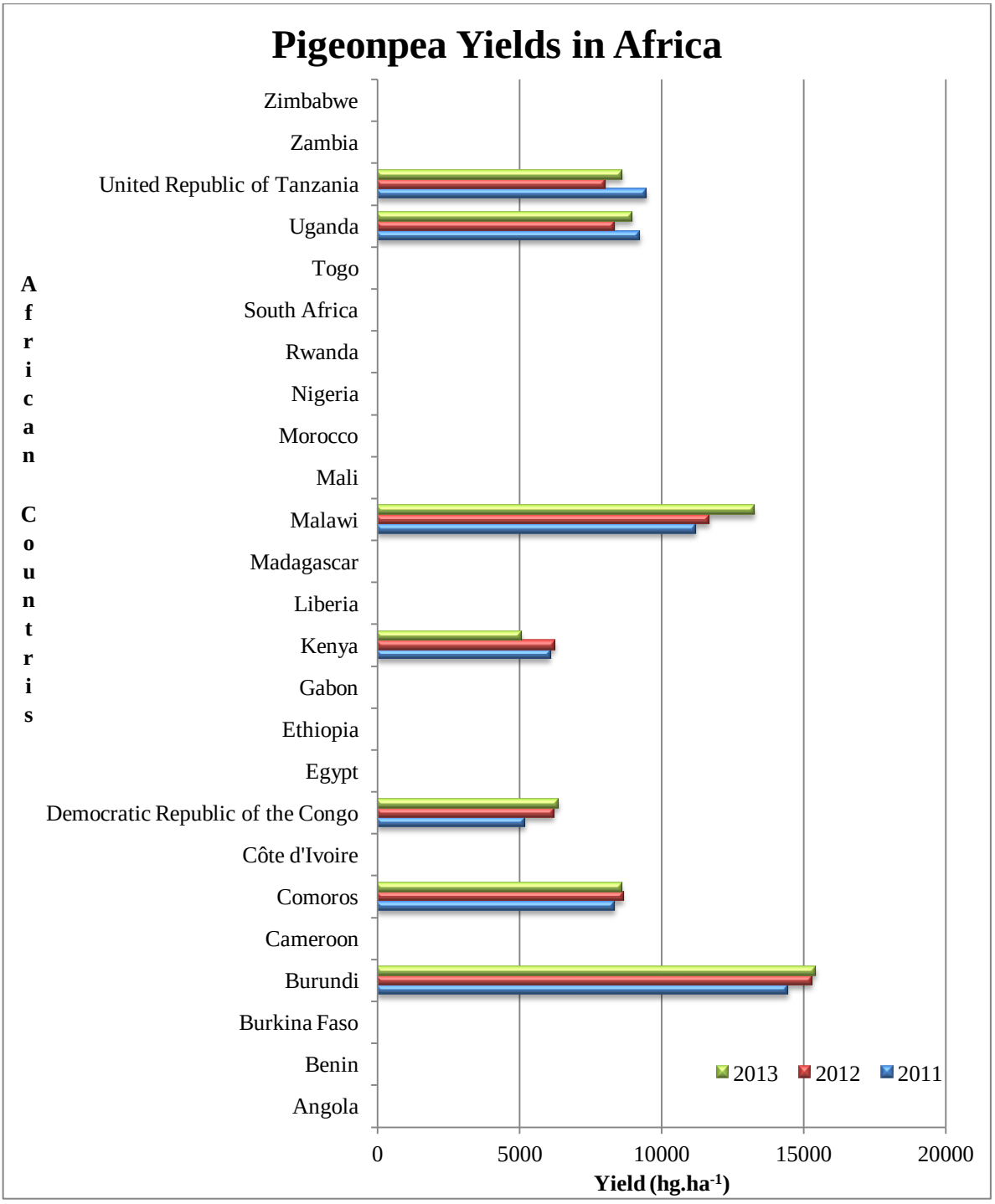

Figure 1.6: Pigeonpea yield per hectare amongst African countries for the period 2011 to 2013 (FAO, 2015).

The largest market for pigeonpea lies in India (Müller, et al., 1990). Pigeonpea has unrealised potential as a crop both in generating adequate nutrition and in its ability to tolerate environmental stress (Odeny, 2007). This crop is often referred to as an "orphan" crop

because limited research has been done on this legume. However, it is a very drought tolerant crop, with the ability to adapt to several environments and is typically grown in semi-arid climates (Troedson, et al., 1990). Seven countries in Africa are producers of pigeonpea (Figure 1.6). Pigeonpea in Africa is used primarily as a no-export cash crop, with large yields being noted in several countries (Shanower, et al., 1999). It is not a crop often sought after in Africa, but it has the ability to fix around 2350 $hg.ha^{-1}$ of nitrogen. It also has the capacity to generate more nitrogen per unit area from plant biomass than many other legumes (Peoples, et al., 1995). Cultivars of pigeonpea in Africa are characteristically medium to late maturing and generally intercropped with cereals or other vegetable legumes (Sakala, et al., 2000, Atachi and Machi, 2004).

1.2.5 Dry Bean

Dry bean or common bean (*Phaseolus vulgaris* L.) is a primary source of protein and an important source of calories in Latin America, and Southern and Eastern Africa (Pachico, 1993, Beebe, et al., 1999). It is an adaptable crop, capable of growing under various environmental conditions. Most parts of the plant are consumed including: the mature grain, immature seed, pods and leaves (Broughton, et al., 2003). There are a number of agro-ecosystems in Africa which support the growth of dry beans. Intensive bean production occurs typically in moderate to high population areas (Allen and Edje, 1990, Wortmann, et al., 1998). Poor soil fertility in conjunction with poor nodulation and inoculation of field experiments has limited yields in Brazil (Hungria, et al., 2003). High populations of inefficient indigenous rhizobia in soil limit symbiosis (Graham, 1981, Thies, et al., 1991). The symbiotic relationship between dry bean and rhizobia is susceptible to environmental stresses such as high temperatures and low soil moisture content (Hungria, et al., 1997, Hungria and Vargas, 2000). Nitrogen fixation is affected more by phosphorus deficiency in dry beans than in crops such as soybean (Israel, 1987, Vadez, et al., 1999).

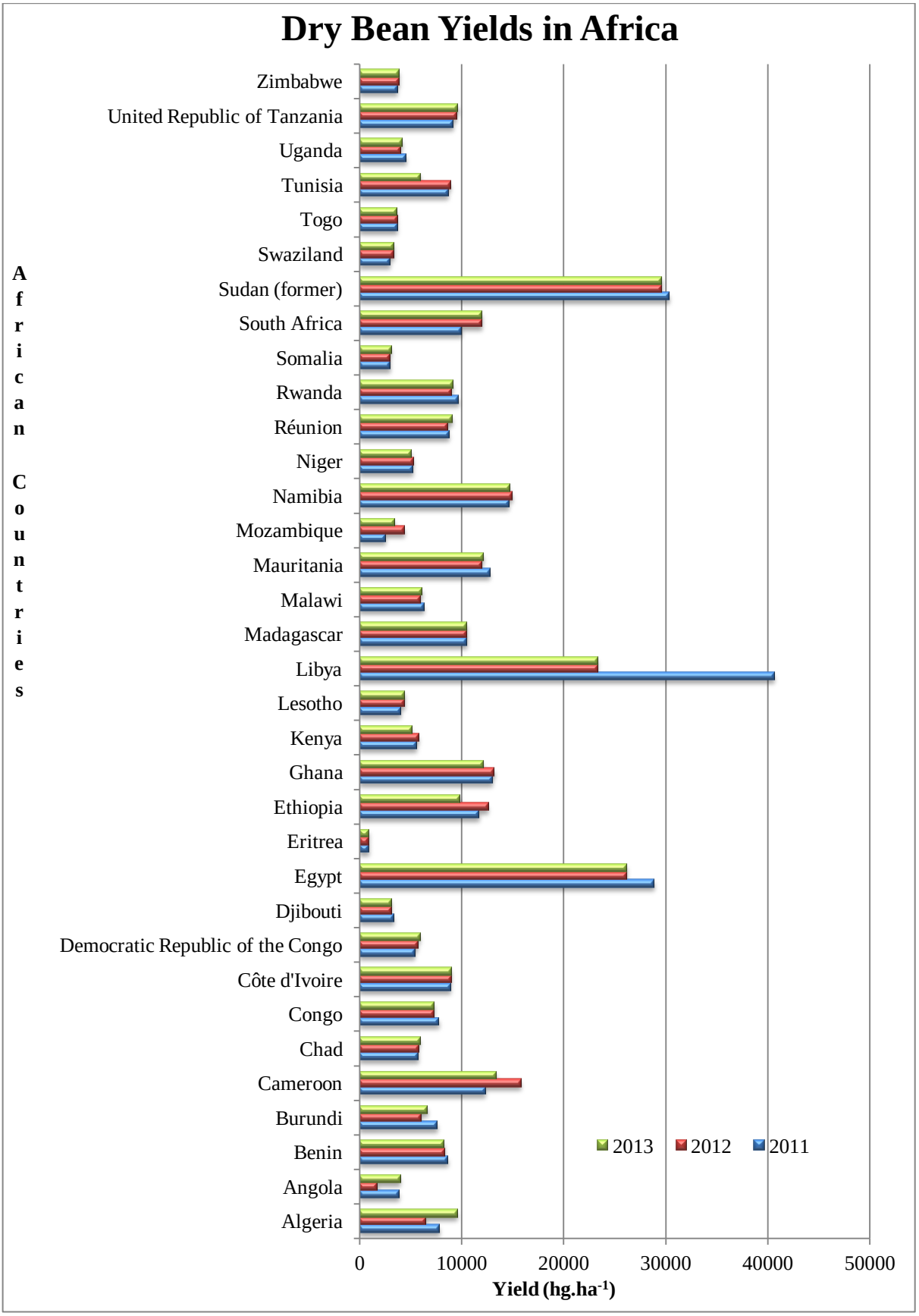

Figure 1.7: Dry bean yield per hectare in African countries for the period 2011 to 2013 (FAO, 2015).

Dry bean is an important cash crop to small scale farmers. According to Wortmann, et al. (1998) east African countries, mid-altitude African countries and southern African countries produce 38%, 24% and 32% of dry bean, respectively. Egypt, Libya and former Sudan are the largest producers of dry bean in Africa (FAO, 2015). There are 9 morphological types of dry bean that are commonly grown in east and southern African countries, these include: Calimas (or Rosecoco); reds (small and medium sized); red (large and kidney); yellow (and tans); creams; navy; white (large and medium sized); purples and black (Wortmann, et al., 1998).

1.2.6 Green Manures

A green manure is defined as a crop which is used as a soil amendment or nutritional source for succeeding crops (Cherr, et al., 2006). Legumes are of particular use in industrial scale intercropping systems to improve fallows and as cover crops (Borget, 1992). Green manures have several benefits, these include:

1. Slow release of nitrogen by decomposing green manures can be taken up more efficiently and reduce leaching in comparison with chemical nitrogen fertilizers (Augustin, et al., 1981, Abdul-Baki, et al., 1996, Aulakh, et al., 2000, Cline and Silvernail, 2002).
2. Increase soil organic matter and microbial biomass (Goyal, et al., 1992, Chander, et al., 1997, Biederbeck, et al., 1998, Goyal, et al., 1999).
3. Create a niche for beneficial organisms (Bugg, et al., 1990, Casswell, et al., 1991).
4. Improve nutrient retention, nitrogen uptake and reduce soil erosion (Dapaah and Vyn, 1998).
5. Improve cation exchange capacity and improve soil water retention (Turner, et al., 1994, Paino, et al., 1996, Serra-Wittling, et al., 1996).

1.2.6.1 Sunnhemp

Sunnhemp (*Crotalaria juncea* (L.)) has the ability to adapt to its tropical environments rapidly (Rotar and Joy, 1983). Sunnhemp is alternated with grass species to allow for slower nutrient release from the green manure residues, making equivalent nutrients available for the

succeeding crops (Perin, et al., 2006)This crop can also be used as forage for animals (Rotar and Joy, 1983). Other beneficial characteristics include: it is fast growing, high nitrogen fixing crop which increases biomass, is resistant to nematodes and can grow in low fertile soils (Anonymous(b), 1999).

1.2.6.2 Dolichos

Dolichos (*Lablab purpureus*) is used as a green manure or forage crop in Australia (Murtagh and Dougherty, 1968). Dolichos is useful on many levels including: it can grow under drought conditions, is adaptable to various environmental conditions, can grow in acid soils, is fast growing and is used as a feed supplement for animals (Aganga and Tshwenyane, 2003). Due to dolichos' ability to grow in acid soils, it allows for screening of *Rhizobium* spp. strains that are acid tolerant (Zaroug and Munns, 1979). Dolichos has a deep tap root allowing it to access nutrients that are not easily available to other annual crops and helps in reducing soil erosion and reduce runoff (Murphy and Colucci, 1999).

1.3 *Rhizobium* spp. strains

The taxonomy of rhizobia has changed over the past 20 years. The α-Proteobacteria members of the genus *Rhizobium*, are now subdivided into 7 genera (Willems, 2006). This increase in the number of species and genera is for two fundamental reasons. Firstly, of the 650 genera of legumes, only 57% have been studied for nodulation (Sprent, 1995). Secondly, the evolution of taxonomic research using DNA and RNA has allowed for genomic studies, which has increased the number of rhizobial genera (Willems, 2006). The interaction between rhizobia and legumes are specific, complex and can result in the formation of nodules. In the case of genus *Bradyrhizobium*, there are minute differences implying a high degree of similarity of 16S rDNA sequences. Some differences ranging from 0.1%-2.0% divergence in the sequences between most species of *Bradyrhizobium* and up to 4% difference with *Bradyrhizobium elkanii*, in comparison to other *Bradyrhizobium* spp. have been reported (Willems, et al., 2001). Lectins found on the seed have the ability to bind to specific sugars secreted by rhizobia, which allow for initial attachment of bacteria to root epidermal cells (De Hoff, et al.,

2009). The lectin binding hypothesis was formulated to explain why crops such as soybean form symbiotic relationships with *Bradyrhizobium japonicum* or alfalfa with *Sinorhizobium meliloti* (Hirsch, et al., 2001).

1.3.1 Specific Strains

Some strains have a limited host range, for example, *Rhizobium leguminosarum* bv *trifolii* only nodulates on *Trifolium* species (Dénarié, et al., 1992). Specificity may occur as a result of incompatibility between the nitrogen-fixing strain and legume resulting in no infection. Other effects include: nodules do not develop; the infection thread does not form; the bacteroid cells do not fix nitrogen (Miller, et al., 2007). Specificity is governed by many factors, the main factor being Nod Factors, which elicit root hair deformation, cortical-cell divisions and nodule-like outgrowths (Heidstra and Bisseling, 1996, Spaink, 1996). Host-specificity is determined by *nod* genes, which alters the basic acylated Nod Factor structure (Downie, 1998). Depending on the strain, the symbiotic relationship is host-specific or can infect a number of hosts. Released flavonoids can act as inducers for certain rhizobia and increase rhizobial infection, as in the case of *Bradyrhizobium japonicum* and *Rhizobium* sp. NGR234. However, these same flavonoids can act as antagonists, as in the case of *Rhizobium leguminosarum* bv *trifolii* and bv *viciae* (Bolanos-Vasquez and Werner, 1997, Begum, et al., 2001).

1.3.2 Multi-host Strains

Multi-host or promiscuous strains are strains of Rhizobia that nodulate a wider host range of legumes. Well known multi-host strains include: *Rhizobium* sp. NGR234; *Rhizobium tropici*; *Rhizobium fredii*; *Bradyrhizobium japonicum*; *Bradyrhizobium elkanii* (Van Rhijn and Vanderleyden, 1995). *Rhizobium* sp. NGR234 is able to nodulate more than 112 genera of legumes. Streit, et al. (2004) researched the link between broad-host range *Rhizobium* sp. NGR234 and narrow-host range *Rhizobium meliloti* strain 1021 (also known as *Sinorhizobium meliloti*). A 594 kb sequence from both strains of Rhizobia were analysed for similarities or

differences. A minimum of 28% of the entire sequence or 19 conserved gene clusters have a high degree of similarity implying a common ancestor for both strains.

1.4 Factors Affecting Nodule Formation and Nitrogen Fixation

There are many factors that impact on the infection, formation and function of *Rhizobium* spp. nodules. Abiotic and biotic factors can either enhance or diminish the capacity of rhizobial strains to infect legumes. Symbiosis between *Rhizobium* spp. and legume root requires a series of factors to achieve nodule formation. Light plays a role in inhibition of nodule development (Lie, 1969, Grobbelaar, et al., 1971). Malik, et al. (1984) showed *Rhizobium* spp. inoculated soybean cotyledons and hypocotyls (hypocotyledonous stem) exposed to light at varying degrees before planting had decreased nodulation with longer exposure to light. Maximum nodulation was at 1 day after exposure, 2 days after exposure nodulation decreased by 50%, and 7 days after exposure there was 70% less nodulation. Sironval, et al. (1957) indicated that the day length affected not only the amount of amount of nodulation, size and colour. Short day length produced smaller nodules that were red. However, any larger nodules found were pink in colour when excised. Longer day lengths produced larger nodules that were heavy and prominently red in colour when excised.

Table 1.1 lists the various factors together with the different mechanisms of impact on nodule formation and eventual nitrogen fixation.

Table 1.1: Factors affecting nodule formation and nitrogen fixation of rhizobial species in legumes

FACTOR	MECHANISM OF IMPACT	REFERENCE
Ethylene	Hormone secreted by legume plants affects the nodule number.	Penmetsa and Cook (1997)
Enzyme 1aminocyclopropane-1-carboxylate (ACC) oxidase	Catalyses the synthesis of ethylene to blocks cortical cell division.	Lee and LaRue (1992)
Dioxygen (O_2)	Presence of dioxygen at atmospheric levels inhibits nitrogenase activity.	Becana and Klucas (1992)
Boron and Calcium deficiencies	Inhibits induction of nod genes and degree of adherence of bacteria to the surface of legume roots.	Redondo-Nieto, et al. (2003)
High temperature	Affect transport and storage of *Rhizobium* inoculants. Kills *Rhizobium*.	Abdel-Gadir and Alexander (1997)
pH	Decreases in pH suppress nod genes. Decreases replication and infection of *Rhizobium*. Restricts distribution and colonization in soils.	Richardson and Simpson (1988) Evans, et al. (1988) Richardson and Simpson (1988)
Herbicide Application	Affects nodulation.	Kumer, et al. (1981)
Fungal Pathogens	Decreases *Rhizobium* population in soil rhizosphere.	Bhattacharyya and Mukherjee (1990)
Fungicide Application	Affects *Rhizobium* growth and nodulation.	Durgesha (1994)
Fertilizer Application	Directly affects nodulation of legumes by *Rhizobium* spp.	Satapathy, et al. (1992)
Salinity	High salt concentrations inhibit the interaction between rhizobia and legume, preventing nodulation.	Singleton and Bohlool (1984)

1.5.1 Role of Mineral and Nutrient Uptake and its Effect on Rhizobium

Bacteria from the family *Rhizobiaceae* can survive in a range of environmental conditions. *Rhizobium* spp. can exist on inorganic and organic nitrogen sources. These include molecular nitrogen, nitrates, nitrites, amino acids and ammonium. *Rhizobium* spp. can also utilize a series of carbon compounds, including sugars, organic acids, amino acids and phenolics,

indicating the diversity of nutrients in which rhizobia can utilize in various metabolic pathways (Kahn, et al., 1998). The involvement of mineral nutrients such as boron and molybdenum in legume-*Rhizobium* symbiosis is slightly more complicated. This is due to the influence that mineral nutrients have on *Rhizobium* spp. populations, infection and nodulation (Smith, 1982). Plants need and utilize seventeen different mineral elements for plant growth. These include carbon, hydrogen, oxygen, sulphur, magnesium, phosphorus, zinc, potassium, calcium, iron, copper, manganese, nickel, cobalt, selenium, boron, molybdenum, chlorine and nitrogen. Macronutrients, are nutrients required in large quantities, whereas with micronutrients (trace elements) or minor elements are needed in small quantities (Fageria, 2009).

1.5.1.1 Macronutrients

Macronutrients are mineral nutrients which satisfy three criteria: firstly the nutrient is required for a plant to complete its life cycle or forms an intrinsic part of the structure or aids in the metabolism of the plant; secondly, no other element can be substituted in its place. Lastly, all plants require macronutrients; a lack of this element results in abnormality in growth and development (Epstein and Bloom, 2005, Barker and Pilbeam, 2007). Macronutrients include oxygen, nitrogen, calcium, phosphorus, sulphur, potassium and magnesium (Bonilla and Bolaños, 2009).

1.5.1.1.1 Nitrogen

Nitrogen is required for formation of amino acids, proteins, enzymes, hormones, phytoalexins, phenolics and other cellular components (Huber and Thompson, 2007). Nitrogen can be in the form of many compounds that can be metabolized by plants. These include nitric acid, nitrate, pernitric acid, ammonia and ammonium (Barker and Bryson, 2007). High nitrogen losses are due to leaching, denitrification, volatilization, soil erosion and surface runoff, making soil nitrogen a major limiting factor in agricultural systems (Fageria, et al., 2005). The relationship between nitrogen and rhizobia is an indirect one. The presence of nitrogen negates the effectiveness and infection of rhizobia (Bonilla and Bolaños, 2009). Application of soil nitrogen in the presence of rhizobia affects nodulation, decreased infection, reduces leghaemoglobin synthesis and induces nodule senescence (Munns, 1968,

Bisseling, et al., 1978, Becana and Sprent, 1987, Abdel-Wahab and Abd-Alla, 1995). A deficiency in nitrogen results in stunted growth of roots, stems, leaves, flowers, fruits and seeds. Typical nitrogen deficiency symptoms include uniform chlorosis of leaves. Prolonged deficiency results in overall yellowing of the plant and stunting. Particularly during the growth stages, accumulated nitrogen is relocated to newer leaves, resulting in older leaves yellowing and eventually turning brown and dropping off (Tucker, 1984).

1.5.1.1.2 Phosphorus

Phosphorus (P) is an essential nutrient for *Rhizobium* spp. In highly weathered soils with a high clay content the phosphate can be bound to clay particles, creating a deficit in this nutrient, leading to reduced nodulation and competition amongst various *Rhizobium* spp. (Reisenauer, 1966). Concentrations of phosphates in soil solution range from infertile soil containing 0.001 $mg.L^{-1}$, to fertile soils containing $1mg.L^{-1}$ (Brady and Weil, 2002). In order for phosphate to be taken up, a process known as mineralization must occur. This process entails the conversion of organic matter to orthophosphate anions. It is absorbed through plant roots as anions in the form of $H_2PO_4^-$ and HPO_4^{-1} (a variation of phosphoric acid). Mineralization depends on soil temperature, soil moisture, oxygen availability and soil pH. Phosphate ions are strongly absorbed by silicates in solution when soil pH is near neutral. Alkaline soil causes phosphates to become relatively insoluble compounds when it binds with calcium, whilst in acid soils insoluble complexes can arise due to the abundance of dissolved heavy metals such as manganese, iron and aluminium (Daroub and Snyder, 2007).

Plant phosphates exist in four forms: firstly, as free inorganic orthophosphates (Pi), taken up by roots; secondly, as energy-rich pyrophosphate bonds (ATP) or other nucleotide triphosphates; thirdly, as inorganic orthophosphates attached via a single phosphate bond to a hydroxyl group or organic sugars or alcohols to form glucose or glucose-6-phosphate; lastly, inorganic orthophosphates can form diester bonds between organic molecules to form RNA and DNA (Rice, 2007). The more extensive the root system of leguminous plant increases phosphate absorption and this decreases the need to add phosphate fertilizers (Cassman, et al., 1981). In cereals, phosphorus is required to increase the number of tillers and panicles (heads) and grain yield (Fageria, 2002, Fageria and Baligar, 2002). Phosphorus is an important element required by plants for energy transfer and the metabolism of proteins (Prabhu, et al., 2007). Phosphate forms part of phospholipids, nucleotides, coenzymes, nucleic acids,

phosphoproteins and is used to create phosphate bonds (Sanchez, 2007). Leguminous plants require phosphorus to increase the number of pods, grain weight and grain yield (Fageria, 2002, Fageria and Baligar, 2002). Phosphorus is required for nodulation and nitrogen fixation (Ssali and Keya, 1983). Deficiencies in phosphorus causes stunting, reduced yields and discolouration of older leaves to reddish to purple colour. Phosphorus is not a component of chlorophyll. However, phosphorus deficiency results in the increased concentration of chlorophyll in leaves, particularly the younger leaves, which display as dark green (Prabhu, et al., 2007). Deficiencies in phosphate can be attributed to several factors: Firstly, high immobility or high fixation capacity due to acidic soils; secondly, deficit of naturally occurring phosphorus in soil; thirdly, high uptake by modern crop cultivars; fourthly, deficiency cause by soil erosion; fifthly, low input of phosphate into soils by farmers particularly in developing countries; and sixthly, biotic stresses, i.e., infestations due to insects, diseases and weeds (Fageria, 2009).

1.5.1.1.3 Calcium and Hydrogen Ion Concentrations

Temperate and tropical legumes create interactions between calcium and hydrogen ion concentrations. Calcium plays an important role in the nodulation and nitrogen fixation of leguminous plants. The requirements for calcium are increased at the stage of root infection and nodule initiation more than during legume development or nodule formation in the presence of fixed nitrogen. With varying levels of calcium, higher concentrations of calcium increase levels of nodulation (Lowther and Loneragan, 1968). Soil acidity affects rhizobia, by affecting growth and colonization in the rhizosphere, infection, and the generation of nodules (Munns, 1976, 1977, 1978). The pH range of acidic soils is between a pH of 4.0 and 7.0. Whilst above a pH of 7.0 is considered to be alkaline soils, a soil pH of around 7.0 is considered to have a buffering capacity (Coleman and Thomas, 1967). Calcium is a secondary macronutrient required by plants for cell elongation and division, more specifically, root and leaf development, cell membranes, formation of cell wall compounds and activation of several plant enzymes (Mullins and Hansen, 2006). Calcium deficiency results in deformed cell walls, indicated by vacuolated and swollen cells, and vulnerability to lysis or antibody absorption (Vincent and Humphrey, 1968, Wendt, 1971). Limited growth at low soil pH is the result of high hydrogen ion concentrations and their activity rather than the heavy metals present in soil (Clark, 1982). Lectin binding of legumes with rhizobia depends on the presence of calcium ions and transitional metals (Hardman, et al., 1982). The calcium

content in the rhizosphere of soil inhabited by legumes affects the ability of legumes to take up phosphates (Beck and Munns, 1985).

1.5.1.1.4 Potassium

Potassium is a highly absorbed nutrient. Potassium flows from older leaves to younger leaves, thus potassium deficiencies are seen in older leaves first (Prabhu, et al., 2007). Potassium is essential for plant growth for the following reasons (Fageria and Gheyi, 1999):

1. It improves root growth, water and nutrient uptake.
2. It aids in the activation of at least 60 different enzymes active in plant growth.
3. It reduces respiration and energy losses.
4. It helps photosynthesis and food formation.
5. It aids in translocation of starches and sugars.
6. It increases protein in plants.
7. It reduces water loss and wilting.
8. It aids in reduction of crop diseases.
9. It is thought to be an essential element for the formation of chlorophyll, because potassium deficiency results in chlorosis of leaves.
10. It can inhibit nitrogen fixation by limiting plant growth.
11. It neutralizes acids formed in plant cells during the metabolism of carbohydrates.
12. It is involved in the opening and closing of stomata.
13. It aids in uptake and transport of iron to plants.

Potassium deficiency symptoms include slower plant growth, reduced root growth, weakened stalks, shrivelled fruit and seeds, and increased susceptibility to disease (Fageria, 2009).

1.5.1.2 Micronutrients

The term micronutrient or trace element refers to elements such as boron, chlorine, copper, iron, manganese, silicon and zinc. All of these are needed for healthy plant growth and development at a fraction of the amount required for macronutrients (Benton Jones Jr., 1998). Factors affecting micronutrient deficiencies include: prolonged intensive cropping, soil

erosion, leaching, decreased farm manure application in comparison to chemical fertilizers, purer chemical fertilizers, and modern crop cultivars (Zia, et al., 2006).

1.5.1.2.1 Silicon

Silicon is not typically considered essential as a nutrient for plant growth, but its application has proven beneficiary to plants (Mali and Aery, 2008). Benefits of the accumulation of silicon have been noted in silicon accumulating and non-silicon accumulating plants (Epstein, 1999). Silicon is generally absorbed from soil solutions and its concentration may vary from 1- 40 $mg.L^{-1}$ (Hallmark, et al., 1982). Silicon is readily taken up by plants in the form of monosilicic acid/orthosilicic acid (H_4SiO_4). It is very reactive to aluminium, iron and manganese, forming insoluble silicates. Monosilicic acid can replace the anions of phosphate (HPO_4^{2-}) and form bonds with calcium, magnesium, aluminium and iron, and thus making more phosphates available for plants (Daroub and Snyder, 2007). Mali and Aery (2008) described a correlation between the use of silicon with *Rhizobium* spp. and infection of leguminous plants. Application of silicon at 100 $\mu g.g^{-1}$ produced higher nodule numbers, higher dry yield, root and shoot weights. Accumulation of silicon in plant cell walls provides added rigidity alongside plant lignins (Weiss and Herzog, 1978). These results concurred with experiments carried out by Nelwamondo and Dakora (1999) who found that application of silicon in the forms of metasilicic acid (H_4SiO_3) or silicic acid (H_4SiO_4) significantly increased the number of nodules, the nodule dry mass and nitrogen fixation per plant, under hydroponic conditions and when grown in sand. Other findings included significant increases in total dry matter at higher silicon concentrations. Application of silicon positively affects the bacteroids and symbiosomes in nodules of nitrogen fixing cowpeas (Nelwamondo, et al., 2001).

1.5.1.2.2 Boron

Boron is classified as an essential micronutrient for plants. Initially its importance was highlighted in an article published by Brenchley and Warington (1927). Deficiencies of this element results in anatomical, biological and physiological changes, found as secondary symptoms (Blevins and Lukaszewski, 1998). The effect of boron on legumes and biological nitrogen fixation was first investigated by Brenchley and Thornton (1925), who showed that boron deficiency resulted in ineffective nodules. Boron also stimulates the germination of

pollen tubes (Garía-Hernández and Cassab López, 2005). Low concentrations of boron have shown a decrease in infection and colonisation by *Rhizobium leguminosarum* (Bolaños, et al., 1996). This effects *Rhizobium* spp. by the degeneration of the cell wall and peribacteroid membrane surrounding intracellular bacteroids impacting the ability to form nodules and the ability of the *Rhizobium* spp. to fix nitrogen (Bolaños, et al., 1994).

1.5.1.2.3 Cobalt

Cobalt is a micronutrient that has been associated with animals, mainly as a constituent of Vitamin B12. Further study revealed that some microorganisms require preformed Vitamin B12 whilst others require the presence of cobalt to form Vitamin B12. Nitrogen-fixing bacteria fall under the latter where Vitamin B12 has been found in nodules of leguminous plants (Hutner, et al., 1950, Anderson and Andrews, 1952, Marston and Lee, 1952, Darken, 1953, Holm-Hansen, et al., 1954, Levin, et al., 1954). Cobalt deficiency hinders nodule formation, and function (Dilworth, et al., 1979). Due to the direct impact of cobalt deficiency on nitrogen fixation, crops, particularly legumes, show nitrogen deficiency when grown in cobalt-deficient soils (Robson and Snowball, 1987).

1.5.1.2.4 Molybdenum

In acid soils molybdenum binds to iron hydrous oxides and hydroxides. The benefits of molybdenum are only available to plants such as common beans at a soil pH above 5.5 (Franco and Day, 1980). Like cobalt, molybdenum has a direct effect on nitrogen fixation, and molybdenum deficiencies appear to be nitrogen deficiencies in legumes. This is because molybdenum forms a component of nitrate reductase, which is used in the conversion of nitrate to ammonia, and is found in several other enzymes (Tiaz and Zeiger, 1998). Molybdenum is also thought to aid resistance to plant viruses (Verma and Verma, 1967). Molybdenum can be taken up by plants in high concentrations without becoming toxic to the plant, but can be harmful to cows and other foraging livestock. Molybdenum is easily absorbed in the form of molybdate (MoO_4^{2-}) with a ten-fold increase in absorption with the increase in pH by one unit (Benton Jones Jr., 1998).

1.5.1.2.5 Nickel

Nickel is a recent addition to the list of essential micronutrients required for plant growth and development (Fageria, 2014). The effect of nickel as a micronutrient was discovered in legumes. Thereafter its importance as an essential micronutrient was established in cereal crops (Welch, 1981, Eskew, et al., 1983, Eskew, et al., 1984, Fageria, et al., 2011). Symptoms of nickel deficiency include chlorosis and patchy necrosis in and decreased iron concentrations young leaves of graminaceous plants. The effect of nickel on the control fungal disease such as rust was shown by Wood and Reilly (2007) and Graham (1983). Nickel plays a role in nitrogen fixation. It is a constituent of the enzyme urease and protects against inactivation of the enzyme nitrogen reductase (Srivastava and Gupta, 1996, Heckman, 2007, Alloway, 2008). Soybean cannot grown with urea as a nitrogen source in the absence of nickel (Polacco, 1977). The enzyme hydrogenase is essential for nitrogen fixation but is dependent on nickel (Cammack, 1995, Maroney, 1999). Application of nickel to soil resulted in significant increases in nodule weight and seed yield of field grown soybean plants (Bertrand and de Wolf, 1967, Brown, 2007).

1.6 Economic Importance of Rhizobium

As much as 200 to 300 kilograms of nitrogen per hectare can be generated by biological nitrogen fixation (BNF). Globally, approximately 70 million tons of BNF nitrogen is generated per year (Brockwell, et al., 1995, Buehring, et al., 2003). Reduction in the usage of nitrogen fertilizer from 88 million tons and its effect on the environment and its economic impact requires the development of effective, reliable and user friendly BNF (Peoples, et al., 1994). Brazil is a leader in the use of BNF. Brazil applies far less nitrogen fertilizer than other countries. This saves Brazil about 1.6 billion US$ per application due to the 150 million tons (6% nitrogen content) of nitrogen that is biologically fixed from the atmosphere (Döbereiner, 1997).

In Africa grain legumes and tree legumes are important nitrogen fixers, especially for small scale farmers. Atmospheric nitrogen is fixed at approximately 15-210 kilograms nitrogen per hectare and 43-581 kilograms nitrogen per hectare, respectively. Most African soils are

nutrient deficient, limited typically in elements such as phosphates, potassium, sulphur, molybdenum and zinc (Dakora and Keya, 1997).

1.7 Stickers

Stickers or sticking agents are adhesive compounds that enable the inoculant to adhere to the seed more effectively than application of the inoculant only. Stickers act not only as adhesives agents, but also as energy sources and can protect the cells from desiccation (Elegba and Rennie, 1984). There are a number of stickers are available, these include gum Arabic, carboxymethyl cellulose (CMC), methylethyl cellulose (MEC), honey, water and sugar. Gum Arabic can attach approximately 2,5 million rhizobial cells to a soybean seed, ME cellulose attaches 2 million cells, honey 500 000 cells, water 450 000 cells and sugar 400 000 cells per soybean seed (Ajeigbe, et al., 2010). Hoben, et al. (1991) compared the use of mineral, peanut and soybean oil as alternatives to gum Arabic and water. The oil treatments attached fewer rhizobial cells to common bean (*Phaseolus vulgaris*), chickpea, (*Cicer arietinum*), soybean (*Glycine max*) and peanut (*Arachis hypogaea*). However, the survival rate of rhizobial inoculants on seeds coated with oil stickers were similar to those attached with gum Arabic. Oils as adhesives showed no adverse effects on germination and nodulation of the plants. At 34 °C, more chickpea rhizobial cells survived using soybean and peanut oils in comparison to gum Arabic. Gum Arabic promotes root hair infection, early nodulation by rhizobia and increases leaf number during initial nodulation (Subba Rao, et al., 1971).

1.8 Rhizobial Poisons

Rhizobial poisons include herbicides, fungicides and insecticides that inhibit the nitrogen-fixing capability of rhizobia. Pesticides can be applied as seed treatments, foliar sprays or via drenching. According to Drouin, et al. (2010), the effects of pesticides are correlated to the function of the pesticide itself, implying that rhizobia is most susceptible to fungicides and herbicides, with a lesser effect from insecticides. Over the years, there has been conflicting research on the effect of pesticides on rhizobia. Depending on the concentration of the pesticide and the specific pesticide, there may be an effect on the survival and infection of rhizobia (Drouin, et al., 2010, Ahemad and Khan, 2012).

1.8.1 Herbicides

Herbicides adversely impact on nodulation and nitrogen fixation by either affecting the plant or affecting the growth and metabolism of soil microorganisms (Sawicka, et al., 1996, Singh and Wright, 2002). The type of herbicide, the concentration, the *Rhizobium* spp. or *Bradyrhizobium* spp. strains used for inoculation, and even the weather conditions during application, are all contributing factors that can influence nodulation and nitrogen fixation (Sprout, et al., 1992). Patil, et al. (2012) tested 2,4-D amine salt, glyphosate and atrazine at below, at, and above the minimal inhibitory concentration levels. As concentrations of herbicides increased, there was a noticeable decrease in shoot length, dry weights, nodule number and nodule weight. Similar results were obtained with pre-emergence herbicides tested on green gram (*Vigna radiata*) inoculated with *Bradyrhizobium* sp. (*Vigna*) (Khan, et al., 2006). Increases in atrazine, isoproturon or metribuzin concentrations resulted in decreases plant vigour, nodulation, chlorophyll content, seed yield and protein content. At 200 $\mu g.kg^{-1}$ of sulfosulfuron, there was a 10% reduction in seed yield. Atrazine and metribuzin were toxic and inhibited the vegetative growth of green gram. Alachlor and metribuzin significantly decreased nodulation, nitrogenase activity and nitrogen content in soybean (Malik and Tesfai, 1985). Repeated applications of herbicides can accumulate in the soil, disrupting the infection metabolic pathways and growth of rhizobia and legumes (Heinonen-Tanski, et al., 1982). Quizalafop-*p*-ethyl and clodinafop herbicides in excess of the

recommended dosage, reacted adversely by decreasing dry matter accumulation, grain yield, symbiotic properties and nutritional status of pea plants (Ahemad and Khan, 2009).

Chlorimuron-ethyl is a broad-spectrum herbicide belonging to the sulfonylurea family of herbicides, used for grasses and broad-leaf weeds affecting soybean crops. Sulfonylurea is considered to be among the most active herbicides together with the imidazolinones family of herbicides (Stridham and Singh, 1991). These two herbicide families generates a phytotoxic effect by the inhibition of acetolactate synthase (ALS) or otherwise known as acetohydroxy acid synthase (AHAS), an enzyme that reacts with a branched chain of amino acids consisting of valine, leucine and isoleucine, which are present in both plants and microorganisms (Zawoznik and Tomaro, 2005).

Glyphosate, the active ingredient in RoundUp®, is a broad-spectrum, non-selective herbicide which does not move down to soil profile to groundwater and has limited persistence (Duke, 1988, Franz, et al., 1997). Rhizobia are affected because glyphosate reduces the concentration of aromatic acids that is required for normal protein synthesis, thereby reducing the microbes' growth. This can be partially reversed by the addition of tyrosine, tryptophan and phenylalanine (Hoagland, 1980). Moorman, et al. (1992) suggested two mechanisms by which glyphosate can affect nitrogen fixation. Firstly, flavonoid-type compounds that are responsible for symbiosis between legume and rhizobia become physiologically and ecologically disrupted by glyphosate. Secondly, phenolic acids can accumulate and disrupt the process of nitrogen fixation from within the nodule. Glyphosate is also a chelating agent and binds essential cations such as molybdenum and cobalt. Hernandez, et al. (1999) showed that glyphosate causes a disturbance of the nodule metabolism by blocking the shikimate pathway in the nodules leading to high levels of shikimate and protocatechuic acid (PCA) accumulated within the nodules. 2.5 mM glyphosate exposure on *Lupinus albus* cv. Multolupa inoculated with *Bradyrhizobium* sp. (*Lupinus*) resulted in progressive cellular degradation on plant and bacteroidal cytosol, and breach of the bacteroidal membrane (de María, et al., 2005). Aamil, et al. (2004) showed that the use of glyphosate resulted in only a 12.5% increase in seed yield at 0.5 $\mu g.ai.g^{-1}$, whilst metribuzin decreased seed yield by 33% and 55% at 0.5 $\mu g.ai.g^{-1}$ and 1 $\mu g.ai.g^{-1}$, respectively. At a higher rate of 2 $\mu g.ai.g^{-1}$: glyphosate, fluchloralin and 2,4-D significantly reduced the nodule numbers and nodule mass. de María, et al. (2006) had found that after 24 hours exposure to glyphosate, the nitrogenase activity in nodules had decreased and after 5 days there were decreases in nodular starch and sucrose synthase of nodules.

1.8.2 Insecticides

Insecticides can be applied as soil, seed or foliar treatments. Repeated applications results in disturbances in changes in physiological soil properties including pH, salinity and even affect the soil microorganisms (Sarnaik, et al., 2006). Pham, et al. (2004) showed that insecticides interact with rhizobia and other microorganisms, causing damage to DNA, protein and membranes. Similar results were obtained by Fox, et al. (2007) who found that insecticides inhibited symbiosis by delaying the attachment of rhizobia to plant roots, delaying the infection process and thereby reducing the number of nodules formed, and by reduced nitrogenase activity and yield. Carbaryl at 450 µg per filter paper disk affect the growth in 10% of *Mesorhizobium* spp, 14% of *Rhizobium* spp. and 30% of *Bradyrhizobium* spp. (Drouin, et al., 2010). In contrast, Sarnaik, et al. (2006) showed that several insecticides applied to soybean did not affect the rhizobial count but decreased the numbers of phosphate solubilizing bacteria. The greatest effects were noted with the application of chlorpyriphos 20 EC, imidacloprid 200EC and thiomethoxam 25WG, when they were applied as foliar spray. Thiomethoxam 70 WS, imidacloprid 70 WS, phorate 10G, carbofuran 3G and chlorpyriphos 20EC, applied as seed treatments, caused a decrease in phosphate solubilizing bacteria.

1.8.3 Fungicides

Many fungicides hinder the performance of rhizobial inoculants (Kaur, et al., 2007). Viable *Bradyrhizobium japonicum* cells were reduced by 61% on soybean seeds after application of Apron (metalaxyl) and pentachloronitrobenzene (PCNB) decreased by 18% and 78% respectively (Revellin, et al., 1993). Other fungicides, including carbendazim, thiram, captan, and mancozeb have all been associated with reduced viability, chlorophyll content and decreased nodulation (Curley and Burton, 1975, Bhattacharyya and Sengupta, 1984, Aamil, et al., 2004). Mancozeb can reduce the growth rate of *Bradyrhizobium* sp. USDA 3187 by 50% and can affect the symbiotic relationship between the rhizobia and legumes. This fungicide alters the biochemistry of polysaccharides, polyamines and membranes of the rhizobial cells (Fabra, et al., 1998). Drouin, et al. (2010) found that captan and mancozeb caused a 91% to 100% inhibition of strains of *Sinorhizobium* spp.; *Mesorhizobium* spp.; *Rhizobium* spp. and *Bradyrhizobium* spp. at 45 µg and 450 µg per filter paper disk.

1.8.4 Acid Soils

30-40% of potentially arable land in the world are acidic soils (Van Wambeke, 1976, Von Uexköll and Mutert, 1995). Soils become acidic due to leaching of magnesium, potassium, sodium and calcium, thus reducing the buffering capacity of the soil and therefore the pH of the soil (Kidd and Proctor, 2001). Acid rain is an important factor, comprising of nitric and sulphuric acids, a combination of acids that increases soil acidification (Samac and Tesfaye, 2003). In acid soils plants exhibit nitrogen, phosphorus, calcium, magnesium and potassium deficiency along with manganese and aluminium toxicities (Hartel and Bouton, 1989, Kinraide, 1991, Sumner, et al., 1991). The distal elongation zone of the root-tip accumulates a higher aluminium concentration than other parts of the plant (Ryan, et al., 1993, Kochian, 1995, Čiamporová, 2002, Samac and Tesfaye, 2003). Legumes are affected because as rhizobia are acid sensitive. Neutral pH soils to slightly acidic soils are preferred for many legumes. However, the response of legumes and rhizobia to acidic environments varies depending on the crop and strain (Correa and Barneix, 1997). Research done by Watkin, et al. (2003) showed there is a difference between acid-soil tolerant and acid-soil sensitive strains of *Rhizobium leguminosarum* bv *trifolii*. When acid-tolerant strain WSM409 was exposed to acidic environments it showed an effect on the size and morphology of the bacterial cells. Furthermore, there was a greater accumulation of phosphorus in both acid-tolerant and acid-sensitive strains, with a lower degree of calcium and magnesium associated with cells in acidic environments than on the same strains grown on media at pH 7.0. The typical effect of an acidic environment to rhizobia in both temperate and tropical climates is a lowered survival of cells, reduced persistence of cells in soils and decreased nodulation (Graham, et al., 1982, Munns, 1986, Brockwell, et al., 1991, Ibekwe, et al., 1997).

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Chapter 2: Isolation of freshly isolated multi-host nitrogen-fixing *Rhizobium* and *Bradyrhizobium* species from cowpea, soybean, groundnut and pigeonpea nodules

Abstract

Multi-host or promiscuous Rhizobia are capable of infecting and inducing nitrogen fixation due to their capacity to infect a wide host range. The purpose of this study was to isolate and store freshly isolated multi-host *Rhizobium* and *Bradyrhizobium* spp. with the capacity to fix nitrogen. Cowpea (*Vigna unguiculata* (L.) Walp.), soybean (*Glycine max* (L.) Merrill), groundnut (*Arachis hypogaea* (L.) Kohler) and pigeonpea (*Cajanus cajan* (L.) Millsp.) were planted in a glasshouse in an artificial potting mix and under field conditions for a period of 30 days. Nodules from infected plants were washed, surface sterilized and cut to expose the presence or absence of leghaemoglobin. Those nodules containing the leghaemoglobin were surface sterilized and crushed to extract the bacterium. This solution was inoculated onto Congo Red Yeast Mannitol agar and grown at 28 °C for 7-14 days. Individual colonies were subsequently re-cultured and stored in double sterilized water at room temperature and in a 40% glycerol solution at -80 °C. A total of 31 strains were isolated. Two commercial strains, WB74 and XS21 were obtained from the Agricultural Research Council as commercial multi-host controls. Several isolates were obtained from cowpea, soybean and pigeonpea, plants and two were obtained from groundnut G663. No isolates were obtained from groundnut G665 due to poor germination, seed age and limited number of seed. All isolates tested were Gram negative, medium rods. All isolates were identified morphologically as rhizobia.

2.1 Introduction

Rhizobium spp. are characterised as small to medium rods that stain Gram negative and are classified as either fast or slow growing. Rhizobia are strict aerobes with certain strains of *Bradyrhizobium* able to grow autotrophically on atmospheric hydrogen (H_2) (Schlegel, 1993).

Rhizobia exist in three types of environments: as free living bacteria in soil; in infected root nodules of leguminous plants; and in the rhizosphere of leguminous plants (Slattery, et al., 2001).

Crops infected with nitrogen-fixing *Rhizobium* or *Bradyrhizobium* strains are capable of fixing atmospheric nitrogen, thus enabling sustainable agricultural systems (Graham and Vance, 1993). There are two types of symbiotic associations that can be made, i.e., specific or multi-host (or promiscuous). Multi-host *Rhizobium* spp. and *Bradyrhizobium* spp. are capable of infecting a wider legume host range as oppose to specific strains which only infect a specific crop (Pueppke and Broughton, 1999). Multi-host genotypes of legumes have the capacity to be infected by more than one *Rhizobium* spp. or *Bradyrhizobium* spp. strain (Pulver, et al., 1985, Gwata, et al., 2004). The greatest limitation of *Rhizobium*-legume symbiosis is that not all strains of Rhizobia are effective at infection, and others do not fix nitrogen due to a restricted host range (Shantharam and Wong, 1982).

Nodule organogenesis is the development of the nodule. Nodule development requires four stages. Firstly, flavonoids attract the bacteria to plant roots and stimulate nodulation and gene expression. This leads to the secretion of nod factors (Dixon and Wheeler, 1986, Caetono-Anollés and Gresshoff, 1991, Dénarié, et al., 1996, Spaink, 2000, Ferguson, et al., 2010). Secondly, infection leads to morphological changes of the root hair (Calvert, et al., 1984, Dixon and Wheeler, 1986). Thirdly, differentiation of the host cell organelles leads to the production of an infection thread (Dixon and Wheeler, 1986, Werner, et al., 1992). Lastly, a supply of carbon sources is established into the nodule for the assimilation of nutrients from the host plant. Nodule formation occurs in approximately six days (Calvert, et al., 1984, Werner, et al., 1992). There are two types of nodules, determinate or spherical and indeterminate or cylindrical nodules. The difference between these nodules lies in the site of initial cell division. For indeterminate nodules, the initial cell division occurs perpendicular to the inner cortex. Parallel divisions occurs, in the endodermis and pericycle, forming the nodule primordia and producing a cylindrical shape (Bond, 1948, Libbenga and Haaker, 1973, Newcomb, 1976, Newcomb, et al., 1979, Ferguson, et al., 2010). Determinate nodules initial internal cell division and maintenance occurs in the meristematic region and forms mature spherical nodules (Newcomb, et al., 1979, Gresshoff and Delves, 1986, Rolfe and Gresshoff, 1988, Ferguson, et al., 2010).

Isolation of multi-host *Rhizobium* and *Bradyrhizobium* spp. allows for a broader spectrum of bacterial sugars to bind to lectins from various legumes increasing root infection, nodulation and possibly nitrogen fixation. Due to the high degrees of similarity of 16S rDNA sequences of *Bradyrhizobium* spp., the use of genotypic methods, are preferred for accurate identification (Willems, et al., 2001). The objective of this study was to isolate freshly isolated *Rhizobium* and *Bradyrhizobium* strains that have multi-host properties, from two soil sources using four varieties of legumes.

2.2 Materials and Methods

2.2.1 Origins of Seed Samples

Table 2.1: Shows the variety of seeds, scientific names and supplier of seed used in experimentation.

Common Name	Scientific Name	Supplier
Soybean	*Glycine max* (L.) Merrill cv. LS6161RR	Link Seed, Greytown, South Africa
Cowpea	*Vigna unguiculata* (L.) Walp. cv. Glenda	Agricol, Cato Ridge, KwaZulu-Natal
Groundnut	*Arachis hypogaea* (L.) Kohler cv. G663 and G665	Proseeds, Pietermaritzburg, South Africa
Pigeonpea	*Cajanus cajan* (L.) Millsp. cv. ICPL 87091; ICPL 87051; ICPL 93015; ICEAP 00040, Royes and Ukulinga	Proseeds, Pietermaritzburg, South Africa

Rhizobia were isolated from unsterilized potting mix under glasshouse conditions and from soil in the University of KwaZulu-Natal (UKZN) Plant Pathology Disease Garden. The crops were watered with nitrogen-free fertilizer solution and harvested after 30 days (Table 2.1). Nodules on plant roots containing leghaemoglobin were selected for the isolation process.

2.2.2 Isolation, screening and media

After 30 days, nodules were carefully removed from each plant, washed, surface-sterilized using a 10% CaHOCl solution and thoroughly rinsed. Each nodule was dissected and only the leghaemoglobin containing nodules were selected. A dissected red nodule was placed into a sterilized McCartney bottle with 1ml of double-sterilized water and crushed using a flamed glass rod. A loopful of the resulting solution was three-way streaked onto Congo red Yeast Mannitol Agar (YMA) plates. The inoculated plates were incubated at 28 °C for 7-14 days, depending on the isolate growth rate.

2.2.3 Storage

Single colonies were cultured and stored using two methods. Firstly, a set of isolates grown on YMA plates were scrapped off and transferred to sterilized McCartney bottles containing a 40% glycerol solution, mixed and finally pipetted into sterilized culture cryotubes for storage at -80 °C. Secondly, a set of isolates were stored in McCartney bottles containing double-sterilized water. A total of 31 freshly isolated *Rhizobium* and *Bradyrhizobium* isolates were isolated and stored for experimentation. Cultures of strains WB74 and XS21 were obtained from the Agricultural Research Council (114 Chris Hani Street, Potchefstroom, South Africa) and were likewise stored.

2.3 Results

Isolation of nitrogen fixing nodules from four varieties of legumes using potting mix and UKZN Plant Pathology Disease Garden allowed for the isolation of multiple nitrogen-fixing isolates with a wider host range. A total of 31 isolates were obtained from cowpea, soybean, pigeonpea and groundnut varieties. Two known “multi-host” strains, i.e. WB74 and XS21 from soybean, were used as commercial controls. These two strains were not listed in Table 1 as they were purchased and were not isolated from the crops utilized in the experiment.

Figure 2.1A: Cowpea root with nodules. Figure 2.1B: Nodules containing red leghaemoglobin.

Nodules were located on the taproot and lateral roots as shown in Figure 2.1A. Clusters of nodules were noted around the tap root, varying from the base of the tap root to a few centimetres below the base of the taproot for cowpea plants. In contrast, soybean, groundnut and pigeonpea plants had a greater distribution of nodules amongst the lateral roots and fewer on the taproot. These nodules were removed, surface sterilized and the leghaemoglobin containing were used for isolation and storage (Figure 2.1B). Nodule size varied amongst each crop, variety and individual plant. However, leghaemoglobin was found in most nodules, regardless of the size. The nodules were all determinate in shape.

Table 2.2: List of isolates obtained from cowpea, soybean, pigeonpea and groundnut plants grown under greenhouse and field conditions after 30 days.

Crop	Cultivar	Referred Name	Gram Stain	Size	Shape	Colony morphology			
						Form	Texture	Elevation	Margin
pigeonpea	Royes	Royes I3/3	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S28A	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	Royes	Royes I1/2	Negative	Medium	Rods	Circular	mucoid	convex	entire
cowpea	Glenda	CCI3	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S30	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S33A	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	SCI	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	ICPL 87051	87051 I2/1	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S20B	Negative	Medium	Rods	Circular	mucoid	convex	entire
cowpea	Glenda	CI1/G	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S18	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S28B	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S33B	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	ICEAP 00040	00040 I3/3	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S26	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	ICPL 87091	87091 C1/2	Negative	Medium	Rods	Circular	mucoid	convex	entire
cowpea	Glenda	CT2/2	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S16	Negative	Medium	Rods	Circular	mucoid	convex	entire
cowpea	Glenda	CI3/2	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	ICPL 87051	87051 I3/1	Negative	Medium	Rods	Circular	mucoid	convex	entire
groundnut	G663	G663 I1/1	Negative	Medium	Rods	Circular	mucoid	convex	entire
groundnut	G663	G663 I3/1	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	ICEAP 00040	00040 I2	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	ICPL 93015	93015 T1/1	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	Ukulinga	Ukulinga I1/1	Negative	Medium	Rods	Circular	mucoid	convex	entire
pigeonpea	ICPL 87091	87091 T1/2	Negative	Medium	Rods	Circular	mucoid	convex	entire
cowpea	Glenda	CI/2	Negative	Medium	Rods	Circular	mucoid	convex	entire
cowpea	Glenda	CTI/6	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S21	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S20A	Negative	Medium	Rods	Circular	mucoid	convex	entire
soybean	LS6161R	S29	Negative	Medium	Rods	Circular	mucoid	convex	entire

List of isolates obtained from cowpea, soybean, groundnut and pigeonpea cultivars.

A total of 31 isolates were obtained from the four crops. Groundnut variety G665 was the only crop variety not to grow. Pigeonpea, cowpea and soybean varieties yielded a number of isolates, with groundnut producing only two isolates, as presented in Table 2.2.

2.4 Discussion

Symbiosis between rhizobia and their host plants results in the production of ammonia via nitrogen fixation. The ammonia is exported and assimilated by the host plant. Photosynthates from the host are supplied to the rhizobia which require them as an energy source for the nitrogenase reaction (Mylona, et al., 1995). Isolation from two soil sources using cowpea, soybean, groundnut and pigeonpea varieties allowed for a greater chance of finding rhizobia with multi-host properties. Several isolates were found for cowpea, soybean and pigeonpea, indicating either the isolates obtained are multi-host or that the crop varieties used for isolation had multi-host traits. Different *Rhizobium* and *Bradyrhizobium* strains have the capacity to infect the same plant at various locations of the root and may even infect within the same nodule (Sessitsch, et al., 1996). Groundnut cultivar G665 failed to grow due to the age of the seed. Two isolates were obtained from the groundnut variety G663. Cowpea was the first of the four crops to show *Rhizobium* infection, due to the change in leaf colour (light green to dark green) in the early stages of plant development. Soybean and pigeonpea also turned dark green in later growth stages. Ubiquitous cowpea interactions with rhizobia have been found in tropical soils in Africa (Kueneman, et al., 1984). All the isolates were Gram negative, medium rods forming mucoid, convex colonies on yeast mannitol agar. Many of the isolates took two weeks to form colonies, whist others were fast growing. Graham (1964) found a clear distinction between the fast (*Rhizobium* and *Agrobacterium* spp.) and slow growing rhizobia in the genus *Bradyrhizobium* (Jordan, 1982).

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Chapter 3: Visual scales of the growth stages of four legumes and the effect of different levels of nitrogen fertilization on the total chlorophyll and leaf nitrogen contents of these legumes

Abstract

All plants undergo vegetative and reproductive growth phases. These phases are affected by genetic, environmental and agronomical conditions, which influence the height, mass, photosynthetic capacity and yield potential of crops. The purpose of this experiment was firstly to observe and document the first four vegetative (V1-4) and the reproductive (R1; R3; R6 and R7) growth stages of each crop, based on the growth scale established by Fehr, et al. (1971) for soybean. Secondly to use a non-destructive method, specifically the use of a chlorophyll meter reader (Opti-Sciences CCM 200 plus), and a destructive method (LECO-Trumac CNS Analyzer) at four vegetative and reproductive growth stages to determine the total leaf nitrogen content and the total chlorophyll content as a result of the application of different levels of nitrogen fertilizer on four legumes. Soybean (*Glycine max* (L.) Merrill), cowpea (*Vigna unguiculata* (L.) Walp.), dry bean (*Phaseolus vulgaris* var. Gadra (L.) and dolichos (*Lablab purpureus* (L.) Sweet) were the test crops. For the observational growth stages, all crops were subjected to optimal growing conditions and each growth stage was illustrated. Each crop showed similar growth patterns for the vegetative growth stages. However, cowpea and soybean did not develop many vegetative growth stages before entering the reproductive phase of growth, in comparison to dolichos and dry bean. The chlorophyll and leaf nitrogen content treatments included 0% nitrogen; 25% of optimum nitrogen; 50% of optimum nitrogen; 75% of optimum nitrogen and 100% of optimum nitrogen for each test crop. The data collected was analysed using Genstat 14 and the area under the chlorophyll content index progress curve (AUCCIPC), the area under the nitrogen content progress curve (AUNCPC) and their linear regressions were calculated. Of the four crops tested for significant differences in total chlorophyll content at varying levels of nitrogen, only dolichos and dry bean confirmed the hypothesis. However, cowpea, dolichos and dry bean showed strong correlations between the total chlorophyll content and varying

levels of nitrogen fertilization. There were significant differences in total leaf nitrogen at varying levels of nitrogen for all crops except dolichos. However, based on the R squared values, all the crops showed strong correlations between the total leaf nitrogen content and the varying levels of nitrogen fertilization. The physiological properties varied with ontogeny, functional type and nitrogen flux for each crop. Soybean plants were attacked by powdery mildew (*Erysiphe polygoni* D.C.), mealy bug (*Pseudococcus filamentosus* Guen.) and red spider mite (*Tetranychus urticae* Koch.), which led to chlorosis and defoliation, which also affects chlorophyll levels.

Keywords: *vegetative growth stages; reproductive growth stages*; *total chlorophyll content; total leaf nitrogen content; chlorophyll meter; CNS analyzer; soybean; cowpea; dolichos; dry bean*

3.1 Introduction

There are two distinct growth phases of plant development, namely, the vegetative and reproductive phases. The vegetative or developmental phase allows the plant to increase in height, mass and photosynthetic capacity. The reproductive phase is induced by photoperiodic induction that allows for the production of reproductive structures (Huijser and Schmid, 2011). Leaf production is an integral part of plant development and is determined by several factors that are all controlled genetically, environmentally or agronomically (Hay and Walker, 1989):

- The time of crop emergence;
- The rate of unfolding in leaf production (dicotyledonous plants) or appearance (monocotyledonous plants);
- The rate of leaf expansion and the duration of leaf expansion;
- The rate of branching/tillering and the rate of leaf senescence, damage or removal.

Environmental factors such as water deficiency have a greater impact on the reproductive stages than on the vegetative stages of cereals (Yoshida, 1972). Nutrient availability, climate, soil temperature, soil-water holding capacity, soil structure, soil pH and soil microorganisms also play important roles in plant development (Fageria, 2009). Wheat varieties that are

vernalization-responsive, flower earlier when the seed is exposed to temperatures narrowly above freezing. This hastens the flowering process and reduces the duration of the vegetative stages (Gott, 1961, Halse and Weir, 1970, Rawson, 1970).

There are several differences between monocotyledonous and dicotyledonous plant development. Firstly, dicotyledonous plants produce leaves at each node, and they are not concealed by encircling sheaths of older leaves as found with monocotyledonous plants. Secondly, dicotyledonous species are capable of continual leaf production even after the reproductive phase has begun, whereas once the spikelet is initiated, monocotyledonous plants stop producing leaves. Finally, dicotyledonous plants have a larger node range to produce branches whereas tillering constricts monocotyledonous plants to a few basal nodes (Hay and Porter, 2006).

Many studies have been conducted, depicting the growth stages of various crops, particularly monocotyledonous plants, each varying slightly in their definition of each growth stage but all are based on the Feekes and Zadoks scales (Feekes, 1941, Zadoks and Schein, 1979, Lancashire, et al., 1991, Counce, et al., 2000, Meier, et al., 2009).

Nitrogen use efficiency (NUE) is the effective uptake and usage of soil nitrogen for maximum plant development and yield (Hodge, et al., 2000). Nitrogen usage involves several steps which include: uptake; assimilation; translocation during the vegetative growth and early reproductive stages; and recycling and remobilization of nitrogen during the reproductive growth stages (Masclaux-Daubresse, et al., 2010). The ability of plants to extract nitrogen from soil is dependent on the soil type, the environment and the plant species. An estimated 50%-70% of inorganic nitrogen fertilizer applied to the soil is lost (Hodge, et al., 2000, de María, et al., 2006, Masclaux-Daubresse, et al., 2008). In plants fed inorganic nitrogen, nitrogen uptake seems to control nitrogen assimilation (Warner and Huffaker, 1989, Wilkinson and Crawford, 1993). Kichey, et al. (2007) stated that grain yield is not solely dependent on nitrogen uptake before flowering but also on nitrogen remobilization during seed maturation. Grain filling triggers nitrogen remobilization, with each leaf contributing equally to seed filling (Masclaux-Daubresse, et al., 2008). Nitrogen remobilization occurs earlier in plants with low levels of nitrogen fertilizer as compared to plants treated with high levels of nitrogen fertilizer (Ta and Weiland, 1992). Likewise, the rate of nitrogen remobilization is increased when the level of nitrogen fertilizer is decreased or is limited due

to environmental conditions (Uhart and Andrade, 1995). Therefore the level of leaf nitrogen depends on the environmental conditions, cultivar and the growth stage of the plant.

Leaf pigments such as chlorophyll, carotenoids and anthocyanins absorb light at specific wavelengths and are therefore optically detectable using spectral reflectance. However, photosynthetic activity varies with the leaf type (Gamon, et al., 1997). Gamon and Surfus (1999) described several critical leaf properties that affect pigment content and therefore photosynthesis. The photosynthetic capacity of leaves decrease with age (Hikosaka, 1996). Chlorophyll degradation forms an integral part of leaf senescence and fruit ripening. Along with nitrogen flux, senescence allows for chlorophyll to be metabolized into colourless non-fluorescent chlorophyll catabolites (NCCs), which change leaves from green to yellow and eventually brown (Pružinská, et al., 2003, Gray, et al., 2004) . According to Prsa, et al. (2007) the leaf nitrogen content affects not only the capacity for light absorption but also the capacity for light utilization by the photosynthetic electron transport system.

The aim of this experiment was firstly the observational study to observe and document the first four vegetative growth stages (V1-V4) and four of the eight reproductive stages for soybean (*Glycine max* (L.) Merrill); cowpea (*Vigna unguiculata* (L.)Walp.); dry bean (*Phaseolus vulgaris* var. Gadra (L.)) and dolichos (*Lablab purpureus* (L.) Sweet), based on the scale of Fehr, et al. (1971) on soybean and secondly to determine the total leaf nitrogen content and the total chlorophyll content by applying different levels of nitrogen fertilization and measuring the chlorophyll content using a non-destructive method and the leaf nitrogen content using a destructive method. The chlorophyll content and leaf nitrogen contents were measured at the specific growth stages used in the observational trial to allow for the calculation of the total chlorophyll and leaf nitrogen contents.

3.2 Materials and methods

The varieties of legumes used for experimentation. All crops used were of early maturing varieties (Table 3.1).

Table 3.1: Shows the variety of seeds, scientific names and supplier of seed used in experimentation.

Common Name	Scientific Name	Supplier
Soybean	*Glycine max* (L.) Merrill	Link Seed, Greytown, South Africa
Cowpea	*Vigna unguiculata* (L.) Walp.	Agricol, Cato Ridge, KwaZulu-Natal
Dry bean	*Phaseolus vulgaris* var. Gadra (L.)	Proseeds, Pietermaritzburg, South Africa
Dolichos	*Lablab purpureus* (L.) Sweet	Agricol, Cato Ridge, KwaZulu-Natal

3.2.1 Observational growth stage

A fertilizer solution comprising of K_2SO_4 0.43 $g.L^{-1}$; $MgSO_4$ 0.11 $g.L^{-1}$; $CaSO_4$ 0.04 $g.L^{-1}$; Nutrifix® 0.02/20 L; nitrogen (LAN) 0.43 $g.L^{-1}$; phosphorus 0.14 $g.L^{-1}$; was utilized for optimal plant growth. Temperatures were maintained in the glasshouse at 28 °C for a period of approximately 90 days until the reproductive Stage 7 was reached. Four vegetative stages and four reproductive stages were studied, namely: vegetative Stage 1 (V1) is when the first leaves (unifoliate) are formed on the first node after germination. Vegetative Stage 2 (V2) is when the first trifoliate leaves are formed on the second node of the stem. Vegetative Stage 3 (V3) is when the second set of trifoliate leaves are formed on the third node of the stem, and the fourth vegetative Stage (V4) is when the third trifoliate leaves are formed on the fourth node of the stem. The first reproductive stage is when the first flower opens (R1); the third reproductive stage is when the seed pods are first initiated (R3); the sixth reproductive stage is reached when the first seed pod is fully developed (R6); and the final stage is when the pods are mature and the plant reaches the senescent stage (R7). These stages were observed and illustrated for each crop.

3.2.2 Total Chlorophyll and leaf nitrogen levels using different nitrogen levels

All seeds were sterilized using a 5% NaOCl solution (Unilever, South Africa) solution for one minute and rinsing thoroughly in sterile, distilled water, before using a 70% ethanol solution and thoroughly rinsing in sterile distilled water before drying on sterile filter paper in individual sterile Petri dishes. Four seeds per pot were planted into steam autoclaved soil (30 minutes heating time), with three pots per replicate and three replicates per crop for each

treatment (chlorophyll readings) and a repeat trial (harvesting – destructive analysis) using a completely randomized design. The soil pH was 6.10. The soil nitrate concentration was 4.39 $mg.kg^{-1}$ and ammonium was 0.691 $mg.kg^{-1}$. Treatments comprised of a 0% Nitrogen Control (0%N); 25% of the optimum nitrogen (25%N); 50% of the optimum nitrogen (50%N); 75% of the optimum nitrogen (75%N) and 100% (optimum) Nitrogen Control (100%N). All treatments were fertilized with a nitrogen free fertilizer solution (K_2SO_4 0.42 $g.L^{-1}$; $MgSO_4$ 0.11 $g.L^{-1}$; $CaSO_4$ 0.05 $g.L^{-1}$; Nutrifix 0.05/25 L; phosphorus 0.14 $g.L^{-1}$) and limestone ammonium nitrate (LAN) (Blackwoods, South Africa): 25% N (0.11 $g.L^{-1}$); 50% N (0.22 $g.L^{-1}$); 75% N (0.33 $g.L^{-1}$) and 100% N (0.44 $g.L^{-1}$)(Control).

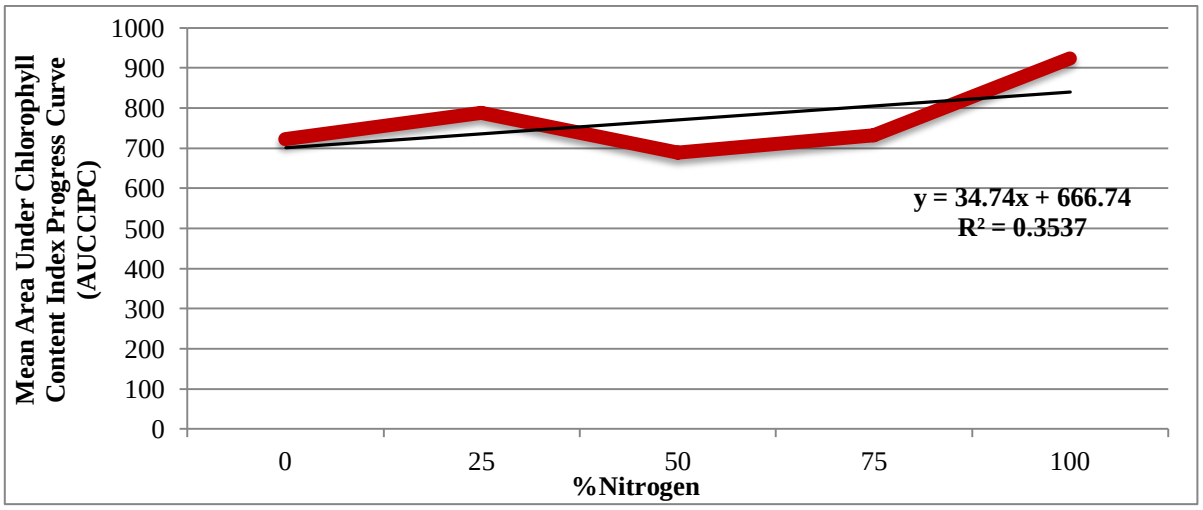
Glasshouse temperatures were maintained at approximately 28 °C for a period of 90 days until the Reproductive Stage 7 was reached and the final chlorophyll content was measured using a chlorophyll meter (Opti-Sciences CCM 200 Plus, USA). Chlorophyll content was measured over four vegetative stages and reproductive stages, namely: first true leaves (V1); first trifoliate stage (V2); second trifoliate stage (V3) and third trifoliate stage (V4); first open flower (R1); initial pod development stage (R3); mature pod and fully viable seed stage (R6) and the initial senescent stage (R7). Nitrogen leaf contents were measured by randomly harvesting 2 sets of 3 plants per treatment per growth stage. The leaves were dried in an oven, crushed and tested for nitrogen content (LECO-Trumac CNS Analyzer, South Africa). Results were analysed using one-way ANOVA and Duncan's multiple comparison test for the means of treatments and stages, and the interactions between treatments and stages (Genstat 14), in order to analyze the area under the chlorophyll content index progress curve (AUCCIPC) and the area under the leaf nitrogen content curve (AULNCC). All plants were treated with pesticides to control powdery mildew (*Erysiphe polygoni*. D. C.); mealy bug (*Pseudococcus filamentosus* Guen.) and red spider mite (*Tetranychus urticae* Koch.), which caused leaf chlorosis, early senescence and defoliation in soybean. Each crop was colour coded in the figures for identification between the crops. The following colours were allocated to the crops: soybean (red); cowpea (green); dolichos (purple) and dry bean (blue).

3.3 Results

Below are the hand drawn visual rating scales for the four crops. Because all the test crops were early maturing varieties, the final vegetative stage was Vegetative Stage 4 (V4) thereafter proceeding into the reproductive growth stages for the visual growth scale (Figures 3.1; 3.4; 3.7; 3.10). The reproductive growth stages were limited to the Reproductive Stages 1; 3; 6 and 7 for the purposes of chlorophyll meter measurements and harvesting the leaves for leaf nitrogen content measurements. The results are displayed per crop, with the visual growth scale followed by the linear regression graphs for the total chlorophyll and total leaf nitrogen content. This is to demonstrate the importance of the growth stages in the determination of total chlorophyll and leaf nitrogen contents.

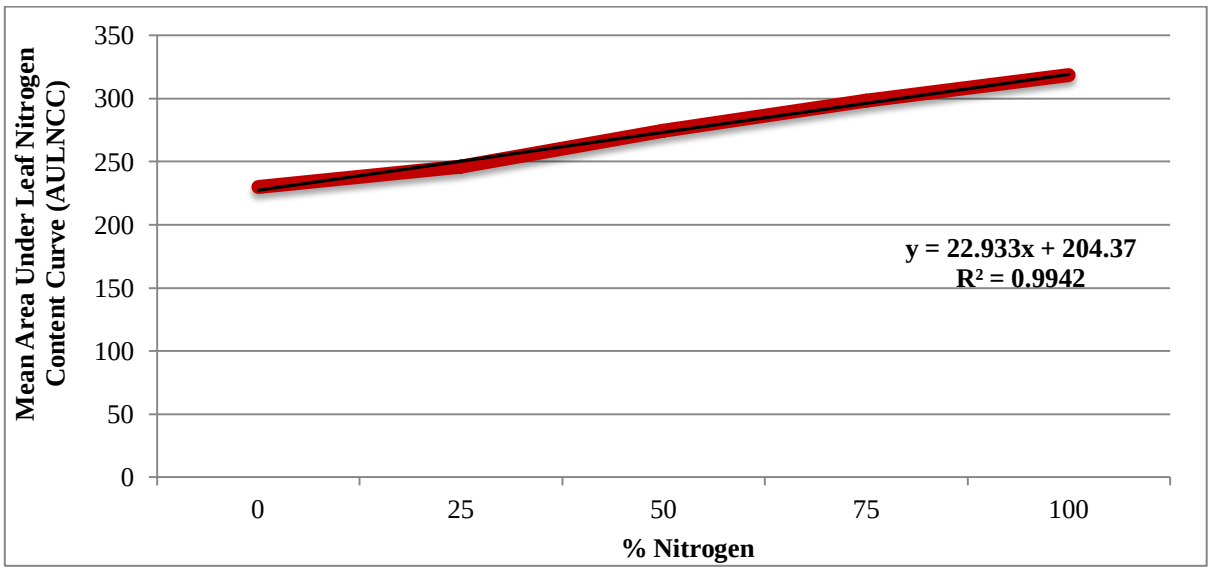
Linear regression was used for analysis of the total chlorophyll and leaf nitrogen contents to show the correlation between the two measurements for each crop (Figures 3.2; 3.3; 3.5; 3.6; 3.8; 3.9; 3.11 and 3.12). The R-squared valued for the total leaf nitrogen showed an above 90% correlation between the increases in leaf nitrogen and the increases in using nitrogen fertilizer. The total chlorophyll content for each of the crops had a similar correlation except for the soybean crop that showed a 35% correlation.

Figure 3.1 showing four vegetative and reproductive growth stages of soybean (*Glycine max* L. Merrill). Vegetative Stage 1 (V1) is when the first leaves (unifoliate) formed on the first node after germination. Vegetative Stage 2 (V2) is when the first trifoliate leaves formed on the second node of the stem. Vegetative Stage 3 (V3) is when the second set of trifoliate leaves formed on the third node of the stem. Vegetative Stage 4 (V4) occurs when the third trifoliate leaves forms on the fourth node of the stem. Reproductive Stage 1 (R1) is when the first flower opens. Reproductive Stage 3 (R3) is indicated by the initial pod development. Reproductive Stage 6 (R6) is when pods develop full viable seed. Reproductive Stage (R7) is at plant maturity and also is the initial senescent stage.



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	102323	25581	0.81	0.547
Residual	10	316049	31605		
Total	14	418373			

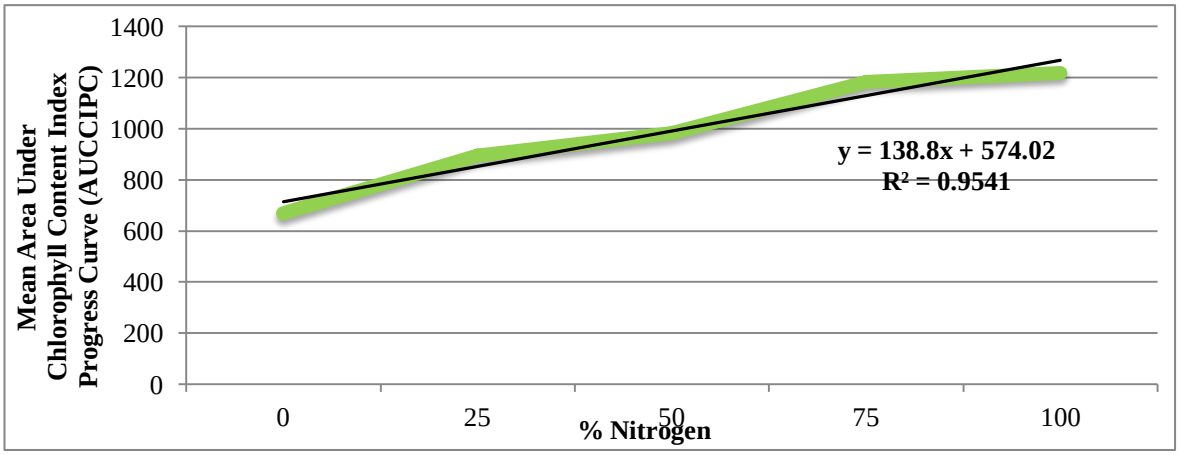
Figure 3.2: Linear regression of the total amount of chlorophyll accumulated during the life of soybean plants (*Glycine max* (L.) Merrill. CV%: 23.1; LSD: 323.42.



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	10579.6	2644.9	14.13	0.006
Residual	5	935.7	187.1		
Total	9	11515.3			

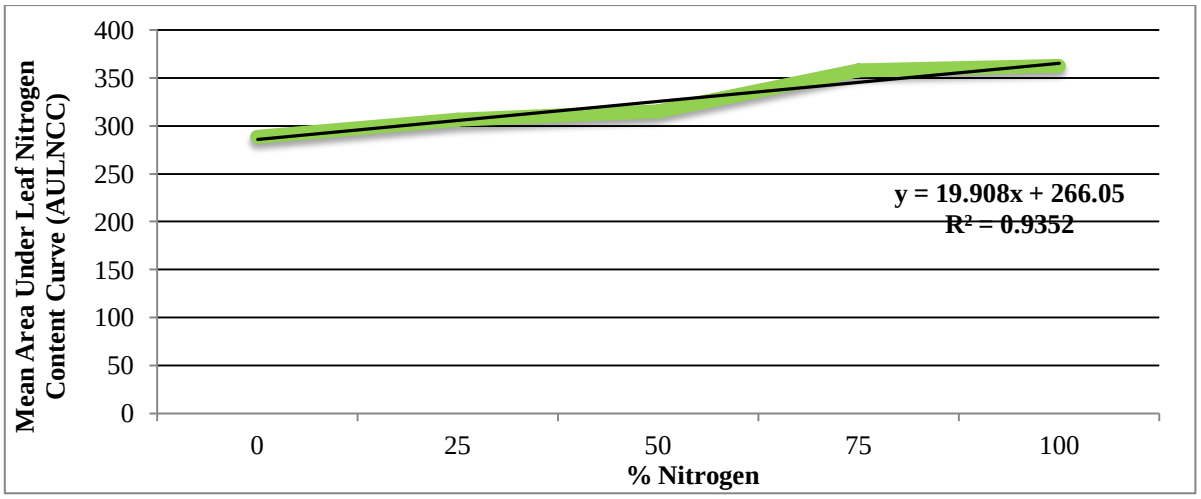
Figure 3.3: Linear regression of the total amount of nitrogen accumulated in leaves during the life of soybean plants (*Glycine max* (L.) Merrill). CV%: 5; LSD: 35.165.

Figure 3.4 showing four vegetative and reproductive growth stages of cowpea (*Vigna unguiculata* L. Walp.). Vegetative Stage 1 (V1) is when the first leaves (unifoliate) formed on the first node after germination. Vegetative Stage 2 (V2) is when the first trifoliate leaves formed on the second node of the stem. Vegetative Stage 3 (V3) is when the second set of trifoliate leaves formed on the third node of the stem. Vegetative Stage 4 (V4) occurs when the third trifoliate leaves forms on the fourth node of the stem. Reproductive Stage 1 (R1) is when the first flower opens. Reproductive Stage 3 (R3) is indicated by the initial pod development. Reproductive Stage 6 (R6) is when pods develop full viable seed. Reproductive Stage 7 (R7) is at plant maturity and also is the initial senescent stage.



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	605866	151467	2.57	0.103
Residual	10	589641	58964		
Total	14	1195507			

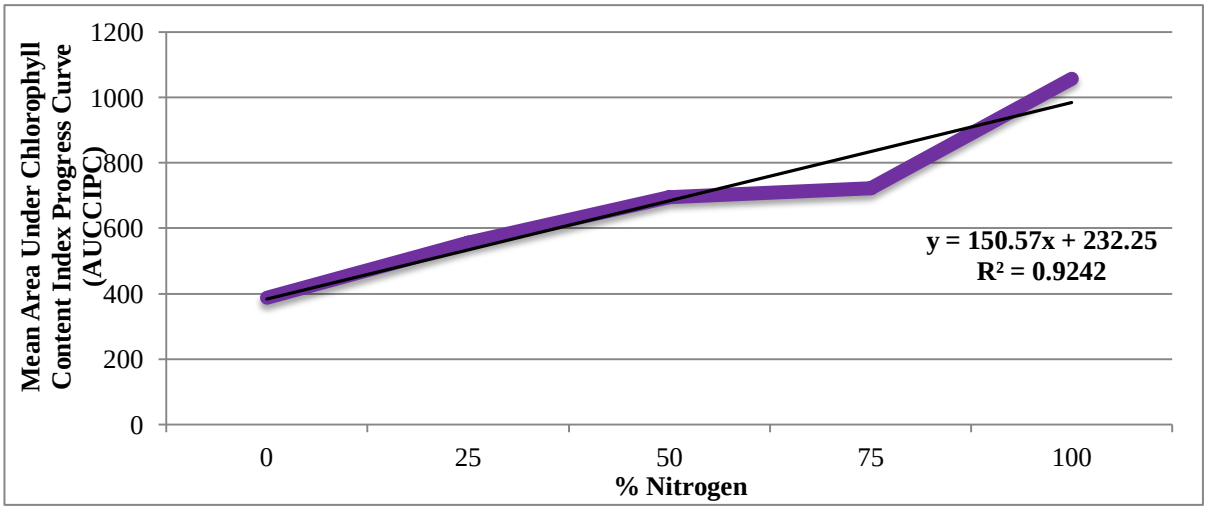
Figure 3.5: Linear regression of the total amount of chlorophyll accumulated during the life of cowpea plants (*Vigna* unguiculata (L.) Walp.). CV%: 24.5; LSD: 441.76.



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	8477	2119.3	18.15	0.004
Residual	5	583.8	116.8		
Total	9	9060.8			

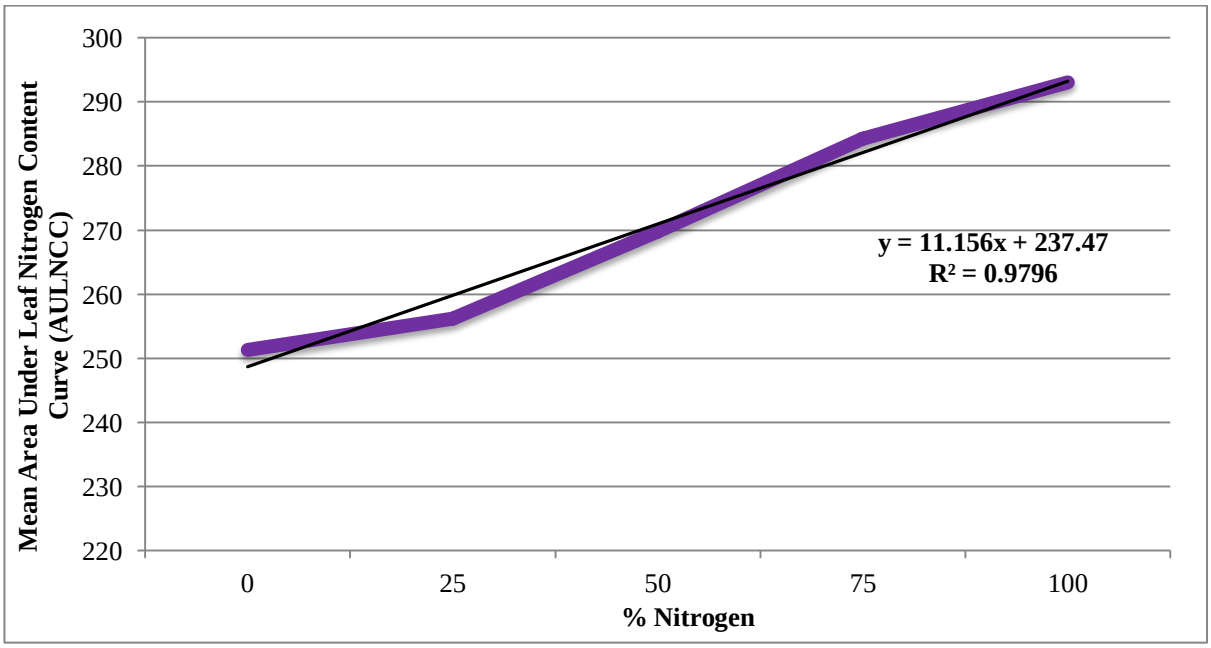
Figure 3.6 Linear regression of the total amount of nitrogen accumulated in leaves during the life of cowpea plants (*Vigna unguiculata* (L.) Walp.). CV%: 3.3; LSD: 27.776.

Figure 3.7 showing four vegetative and reproductive growth stages of dolichos (*Lablab purpureus* L. Sweet). Vegetative Stage 1 (V1) is when the first leaves (unifoliate) formed on the first node after germination. Vegetative Stage 2 (V2) is when the first trifoliate leaves formed on the second node of the stem. Vegetative Stage 3 (V3) is when the second set of trifoliate leaves formed on the third node of the stem. Vegetative Stage 4 (V4) occurs when the third trifoliate leaves forms on the fourth node of the stem. Reproductive Stage 1 (R1) is when the first flower opens. Reproductive Stage 3 (R3) is indicated by the initial pod development. Reproductive Stage 6 (R6) is when pods develop full viable seed. Reproductive Stage 7 (R7) is at plant maturity and also is the initial senescent stage.



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	736039	184010	7.7	0.004
Residual	10	238848	23885		
Total	14	974888			

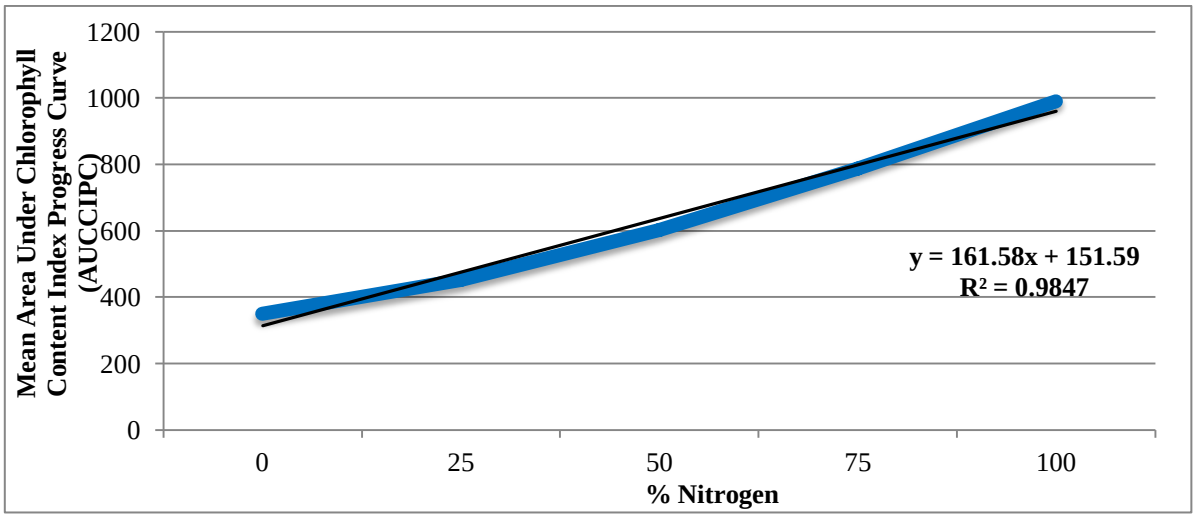
Figure 3.8: Linear regression of the total amount of chlorophyll accumulated during the life of dolichos plants (*Lablab purpureus* (L.) Sweet). CV%: 22.6; LSD: 281.16.



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	2541.2	635.3	1.76	0.274
Residual	5	1805.7	361.1		
Total	9	4346.9			

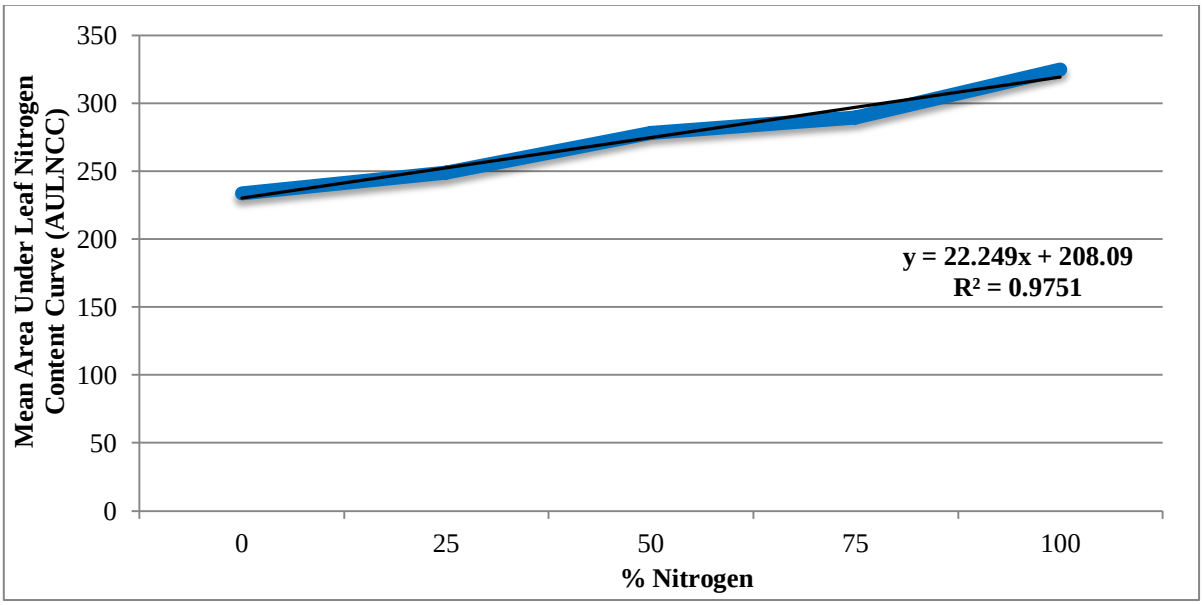
Figure 3.9: Linear regression of the total amount of nitrogen accumulated in leaves during the life of dolichos plants (*Lablab purpureus* (L.) Sweet). CV%: 7.01; LSD: 48.85.

Figure 3.10 showing four vegetative and reproductive growth stages of dry bean (*Phaseolus vulgaris* var. Gadra L.). Vegetative Stage 1 (V1) is when the first leaves (unifoliate) formed on the first node after germination. Vegetative Stage 2 (V2) is when the first trifoliate leaves formed on the second node of the stem. Vegetative Stage 3 (V3) is when the second set of trifoliate leaves formed on the third node of the stem. Vegetative Stage 4 (V4) occurs when the third trifoliate leaves forms on the fourth node of the stem. Reproductive Stage 1 (R1) is when the first flower opens. Reproductive Stage 3 (R3) is indicated by the initial pod development. Reproductive Stage 6 (R6) is when pods develop full viable seed. Reproductive Stage 7 (R7) is at plant maturity and also is the initial senescent.



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	795453	198863	23.1	<.001
Residual	10	86093	8609		
Total	14	881546			

Figure 3.11: Linear regression of the total amount of chlorophyll accumulated during the life of dry bean plants (*Phaseolus vulgaris* (L.). CV%: 14.6; LSD: 168.804.



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	10153.5	2538.4	7.25	0.026
Residual	5	1750	350		
Total	9	11903.6			

Figure 3.12: Linear regression of the total amount of nitrogen accumulated in leaves during the life of dry bean plants (*Phaseolus vulgaris* (L.). CV%: 6.8; LSD: 48.092.

3.4 Discussion

The aim of this experiment was firstly to observe and document the first four vegetative growth stages (V1-V4) and four of the eight reproductive stages for soybean (*Glycine max* (L.) Merrill); cowpea (*Vigna unguiculata* (L.)Walp.); dry bean (*Phaseolus vulgaris* var. Gadra (L.)) and dolichos (*Lablab purpureus* (L.) Sweet), based on the scale of Fehr, et al. (1971). Secondly it was to determine whether the total leaf nitrogen content and the total chlorophyll content correlated to different levels of nitrogen fertilizer, by measuring the chlorophyll content using a chlorophyll meter (non-destructive method) and leaf nitrogen content (destructive method) using a CNS Analyzer on four legumes.

3.4.1. Observational growth stage trial

The vegetative stages, as shown in Figures 3.1, 3.4, 3.7 and 3.10 begin with the unfolding of the unifoliate or true leaves at the first node, which occurs after germination. This is then followed by successive vegetative growth stages to the vegetative *n*th stage (V*n*), categorised by the number of trifoliate leaves produced at the *n*th nodes following the V1 growth stage. The reproductive phase is divided into four reproductive main stages: flower production; pod production; seed production and senescence. There are four reproductive stages which have not been included in this study namely; Reproductive Stage 2 (R2) occurs with the production and opening of all the flowers prior to the production of the first pod. Reproductive Stage 4 (R4), which is when all pods are produced prior to the formation of seeds. Reproductive Stage 5 (R5) is characterised as the initial seed production stage. The final stage is Reproductive Stage 8 (R8), characterized by dry, matured pods with no leaves remaining on the plant (Fehr, et al., 1971). The reproductive stages overlap with one another and therefore the four aforementioned stages were excluded, to show the distinguishable differences between the reproductive growth stages for each crop.

The varieties of legumes used in this study were all indeterminate. This means that, as the reproductive phase began, the vegetative growth continued with leaf production, thereby allowing the plant to increase in size and mass over the reproductive stages of plant development (Sinclair, 1984, Burton, 1997). Indeterminate varieties develop smaller final leaves on each stem, and flowers and pods at different developmental and maturity phases, as

shown in Figures 3.1; 3.4; 3.7 and 3.10 (Burton, 1997, Hay and Porter, 2006). Yield potential of any grain is defined as the seed mass per unit area under the optimum growth conditions (Long, et al., 2006). For legumes yield components are the number of pods per unit area, number of seeds per pod and seed weight (Fageria, 2009). The period of flowering is limited. However, it is aimed at maximum reproductive performance. Flowering determines the number of grains, conditions of growth and maturation of reproductive structures (Davidson and Christian, 1984).

The length of time from sowing to heading in cereals depends not only on the environment but also on the genotype (Davidson and Christian, 1984). Day-length is the total amount of light energy received to ensure growth (MacDowell, 1977). Under shorter day-lengths, the time between the terminal spikelet and the ear formation of wheat is increased and can prevent flowering in sensitive varieties (Rahman and Wilson, 1977), which could explain the small number of vegetative stages observed for soybean and cowpea (Figures 3.1 and 3.4).

Senescence is an age-dependent process signalling the end of a plant's life cycle. It occurs at the cellular, tissue, organ and organism level (Noodén, 1988). Leaf senescence is influenced by various internal and environmental (including biotic and abiotic) factors, which could lead to premature leaf senescence under unfavourable conditions (Lim, et al., 2003, Lim, et al., 2007). Natural leaf senescence begins at the leaf margins and moves inwards towards the leaf base. Leaf senescence allows for the remobilization and relocation of nutrients from structures such as leaves for production of reproductive structures. In monocarpic plants such as soybean, leaf senescence is controlled by the reproductive growth stages, i.e., the removal of the reproductive structures can reverse the effects of leaf senescence (Lim, et al., 2007). These are visible by the loss of the lower leaves, particularly at the R6 and R7 growth stages in Figures 3.1; 3.4; 3.7 and 3.10.

In conclusion, the number of vegetative stages varies amongst different leguminous crops, and this is dependent on a number of environmental and genetic factors. All crops have the same reproductive stages: flowering, pod production, seed production, seed maturity and senescence (plant maturity). The reproductive phase of plant growth is dependent on a number of biotic and abiotic factors, but functions in a way to produce maximum results.

3.4.2. Total chlorophyll and leaf nitrogen levels

Based on the hypothesis that increasing levels of nitrogen fertilizer would increase the total chlorophyll and leaf nitrogen levels, these are the expected outcomes: firstly, that there would be significant differences in total chlorophyll and total leaf nitrogen content between the different nitrogen treatments, and secondly, a that there would be positive linear correlation between the nitrogen fertilizer treatments, and the total leaf nitrogen and chlorophyll content. The mean total chlorophyll contents for all crops were higher than the leaf nitrogen content for each of the nitrogen fertilizer levels.

Soybean (*Glycine max* (L.) Merrill)

Figure 3.2 shows the total chlorophyll content for soybean, which did not vary significantly difference between the treatments. Hence no correlation was established between the total chlorophyll content and the different nitrogen levels. Low level of correlation between the total chlorophyll content and nitrogen fertilizer levels ($R^2 = 0.3537$).

The total leaf nitrogen content for soybean showed significant differences as a result of the different levels of nitrogen fertilization (Figure 3.3). Therefore, there was a positive correlation made between the increasing levels of nitrogen fertilization and increase in leaf nitrogen content using destructive analysis, as indicated by the R-squared value of 0.9942.

Cowpea (*Vigna unguiculata* (L.) Walp.)

The R-squared value of 0.9541 indicated a high degree of correlation between the total chlorophyll content and the levels of nitrogen fertilizer. However, Figure 3.5 shows that the nitrogen treatments did not cause significant variation in the total chlorophyll content of cowpea.

The R-squared value indicated a strong correlation between the leaf nitrogen content and nitrogen fertilizer levels. There were significant differences between the total leaf nitrogen content of cowpea (Figure 3.6).

Dolichos (*Lablab purpureus* (L.) Sweet)

Figure 3.8 shows the significant differences existed between the total chlorophyll content values because of the different levels of nitrogen fertilization. The R-squared value showed a 92.42% correlation between the total chlorophyll content and the nitrogen fertilizer levels.

There were no significant variations in the total leaf nitrogen content for dolichos (Figure 3.9). However, the R squared showed a high correlation between the total leaf nitrogen content of dolichos and the levels of nitrogen fertilizer.

Dry bean (*Phaseolus vulgaris* (L.)

The total chlorophyll content showed significant differences because of the treatments. A strong correlation was shown by a high R-squared value, between the increasing levels of nitrogen fertilization and the increase in the total chlorophyll content (Figure 3.11).

Significant differences were noted for the total leaf nitrogen content for dry bean as a result of the fertilizer treatment (Figure 3.12). A high correlation was shown by an increase in nitrogen fertilizer caused an increase in leaf nitrogen content.

Cartelat, et al. (2005) found that there was a correlation between chlorophyll meter readings of wheat and increases in applied nitrogen, regardless of growth stage and cultivar. Zhao, et al. (2005) found similar results when evaluating the effects of three nitrogen fertilization levels on the photosynthetic rate, chlorophyll content, nitrogen content and hyperspectral reflectance of sorghum. In that study nitrogen deficiency significantly reduced the leaf area, chlorophyll and nitrogen content, and photosynthetic rate. As the level of nitrogen fertilization increased, there was a parallel increase in total chlorophyll content reflected in the high R-squared values for each crop. There was a strong correlation as shown by the R-squared values for all the crops for the total leaf nitrogen content. However, the R-squared value for the total chlorophyll content of soybean was low and therefore there was no correlation between the levels of nitrogen fertilizer and the total chlorophyll content. The total chlorophyll content and leaf nitrogen content varied for all tested crops. There was a general decline in chlorophyll content and leaf nitrogen content during the reproductive stages, as

noted by others (Zaidi, et al., 2005). The following reasons may explain the decline in chlorophyll and nitrogen uptake by the tested crops:

a) The nitrogen treatment supplied was not adequate, or was not effectively taken up and utilized (Counce, et al., 2000).
b) The mealybug, powdery mildew and red spider mite infestations on soybeans impacted on the chlorophyll content and therefore the nitrogen content (Bounfour, et al., 2002, Keller, et al., 2003, de Freitas Bueno, et al., 2009, Kuckenbery, et al., 2009, Broghammer, et al., 2012).
c) The physiological properties vary with ontogeny and functional type for each crop (Sinclair, 1984, Hay and Porter, 2006).
d) Leaf turgor and time of day at which the chlorophyll readings are taken can affect the observed chlorophyll content (Martinéz and Guiamet, 2004).
e) As leaf age increases, so does the rate of decline in SPAD readings of rice plants. However, this rate slows with increasing nitrogen, thereby increasing the duration of the plants growth cycle and possibly increasing yield (Yang, et al., 2014).

Nitrogen flux is the recycling and remobilization of nitrogen from vegetative tissues such as leaves for the production of reproductive structures such as flowers, pods and seeds. Nitrogen flux is dependent on the supply of nutrients and the environmental conditions (Lawlor, 2002). Nitrogen deficiency greatly impacts the size, composition and function of chloroplasts in rice and sugarbeet, respectively (Laza, et al., 1993, Kutík, et al., 1995). This in turn will affect the leaf growth rate and composition and therefore chlorophyll and nitrogen leaf content (Nelson and Dengler, 1997). Yoshida (1972) described leaf senescence as the final stage of leaf development, and noted that it is integral to the nutrient relocation from leaves to reproducing seeds. This implies that seed viability is dependent on the level of nutrients stored in leaves prior to leaf senescence.

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Chapter 4: Screening for phosphate solubilisation, siderophore production and indole-3-acetic acid production by 33 freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp. isolated from four legumes

Abstract

Plant growth promoters have the capacity to affect plant growth directly or indirectly. The ability to solubilize inorganic phosphate allows for increased nodulation, nitrogen fixation and yield. Phosphorus is an important element required by plants for energy transfer and the metabolism of proteins. Phosphate forms part of phospholipids, nucleotides, coenzymes, nucleic acids, phosphoproteins and is used to create phosphate bonds. Siderophore production forms an important part of plant growth promotion, as it allows the microorganism to produce iron binding ligands (siderophores) that bind Fe^{3+} ions and transport them via the electron transport system to chloroplasts and mitochondria. Iron deficiency results in chlorosis of the leaves, by the reduction in the number of thylakoids found in the chloroplast. Indole-3-acetic acid (IAA) is an auxin that allows for metabolism of the amino acid L-tryptophan, resulting in plant growth promotion. Thirty one freshly isolated isolates of *Rhizobium* and *Bradyrhizobium* spp. were isolated from cowpea (*Vigna unguiculata* (L.) Walp.), soybean (*Glycine max* (L.) Merrill), pigeonpea (*Cajanus cajan* (L.) Millsp.) and groundnut (*Arachis hypogaea* (L.) Kohler). These isolates and two commercial controls were tested for phosphate solubilisation and siderophore and, indole-3-acetic acid production. Isolates CT1-6, Ukulinga I1-1, 93015 T1-1, Royes I3-3, Royes I1-2, CI1-G, 00040 I2 and 87051 I2-1 were amongst the highest producers of siderophores, IAA and organic phosphate. However, there were other isolates which tested positive for siderophore production or phosphate solubilisation and tested negative for IAA production.

Keywords: *siderophore production; indole-3-acetic acid; phosphate solubilisation; Rhizobium; Bradyrhizobium; soybean; cowpea; dolichos; dry bean*

4.1 Introduction

Plant phosphates exist in four forms: firstly, as free inorganic orthophosphates (Pi), taken up by roots; secondly, as energy-rich pyrophosphate bonds (ATP) or other nucleotide triphosphates; thirdly, as inorganic orthophosphates attached via a single phosphate bond to a hydroxyl group or organic sugars or alcohols to form glucose or glucose-6-phosphate; lastly, as inorganic orthophosphates that can form diester bonds between organic molecules to form RNA and DNA (Rice, 2007). Leguminous plants require phosphorus to increase the number of pods, grain weight and grain yield (Fageria, 2002, Fageria and Baligar, 2002). Phosphorus is also required for nodulation and nitrogen fixation (Ssali and Keya, 1983). There are two processes involved in phosphate dynamics namely, physiochemical (sorption-desorption) and biological (immobilization-mineralization) (Gyaneshwar, et al., 2002, Hao, et al., 2002, Khan, et al., 2009). The physiochemical process is dependent on the soil organic matter, clay content, iron and aluminium oxyhydroxides, and soil pH (Sharpley, 1983, Nafíu, 2009, Santos, et al., 2011, de Souza, et al., 2014). Phosphorus bound to soil minerals or in complex forms are unavailable for utilization by plants (Ryan, et al., 2001, Gang, et al., 2012). According to Isherword (1998) the efficiency of phosphate fertilizer is 10-25% worldwide. Deficiencies in phosphorus causes stunting, reduced yields and discolouration of older leaves to reddish to purple colour. Phosphorus is not a component of chlorophyll. However, phosphorus deficiency results in the increased concentration of chlorophyll in leaves, particularly the younger leaves, which display as dark green (Prabhu, et al., 2007).

Siderophores are produced in iron-deficient environments. These ferric iron-specific ligands enhance the availability of iron in the rhizosphere (Berraho, et al., 1997). The element iron is required as a component of many enzymes such as nitrogenase but is also found in leghaemoglobin containing nodules (Fett, et al., 1998). Iron also forms part of cytochromes, which contain an iron-porphyrin complex. These complexes form part of the electron transport system in chloroplasts and mitochondria. However, iron deficiencies result in decreased numbers of thylakoid membranes per chloroplast thus inhibiting chloroplast development and resulting in the yellowing of the plant leaves (Platt-Aloia, et al., 1983,

Imsande, 1998). There are many strains of rhizobia capable of producing siderophores such as *Rhizobium meliloti* DM4 (Reigh and O'Connell, 1993).

Some root nodulating bacteria have the capacity to produce to phytohormone indole-3-acetic acid (IAA) (Wang, et al., 1982). Indole-3-acetic acid (IAA) is a physiologically active plant growth hormone (auxin) produced by the metabolism of L-tryptophan. Rhizobia are among the microorganisms with the capacity to metabolize the amino acid L-tryptophan (Datta and Basu, 2000, Ghosh and Basu, 2006, Mandal, et al., 2007). Rhizobia can produce IAA as free living organisms in soil or symbiotically within nodules (Ernstsen, et al., 1987). The objectives of this study were to establish if the freshly isolated strains isolated from cowpea (*Vigna unguiculata* (L.) Walp.), soybean (*Glycine max* (L.) Merrill), groundnut (*Arachis hypogaea* (L.) Kohler) and pigeonpea (*Cajanus cajan* (L.) Millsp.) nodules were capable of solubilizing inorganic phosphate, produce siderophores and indole-3-acetic acid.

4.2 Materials and Methods

Thirty three freshly isolated strains of *Rhizobium* spp. and *Bradyrhizobium* spp. and, two commercial rhizobial strains were grown on yeast mannitol agar (YMA): mannitol (uniVAR, USA) 10 $g.l^{-1}$; K_2HPO_4 (BDH Chem, England) 0.66 $g.l^{-1}$; $MgSO_4.7H_2O$ (Associated Chem Enterprises, South Africa) 0.2 $g.l^{-1}$; yeast extract (Merck, South Africa) 0.4 $g.l^{-1}$; NaCl (Merck, South Africa) 0.1 $g.l^{-1}$; $FeCl_3.6H_2O$ (Merck, South Africa) 0.6$mg.l^{-1}$; bacteriological agar (Merck, South Africa) 15 $g.l^{-1}$ and 10 ml Congo red (Sigma, USA) solution and incubated at 28 °C (MRC Orbital Shaker incubator, Israel) for 1 week.

4.2.1 Phosphate Solubilization

The 33 *Bradyrhizobium* and *Rhizobium* spp. isolates were randomly spotted onto modified Pikovskaya's phosphate medium [10 $g.l^{-1}$ glucose (Merck, South Africa), 5 $g.l^{-1}$ tribasic phosphate (Sigma, USA), 0.5 $g.l^{-1}$ $(NH_4)_2SO_4$ (Merck, South Africa), 0.2 $g.l^{-1}$ KCl (Merck, South Africa), 0.1 $g.l^{-1}$ $MgSO_4.7H_2O$ (Merck, South Africa), 0.5 $g.l^{-1}$ yeast extract (Merck, South Africa), 15 $g.l^{-1}$ bacteriological agar (Merck, South Africa)]. The plates were incubated

(MRC Orbital Shaker incubator, Israel) at 28 °C for a week to allow for visible zones of clearing (Husen, 2003).

4.2.2 Siderophore Production

The 33 *Bradyrhizobium* and *Rhizobium* spp. isolates were inoculated onto modified chrome azurol S medium (CAS). The medium was made up of 4 solutions: Solution 1: CAS agar medium [5.3g NaOH (Merck, South Africa); 30.24 g piperazine-*N*,-*N*'-bis (2-ethanesulfonic acid) (Merck, South Africa); 750 ml distilled water and 20g bacteriological agar (Merck, South Africa)]. Solution 2: 100 ml of stock solution used [3 $g.l^{-1}$ KH_2PO_4 (Merck, South Africa); 5 $g.l^{-1}$ NaCl (Merck, South Africa) and 10 $g.l^{-1}$ NH_4Cl (Merck, South Africa)]. Solution 1 and 2 were mixed and autoclaved at 121 °C for 15 minutes. Solution 3 was filter sterilized and added to CAS medium when cooled to ±50 °C [30 ml Casamino acid/Casein hydrolysate (Merck, South Africa) 10% (w/v); 10 ml glucose (Merck, South Africa) 20% (w/v); 10 ml thiamine (Sigma, USA) (200 µg/ml); 10 ml nicotinic acid (Sigma, USA) (200µg/ml); 1 ml $MgCl_2$ (Merck, South Africa) 1M (2.0 g in 10ml) and 1 ml $CaCl_2$ (Merck, South Africa) 1M (5.55 g in 500 ml)]. Solution 4: CAS-iron-hexadecyltrimethylammonium bromide consists of 60.5 mg chrome azurol S (Merck, South Africa) in 50 ml distilled water; 10ml of 1mM solution (0.14 g $FeCl_3.6H_2O$ (Merck, South Africa) in 500 ml of 10mM solution of HCl (Merck, South Africa) and 72.9 mg of hexadecyltrimethylammonium bromide in 40 ml distilled water. Solution 4 was autoclaved at 121 °C for 15 minutes. When cooled Solution 3 was added to Solution 4 and thereafter mixed into combination of Solution 1 and 2. After thorough mixing, the media was poured and allowed to set to form a relatively deep blue colour. The plates were incubated at 28 °C for 1 week (General Electric Incubator) (Barghouthi, et al., 1989).

Results for the phosphate solubilisation and siderophore production were measured as highly positive (++), slightly positive (+) or negative (-) reactions.

4.2.3 Indole-3-acetic acid test

A loopful of each of the freshly isolated strains of bacteria were inoculated into a modified nutrient broth [5 $g.l^{-1}$ NaCl (Merck, South Africa); 10$g.l^{-1}$ peptone powder (Merck, South Africa) and 10 $g.l^{-1}$ beef extract (Merck, South Africa)] at 28 °C at 150 rpm (MRC Orbital Shaker incubator) for 48 hours. Thereafter 500 µl of each strain was transferred into a minimal salts broth [1.36 $g.l^{-1}$ KH_2PO_4 (BDH Chem, England); 2.13 $g.l^{-1}$ Na_2HPO_4 (Merck, South Africa); 0.2 $g.l^{-1}$ $MgSO_4$ (Associated Chem Enterprises, South Africa) and trace elements], which was modified by adding 5 ml of a L-tryptophan solution [10 g/100 ml glucose (Merck, South Africa); 1 g/100 ml L-tryptophan (Sigma, USA) and 0.1 g/100 ml yeast extract (Merck, South Africa)] filter sterilized (0.2 µm membrane filter) into the autoclaved broth and incubated at 28 °C (MRC Orbital Shaker incubator) for 48 hours. 1.5 ml was removed and placed into sterile Eppendorf tubes and centrifuged (Vacutec Heal-Force Neofuge 13, South Africa) at 12000 rpm for 5 minutes. The supernatant was removed and added to sterile test-tubes with 2 ml Salkowski's reagent ($FeCl_3$-$HClO_4$). The test-tubes were placed in a dark cupboard for 30 minutes for maximum colour development. The colour intensity was measured using a spectrophotometer at 530 nm. The concentrations were extrapolated from a standard curve (Husen, 2003). A standard curve was constructed using different concentrations of commercial indole-3-acetic acid (IAA) (Sigma, USA) dissolved in 95% ethanol. The absorbance for the standard curve was measured using a spectrophotometer at 530 nm.

4.3 Results

Many of the freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp. were capable of producing siderophores and able to solubilize inorganic phosphate (Table 4.1). Sixteen isolates were able to produce IAA, with ten of them producing significant quantities of IAA (Figure 4.3) (Husen, 2003). Sixteen isolates were also able to solubilize inorganic phosphate, as noted by the zone of clearing in Figure 4.1B. However, only six isolates produced significant clearing on Pikovshaya's media (Ahmad, et al., 2008). Eighteen isolates were able to produce siderophores, with fourteen causing significant clearing of the chrome azurol S medium (Barghouthi, et al., 1989).

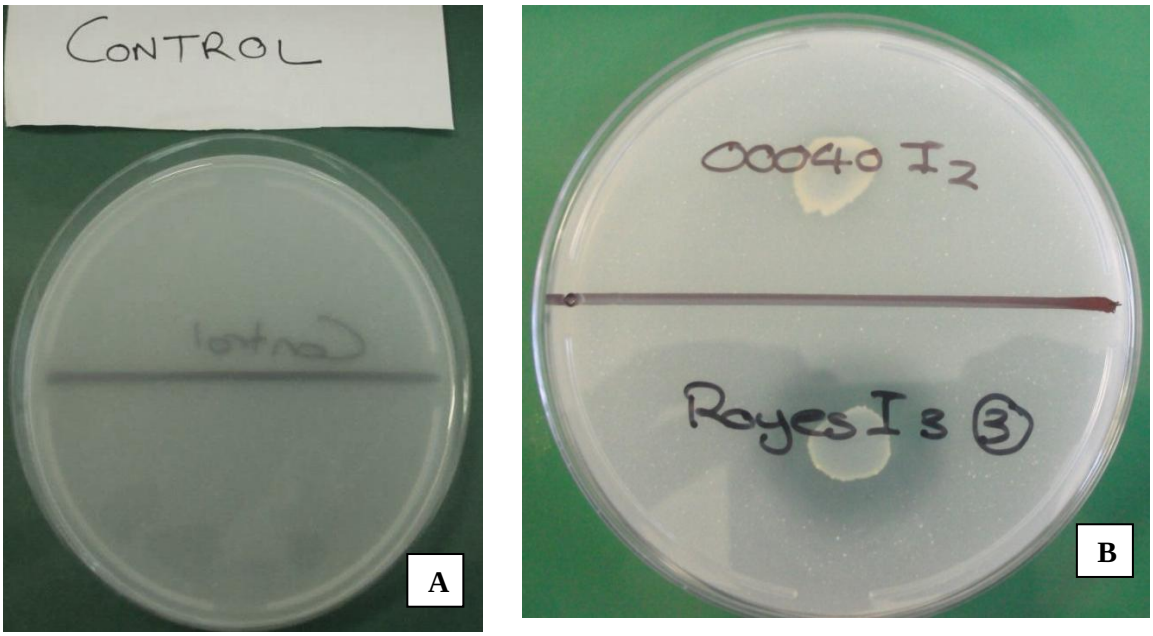

Figure 4.1A (left): Control plate is opaque with no visible clearing.

Figure 4.1B (right): the Royes I3-3 isolate. Positive phosphate solubilisation indicated by the distinct zone of clearing around the Royes I3-3 isolate as indicated by the (++) in Table 4.1. This indicates the ability of the isolate to break down inorganic tricalcium phosphate into soluble phosphate.

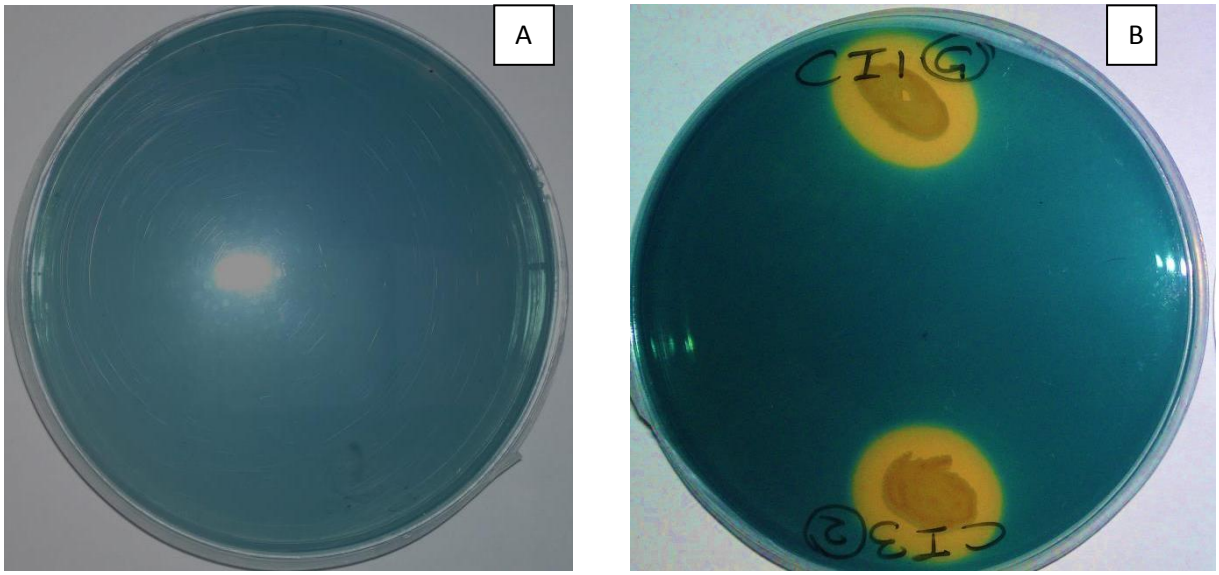

Figure 4.2A (left) Control plate with no orange zones indicated by (-) in Table 4.1.
Figure 4.2B (right) Distinct orange zones (++) by freshly isolated *Rhizobium* and *Bradyrhizobium* spp. isolates CI3-2 and CI1-G as a result of siderophore production. The ability of the isolates to bind free Fe^{3+} ions created the visible orange/yellow halo.

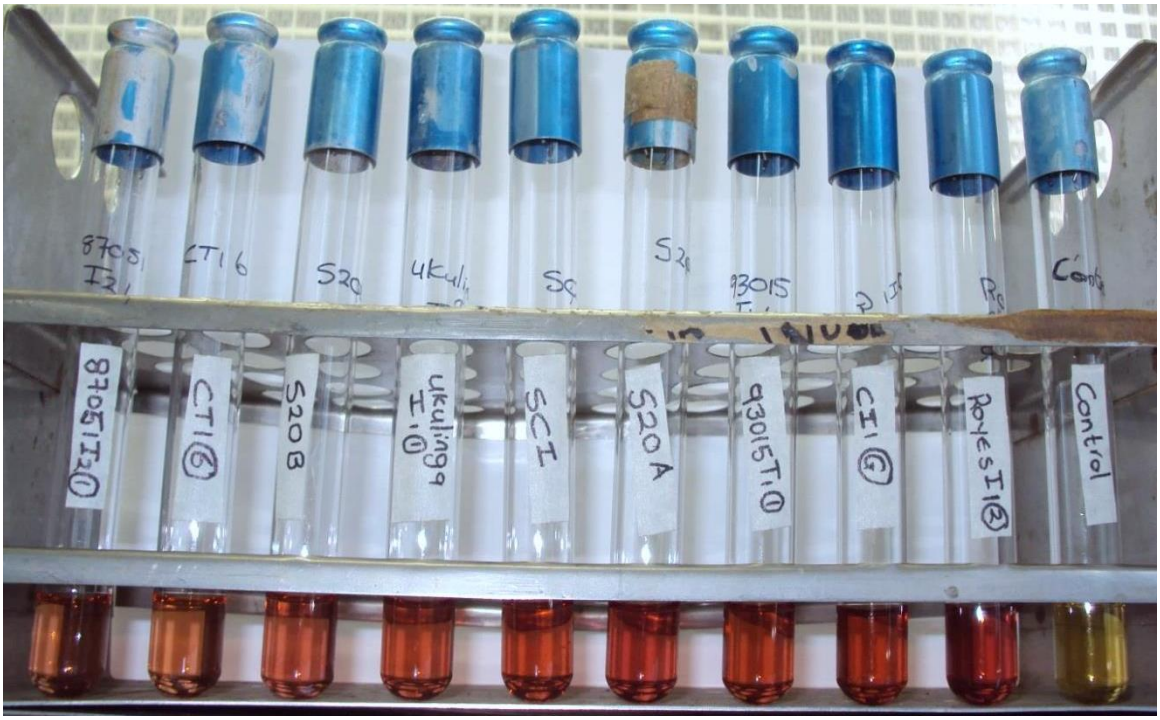

Figure 4.3: Nine of the strongest producers of the plant growth hormone, indole-3-acetic acid (distinct pink colour), in comparison with the Control, which produced no indole-3-acetic acid (distinct yellow colour). CT1-6 and CI1-G were isolated from cowpea, S20B, S20A and SCI were isolated from soybean, whilst Royes I3-3, 93015T1-1, Ukulinga I1-1 and 87051 I2-1 were all isolated from varieties of pigeonpea.

Table 4.1 records the results of the phosphate solubilisation, siderophore production and the indole-3-acetic acid production tests, by freshly isolated *Rhizobium* and *Bradyrhizobium* spp. isolates. (++) indicates high levels of IAA production; phosphate solubilisation and siderophore production. (+) indicates a slight positive reaction and (-) indicates a negative reaction. The IAA concentrations for each isolate were extrapolated from the standard curve. The isolates, which had an absorbance of 0.167 at 530 nm was distinctly pink (Figure 4.3). However, Isolates 00040 I3-3 and S16 had a low absorbance reading but produced a very pale pink colour.

Table 4.1: Phosphate solubilisation, siderophore production and indole-3-acetic acid production of 33 freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp.

Isolate	Phosphate solubilisation	Siderophore production	Indole-3-acetic acid production		
			Positive/ Negative	Absorbance 530 nm	Concentration $g.mol^{-1}$
CT1-6	++	+	++	0.167	1.143
S29	+	-	-	0.008	0.033
Ukulinga I1-1	++	++	++	0.363	2.460
S21	-	-	-	0.027	0.160
93015T1-1	++	++	++	0.498	3.367
CI2	-	++	-	0.017	0.135
Royes I3-3	++	++	-	0.016	0.129
G663 I3-1	-	+	-	0.026	0.154
00040 I2	+	++	++	0.076	0.532
CI3-2	+	++	-	0.013	0.108
Royes I1-2	+	++	++	0.615	4.153
CI1-G	-	++	++	0.642	4.335
S28A	-	-	-	0.028	0.167
CCI3	+	++	-	0.014	0.115
S30	-	-	-	0.029	0.174
S33A	-	-	-	0.027	0.160
SCI	-	-	++	0.484	3.273
87051 I2-1	++	++	++	0.144	0.989
S20B	-	-	++	0.275	1.869
XS21	-	-	-	0.011	0.053
S18	-	-	-	0.026	0.154
S28B	-	-	-	0.029	0.174
S33B	-	-	-	0.027	0.160
00040 I3-3	++	++	+	0.011	0.095
S26	+	-	-	0.031	0.187
87091 C1-2	+	-	-	0.003	0.041
CT2-2	-	++	-	0.023	0.176
S16	-	++	+	0.013	0.108
87051 I3-1	+	++	-	0.007	0.068
G663 I1-1	+	+	-	0.026	0.154
WB74	+	-	-	0.026	0.154
87091 T1-2	-	+	-	0.007	0.026
S20A	-	-	++	0.466	3.152

4.4 Discussion

A study by Antoun, et al. (1998) showed that out of 266 strains of *Bradyrhizobium* and *Rhizobium* spp. tested, 83% produced siderophores, 58% produced IAA and 54% were phosphate solubilizers.

Phosphorus (P) is an essential nutrient for *Rhizobium* spp. In highly weathered soils with high clay content the phosphate can be bound to clay particles, creating a deficit in this nutrient, leading to reduced nodulation and competition amongst various *Rhizobium* spp. (Reisenauer, 1966). In order for phosphate to be taken up, a process known as mineralization must occur. This process entails the conversion of organic matter to orthophosphate anions. It is absorbed through plant roots as anions in the form of $H_2PO_4^-$ and HPO_4^{2-} (a variation of phosphoric acid). Mineralization depends on soil temperature, soil moisture, oxygen availability and soil pH. Phosphate ions are strongly absorbed by silicates in solution when soil pH is near neutral. Alkaline soil causes phosphates to become relatively insoluble compounds bound with calcium, whilst in acid soils insoluble complexes can arise due to an abundance of heavy metals such as iron and aluminium (Daroub and Snyder, 2007). According to Rodrígues and Fraga (1999), *Rhizobium* spp. are amongst the best phosphate solubilizers, which include other genera such as *Bacillus* spp., *Pseudomonas* spp. and *Burkholderia* spp. to name a few. Al-Niemi, et al. (1997) found that under phosphate stress, phosphate can be mobilized by inducing acid and alkaline phosphatases. The phosphate concentration in the nodule can stimulate the expression of these enzymes. Alkaline phosphatases were at the highest concentration during the vegetative stages of the host plant's development and decreased substantially by 75% during the reproductive stages. Sixteen of the 33 freshly isolated isolates were able to solubilize inorganic phosphate, indicating that the lack of soluble phosphate allowed for the stimulation of the enzyme phosphatase to catalyse the inorganic phosphate into a soluble form as shown in Figure 4.1B and Table 4.1.

Siderophores are iron-binding complexes which allow for iron containing enzymes such as nitrogenases in diazotrophs and symbiotic root nodulating rhizobia to compete with the host plant and generate enough iron for leghaemoglobin (Fett, et al., 1998). Eighteen of the thirty three freshly isolated isolates produced siderophores indicating the isolates are capable of forming hemeproteins such as leghaemoglobin (Table 4.1). Environmental factors such as carbon, nitrogen, phosphorus, pH, iron concentrations and even micronutrient concentrations

all play a part in siderophore production (Duffy and Défago, 1999). However, Yeoman, et al. (2000) found that the siderophore mutant gene *fhuA* isolated from *Rhizobium leguminosarum* is capable of creating the orange halo indicative of a positive chrome azurol S reaction but was defective in the uptake of Fe^{3+} ions by accumulating the siderophore vicibactin in the extracellular medium (Figure 4.2B). Stevens, et al. (1999) likewise found that using an *fhuA::gus* fusion was expressed only in the meristematic region of pea nodules and not in the mature bacteroids and some strains of the *R. leguminosarum* contains a *fhuA* pseudogene which has a similar 3' end as the genuine *fhuA* gene. Further study of the genomic make-up of each of the isolates could reveal if the *fhuA* gene or pseudogene is being expressed or if siderophores are genuinely being produced.

Twelve rhizobial isolates of the thirty three tested positive for IAA production as shown in Table 4.1. The production of IAA is not uncommon amongst rhizobia, with host specific strains thought to vary in their cultural and biochemical characteristics. Sridevi and Mallaiah (2008) identified 26 *Rhizobium* strains isolated from *Sesbania* spp. that were able to produce the phytohormone IAA. They also found that the use of 1% mannitol and glucose solutions as carbon sources significantly increased IAA production with the SRS, SPR and SPS isolates tested. Phytohormones are integral to nodule development, with a higher concentration of IAA found within nodules in comparison to uninfected tissues (Thimann, 1936). It is thought that IAA regulates the early development of the legume-*Rhizobium* symbiosis, firstly, by inducing the gene involved in flavonoid signal processing and secondly, by activating the genes involved in motility and attachment (Spaepen, et al., 2009). This implies that the freshly isolated isolates that were capable of IAA production could enhance nitrogen fixation by improving infection and nodule formation. The presence of the auxin precursor L-tryptophan enhances the amount of IAA produced in culture. The production of IAA ultimately results in better root growth and root formation, allowing the host plant to increase its uptake of water and other nutrients (Bhattacharyya and Pati, 2000, Etesami, et al., 2008).

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Chapter 5: Screening of 31 freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp. on four legume crops for multi-host proficiency by measuring the chlorophyll content and yield

Abstract

Chlorophyll is an indicator of a plant's ability to absorb nitrogen. The chlorophyll content in the vegetative and reproductive stages fluctuates, due to nitrogen absorption and relocation, indirectly affecting the dry weight. The aims of this study were to identify firstly which of the 31 strains were capable of being multi-host and to determine when nitrogen fixation was initiated. Secondly, to determine which strains were able to produce a significant amount of nitrogen, by measuring the chlorophyll content and measuring the plant dry weights, in comparison to the effect of commercial rhizobial strains WB74, XS21 and trial fertilizer controls of 100% optimal nitrogen and 0% nitrogen. Freshly isolated isolates of *Bradyrhizobium* and *Rhizobium* spp., and the commercial strains XS21 and WB74, were inoculated onto soybean (*Glycine max* L. Merrill); cowpea (*Vigna unguiculata* L. Walp.); dolichos (*Lablab purpureus* L. Sweet) and dry bean (*Phaseolus vulgaris* L.). Chlorophyll content was measured at the V1, V2, V3, V4, R1, R3, R6 and R7 growth stages. The total chlorophyll content was calculated using area under the chlorophyll content index progress curve and plant dry weights were measured and ranked using the Kruskal-Wallis analysis. Isolates Ukulinga I1-1, Royes I1-2 and 93015 T1-1 produced a total chlorophyll content and plant dry weight higher than the 100%, 0% Nitrogen Controls, and the commercial control, XS21 on soybean. Royes I1-2, 87090C1-2, CCI3 and Ukulinga I1-1 had higher total chlorophyll content than the 100% nitrogen fertilization for cowpea. For dolichos plants treated with *Rhizobium* spp. isolates, CI2 and Royes I1-2 had equally high total chlorophyll content as the 100% Nitrogen Control. Only nodulation with XS21 resulted in a total chlorophyll content similar to that of the 100% Nitrogen Control. Isolates such as S29, CI3-2, Royes I1-2 and 93015 T1-1 all showed that during the V2 and V3 growth stages, the *Rhizobium* and *Bradyrhizobium* spp. began nitrogen fixation, because of exponential increases in chlorophyll content amongst the tested crops. On all the crops tested, rhizobial isolates

00040I2, Ukulinga I1-1, S29, 87051 I3-1, CI2, Royes I1-2 and SCI nodulations resulted in dry weights equal or higher than the 100% optimum Nitrogen Control for each crop. However, during the R1 and R3 growth stages, many of the fragile flowers and pods that had formed fell off and this resulted in fewer flowers forming pods and seeds thus affecting the dry weights and total chlorophyll content in the case of S29 and CI3-2 for soybean and cowpea.

Keywords: *total chlorophyll content; chlorophyll meter; soybean; cowpea; dolichos; dry bean; dry weight; vegetative growth stages; reproductive growth stages*

5.1 Introduction

According to Fischer, et al. (2009) 68% of fertilizer usage is by developing countries. The manufacture of nitrogen fertilizer requires ten times more energy than potassium and phosphate fertilizers (Evans, 1993, Hasegawa, 2003, Fageria, 2014). An estimated 50%-70% of inorganic nitrogen is lost into the soil profile. This depends on the distribution of soil microorganisms to facilitate uptake of nutrients and the proximity of fertilizer application to plant roots (Hodge, et al., 2000, Galloway, et al., 2002). Due to inorganic nitrogen's mobility through the soil profile and its reactive nature, it is susceptible to volatilization, leaching and denitrification (Smil, 1999, Cassman, et al., 2002, Robertson and Vitousek, 2009, Eskin, et al., 2014).

The interactions between rhizobia and legumes are specific, complex and can result in the formation of nodules. There are a high degree of similarity of 16S rDNA sequences, with differences ranging from 0.1%-2.0% divergence in the sequences between most species of *Bradyrhizobium*, and up to 4% divergence between *Bradyrhizobium elkanii* in comparison to other *Bradyrhizobium* spp. (Willems, et al., 2001). Specificity is due to the incompatibility between some nitrogen-fixing strain of rhizobial bacteria and a legume, resulting in the absence of infection. It also inhibits nodule development; therefore the infection thread does not form. This inhibit the bacteroid cells, stopping nitrogen fixation (Miller, et al., 2007). Specificity is governed by many factors, the most important being Nod Factors, which elicit root hair deformation, cortical-cell divisions and nodule-like outgrowths (Heidstra and Bisseling, 1996, Spaink, 1996). Host-specificity is determined by *nod* genes, which alters the

basic acylated Nod factor structure (Downie, 1998). Depending on the strain, the symbiotic relationship is host-specific or can be with a number of hosts. Multi-host or promiscuous strains such as NGR234 are strains of Rhizobia that nodulate a wider host range of legumes (Van Rhijn and Vanderleyden, 1995, Streit, et al., 2004).

The purpose of this study was to determine whether 31 freshly isolated *Rhizobium* and *Bradyrhizobium* spp. strains isolated from groundnut (*Arachis hypogaea* (L.) Kohler); cowpea (*Vigna unguiculata* (L.) Walp.); soybean (*Glycine max* (L.) Merrill); pigeonpea (*Cajanus cajan* (L.) Millsp.) and two commercial strains (XS21 and WB74) are capable of infecting and fixing nitrogen in soybean, cowpea, dolichos (*Lablab purpureus*) and dry bean (*Phaseolus vulgaris*). Chlorophyll meter readings were measured to determine the nitrogen fluxes during various vegetative and reproductive growth stages and were analyzed using ANOVA and total chlorophyll content and the dry weight of each crop per treatment were analyzed using the Kruskal-Wallis analysis.

5.2 Materials and Methods

Table 5.1 The varieties of legumes used for experimentation. All crops used were of an early maturing variety.

Table 5.1: Shows the variety of seeds, scientific names and supplier of seed used in experimentation.

Common Name	Scientific Name	Supplier
Soybean	*Glycine max* (L.) Merrill	Link Seed, Greytown, South Africa
Cowpea	*Vigna unguiculata* (L.) Walp.	Agricol, Cato Ridge, KwaZulu-Natal
Dry bean	*Phaseolus vulgaris* var. Gadra (L.)	Proseeds, Pietermaritzburg, South Africa
Dolichos	*Lablab purpureus* (L.) Sweet	Agricol, Cato Ridge, KwaZulu-Natal

Thirty one freshly isolated strains of *Rhizobium* spp. and *Bradyrhizobium* spp. and two commercial strains, XS21 and WB74, were grown on yeast mannitol agar (YMA): mannitol (Saarchem, South Africa) 10 $g.L^{-1}$; K_2HPO_4 (Saarchem, South Africa) 0.66 $g.L^{-1}$; $MgSO_4.7H_2O$ 0.2 $g.L^{-1}$ (Saarchem, South Africa); yeast extract 0.4 $g.L^{-1}$; NaCl (Saarchem, South Africa) 0.1 $g.L^{-1}$; $FeCl_3.6H_2O$ 0.6 $mg.L^{-1}$; bacteriological agar 15 $g.L^{-1}$; and 10 ml

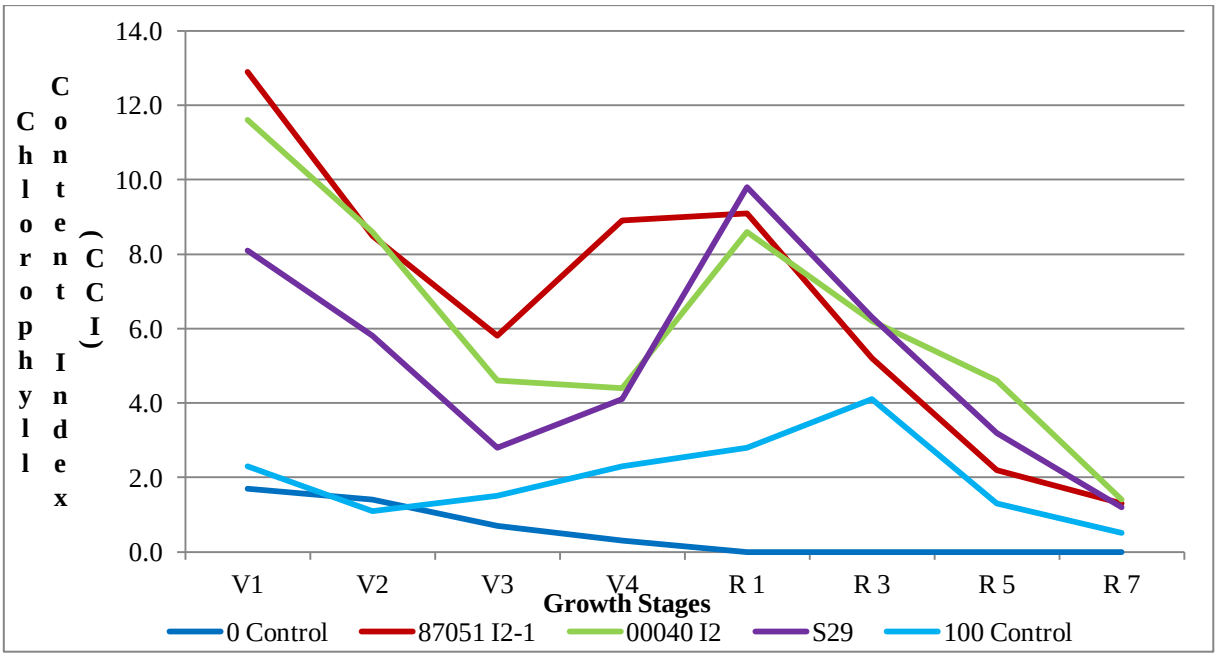
Congo red (Sigma-Aldrich, South Africa), and incubated at 28 °C (MRC Orbital Shaker Incubator, Israel) for 1 week. All seeds were sterilized using a 5% NaOCl solution (Unilever, South Africa) solution for one minute and rinsing thoroughly in sterile distilled water, before using a 70% ethanol solution and thoroughly rinsing in sterile distilled water before drying them on sterile filter paper in individual sterile Petri dishes. Each rhizobial isolate was scraped off inoculated YMA plates and mixed with a 2% gum guar sticker and mixed with sterilized seed of each crop and left to dry. There were two controls, a 0% nitrogen fertilizer Control (0%N) and the 100% (optimum) nitrogen fertilizer Control (100%N). Four seeds per pot were planted into steam autoclaved soil (30 minutes heating time), with two pots per replicates for each treatment (chlorophyll readings) using a completely randomized trial design. The soil pH was 6.09. Plants were treated with a nitrogen-free fertilizer solution (K_2SO_4 0.42 $g.L^{-1}$; $MgSO_4$ 0.11 $g.L^{-1}$; $CaSO_4$ 0.05 $g.L^{-1}$; Nutrifix® 0.05/25 L; phosphorus 0.14 $g.L^{-1}$). Nitrogen was supplied to the 100% Control as limestone ammonium nitrate (LAN) (Blackwood's, South Africa): 25% N (0.11 $g.L^{-1}$); 50% N (0.22 $g.L^{-1}$); 75% N (0.33 $g.L^{-1}$) and 100% N (0.44 $g.L^{-1}$) (Control).

Glasshouse temperatures were maintained at approximately 28°C for a period of 90 days until the Reproductive Stage 7 was reached and the final chlorophyll content was measured using a chlorophyll meter (Opti-Sciences CCM 200 Plus, USA). Chlorophyll content was measured over four vegetative stages and reproductive stages, namely: first true leaves (V1); first trifoliate stage (V2); second trifoliate stage (V3) and third trifoliate stage (V4); first open flower (R1); initial pod development stage (R3); mature pod and fully viable seed stage (R6) and the initial senescent stage (R7). The plants were harvested when pods and seed reached maturity. The plant material was oven dried, and dry weights were taken. Dry weight of total biomass was measured, for each plant for all four crops. Results were analysed using one-way ANOVA and Duncan's multiple comparison test for the means of treatments and stages, and the interactions between treatments and stages (Genstat 14). Kruskal-Wallis analysis of rankings of the area under the chlorophyll content index progress curve (AUCCIPC) and dry weights was done in Genstat 14. All plants were treated with pesticides to control powdery mildew (*Erysiphe polygoni*. D. C.); mealy bug (*Pseudococcus filamentosus* Guen.) and red spider mite (*Tetranychus urticae* Koch.), which caused leaf chlorosis, early senescence and defoliation in soybean.

5.3 Results

The results are based on the chlorophyll content of leaves, which was measured at four vegetative growth stages and reproductive stages to note the changes in chlorophyll content over each legume's growth cycle. All figures (Figures 5.1-5.4) show representative samples of freshly isolated rhizobial isolates in comparison to the 0% Nitrogen Control and 100% Nitrogen Control. See appendices for full data tables. Due to the lack of trial space, the replications for each treatment were limited to two. Therefore, the sample size was not large enough for calculation of the Chi Square and the significance of the results are based on the H-value obtained using the Kruskal-Wallis analysis. The rankings listed in the tables (Tables 5.1-5.4) below are based on the two replicates and due to the heterogeneity of the trial, each replicate was therefore ranked and the mean ranking was used for the analysis in the tables below.

Soybean (*Glycine max* (L.) Merrill)



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	1.0	139.6	139.6	9.4	
REP.*Units* stratum					
Treatment	34.0	2258.3	66.4	4.5	<.001
Stages	7.0	1057.8	151.1	10.2	<.001
Treatment.Stages	238.0	1164.9	4.9	0.3	1
Residual	279.0	4154.3	14.9		
Total	559.0	8774.8			

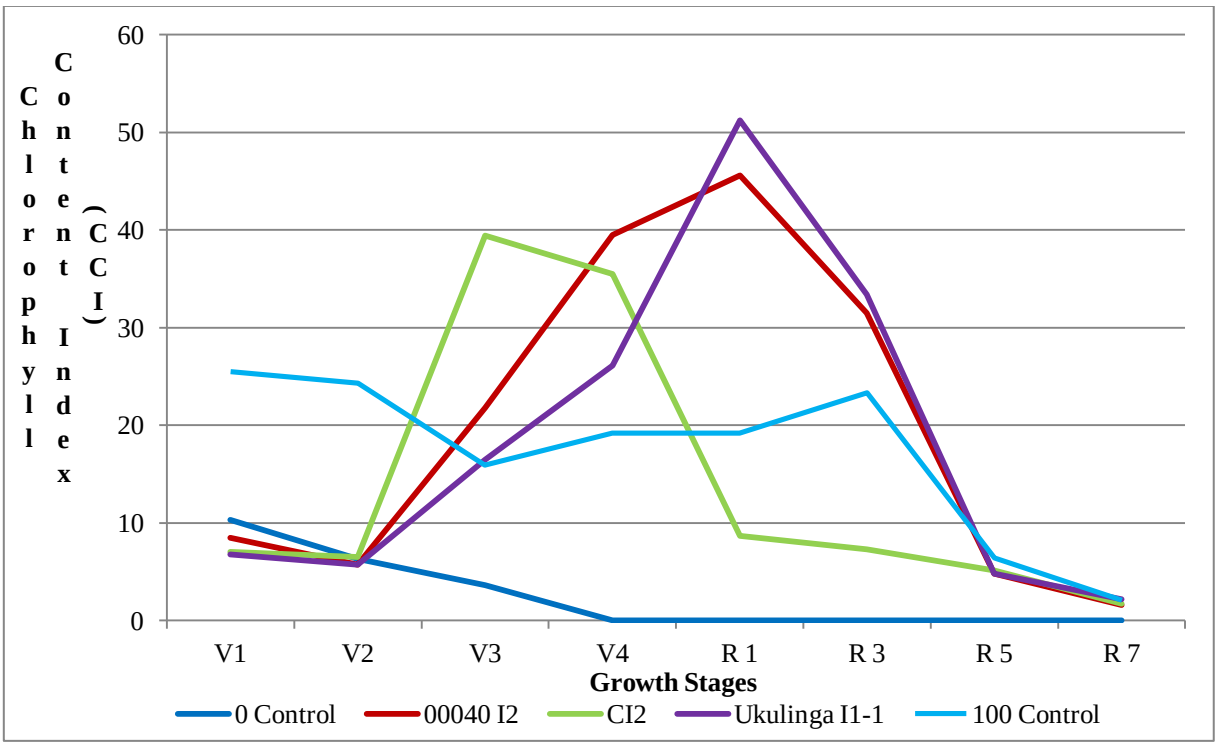
Figure 5.1: The chlorophyll content at four vegetative and reproductive growth stages of soybean (*Glycine max* (L.) Merrill) inoculated with four selected *Rhizobium* and *Bradyrhizobium* spp. strains and two soybean strains as controls. CV%: 125; LSD/DMRT Treatment: 2.7; LSD/DMRT Stages; 1.3; LSD/DMRT Treatment*Stages; 7.6 (See Appendix Table 5.1).

Table 5.2: The Kruskal-Wallis analysis of treatments based on the mean area under the chlorophyll content index progress curve and mean dry weight of soybean (*Glycine max* L. Merrill)

Treatment	Mean AUCCIPC Value	Mean AUCCIPC rank	Mean Dry Weight Value (g)	Mean Dry Weight rank
0% Nitrogen Control	0.00	18.5	0	7
100% Nitrogen Control	134.58	29.25	1.086	48
00040 I2	445.55	50.5	0.884	50.5
00040 I3-3	146.30	32.5	0.395	28.5
87051 I2-1	454.83	59	0.869	51
87051 I3-1	153.48	33	0.181	17.5
87091 T1-2	111.65	28	0.323	23.5
87091 C1-2	231.35	42.5	0.488	29.5
93015 T1-1	471.98	60.5	0.883	49.5
CCI3	318.15	46	0.973	53.5
CI1-G	138.43	30	0.981	38.5
CI2	371.88	52.5	0.765	45.75
CI3-2	103.43	26	0.350	24.5
CT1-6	197.93	34	0.558	35
CT2-2	207.38	34.5	1.189	54.75
G663 I1-1	93.45	25.5	0.701	42
G663 I3-1	0.00	12	0	7
Royes I1-2	243.78	36	0.516	33.5
Royes I3-3	140	31	0.836	44
S16	125.30	27.5	1.075	47
S18	139.48	30.5	0.351	26
S20B	131.60	28	0.276	20.5
S21	466.73	48	0.595	34.5
S26	391.48	40.5	0.605	37
S20A	0.00	12	0.191	16.5
S28A	168.18	33.5	0.305	22.5
S28B	0.00	12	0.355	27
S29	386.58	54.25	1.115	59
S30	164.99	33	0.345	25.5
S33A	376.25	45.5	0.786	36
S33B	144.38	31.5	0.291	24.5
SCI	219.63	35.5	0.783	46
Ukulinga I1-1	468.48	53	1.306	59.5
WB74	246.75	43.5	0.934	53
XS21	156.28	33	0.305	24.5
Sum of ranks		**1242.5**		**1242.5**
H Value		**25.75**		**33.72**
Degrees of freedom		**34**		**34**

Chi square value at $p < 0.05$ is 48.60

Cowpea (*Vigna unguiculata* (L.) Walp.)



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	1	109.18	109.18	2.14	
REP.*Units* stratum					
Treatment	34	21477.7	631.7	12.4	<.001
Stages	7	13418.4	1916.91	37.62	<.001
Treatment.Stages	238	22969.2	96.51	1.89	<.001
Residual	279	14216.8	50.96		
Total	559	72191.2			

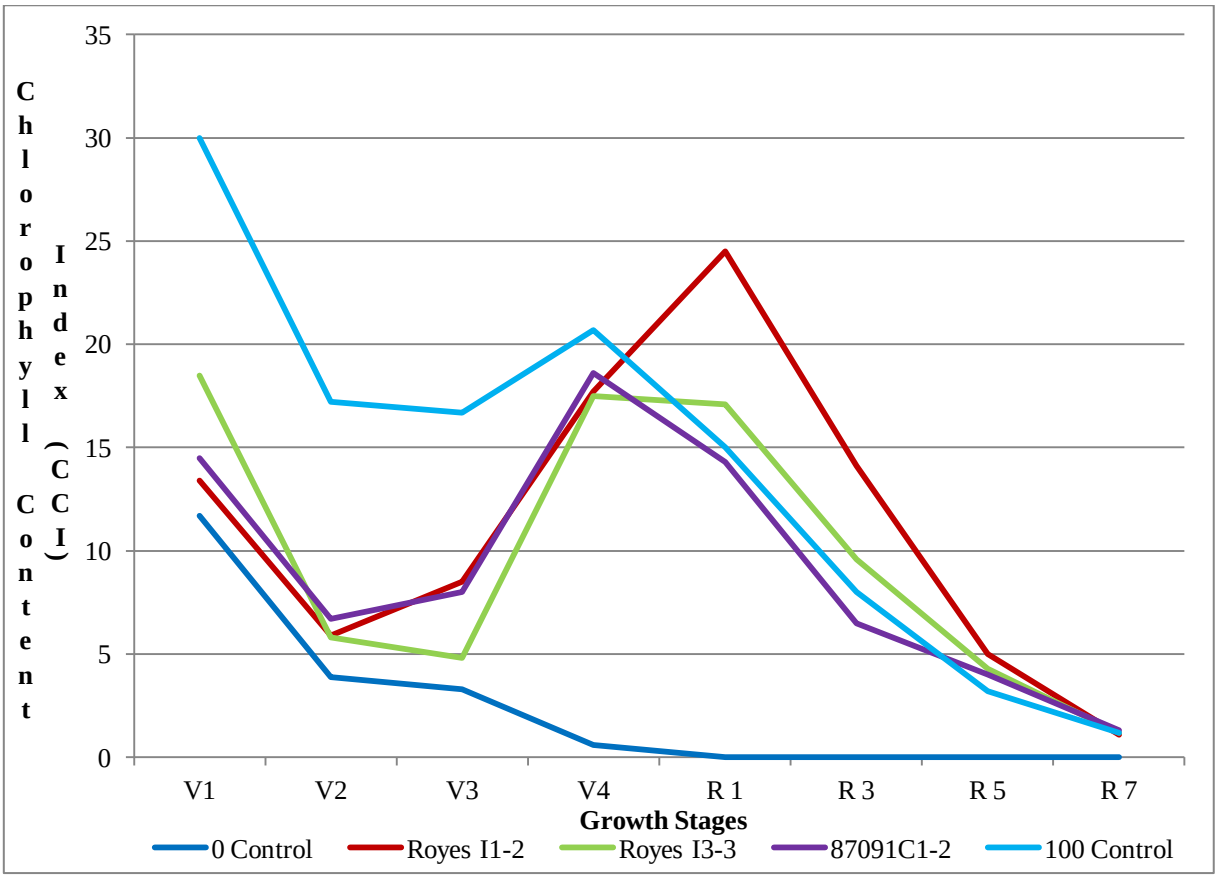
Figure 5.2. The chlorophyll content at four vegetative and reproductive growth stages of cowpea (*Vigna unguiculata* (L.) Walp.) inoculated with four selected *Rhizobium* and *Bradyrhizobium* spp. strains and two controls. CV%: 77.6; LSD/DMRT Treatment: 4.97; LSD/DMRT Stages; 2.38; LSD/DMRT Treatment*Stages; 14.05 (See Appendix Table 5.2).

Table 5.3: The Kruskal-Wallis analysis of treatments based on the mean area under the chlorophyll content index progress curve and mean dry weight of cowpea (*Vigna unguiculata* (L.) Walp.)

Treatment	Mean AUCCIPC Value	Mean AUCCIPC rank	Mean Dry Weight Value (g)	Mean Dry Weight rank
0% Nitrogen Control	105.70	14.5	0.07	14.75
100% Nitrogen Control	1226.40	53	1.52	52
00040 I2	1650.60	62	1.41	49.5
00040 I3-3	134.58	21.5	0.07	16.5
87051 I2-1	102.55	14	0.08	17.25
87051 I3-1	1176.35	53.5	2.23	63
87091 T1-2	73.68	7.5	0.05	9.25
87091 C1-2	1483.30	62.5	1.90	52
93015 T1-1	447.30	33	0.36	29.25
CCI3	1312.68	57.5	1.56	53
CI1-G	1078.18	49.5	1.66	53.5
CI2	1013.08	48.5	1.73	55
CI3-2	1457.40	61	2.51	67.5
CT1-6	820.75	42.5	0.83	39.5
CT2-2	634.20	37	0.57	36
G663 I1-1	124.43	15	0.05	11
G663 I3-1	1176.35	52	1.81	58
Royes I1-2	1552.43	56.5	1.67	55
Royes I3-3	872.55	43.5	1.16	46.5
S16	163.10	23.5	0.11	15.5
S18	762.65	41	1.35	48
S20B	147.18	20.5	0.10	23.5
S21	538.13	29	0.86	33.25
S26	51.45	4.5	0.06	10.75
S20A	265.65	28	0.64	35.5
S28A	312.20	22	0.74	38
S28B	869.58	44	0.81	39
S29	844.52	44	1.08	38.5
S30	122.85	18.5	0.07	15.25
S33A	84.53	11	0.07	15.25
S33B	300.83	18.5	0.27	18
SCI	355.95	21	0.22	18
Ukulinga I1-1	1558.38	64.5	2.08	61.5
WB74	128.45	18	0.07	14.5
XS21	1094.10	50	0.82	39.5
Sum of ranks		**1242.5**		**1242.5**
H Value		**54.23**		**52.73**
Degrees of freedom		**34**		**34**

Chi square at $p < 0.05$ is 48.602

Dolichos (*Lablab purpureus* (L.) Sweet)



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	1	98.36	98.36	5.45	
REP.*Units* stratum					
Treatment	34	4874.38	143.36	7.94	<.001
Stages	7	7188.7	1026.96	56.91	<.001
Treatment.Stages	238	5358.57	22.51	1.25	0.038
Residual	279	5034.5	18.04		
Total	559	22554.5			

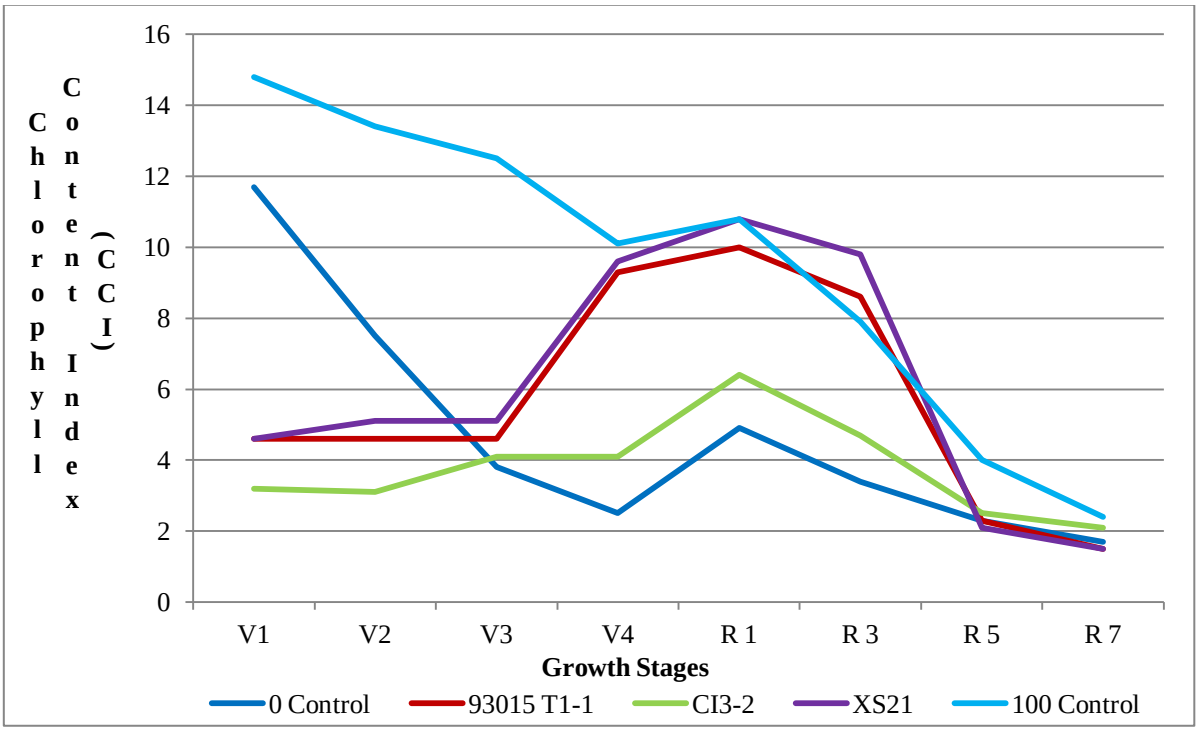
Figure 5.3: The chlorophyll content at four vegetative and reproductive growth stages of dolichos (*Lablab purpureus* (L.) Sweet) inoculated with four selected *Rhizobium* and *Bradyrhizobium* spp. strains and two controls. CV%: 79.2; LSD/DMRT Treatment: 2.96; LSD/DMRT Stages; 1.41; LSD/DMRT Treatment*Stages; 8.36 (See Appendix Table 5.3).

Table 5.4: The Kruskal-Wallis analysis of treatments based on the mean area under the chlorophyll content index progress curve and mean dry weight dolichos (*Lablab purpureus* (L.) Sweet)

Treatment	Mean AUCCIPC Value	Mean AUCCIPC rank	Mean Dry Weight Value (g)	Mean Dry Weight rank
0% Nitrogen Control	97.65	16	0.20	26.5
100% Nitrogen Control	894.78	65.5	2.77	69.5
00040 I2	309.75	38	1.41	55.25
00040 I3-3	75.08	5.5	0.11	10.75
87051 I2-1	91	13	0.16	17
87051 I3-1	500.68	51	0.99	49
87091 T1-2	441.00	34.5	1.14	50.5
87091 C1-2	667.98	57.5	0.49	25
93015 T1-1	73.85	5.5	0.11	10.25
CCI3	120.68	23	0.20	26
CI1-G	464.63	48.5	0.51	41.25
CI2	810.08	60.5	1.54	61
CI3-2	486.50	47.5	0.71	46.25
CT1-6	510.30	51	1.06	53
CT2-2	532.35	51	0.77	47.75
G663 I1-1	370.30	34.5	0.76	44.5
G663 I3-1	343.88	42.5	0.39	32.5
Royes I1-2	884.28	62.5	1.81	62.5
Royes I3-3	720.65	56.5	1.38	54
S16	94.68	13	0.10	8.5
S18	120.58	25.5	0.10	6.5
S20B	167.30	33	0.21	29
S21	302.93	32	0.45	34
S26	206.68	29.5	0.29	31.25
S20A	104.48	18.5	0.11	9.25
S28A	382.55	43.5	0.58	42
S28B	126.18	27	0.20	26.75
S29	117.95	23.5	0.17	20.75
S30	103.60	17	0.13	17.75
S33A	581	43	1.01	34.5
S33B	106.20	17.5	0.16	20.5
SCI	227.85	38	0.68	46
Ukulinga I1-1	581.18	53	1.57	61
WB74	93.63	14	0.13	17.75
XS21	547.40	50.5	1.16	54.5
Sum of ranks		**1242.5**		**1242.5**
H Value		**48.6**		**52.54**
Degrees of freedom		**34**		**34**

Chi square at $p < 0.05$ is 48.602

Dry bean (*Phaseolus vulgaris* (L.)



Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	1	5.118	5.118	1.86	
REP.*Units* stratum					
Treatment	34	798.15	23.475	8.52	<.001
Stages	7	1017.23	145.318	52.75	<.001
Treatment.Stages	238	912.476	3.834	1.39	0.004
Residual	279	768.643	2.755		
Total	559	3501.61			

Figure 5.4: The chlorophyll content at four vegetative and reproductive growth stages of dry bean (*Phaseolus vulgaris* (L.) inoculated with four selected *Rhizobium* and *Bradyrhizobium* spp. strains and two soybean strains as controls. CV%: 42.8; LSD/DMRT Treatment: 1.16; LSD/DMRT Stages; 0.55; LSD/DMRT Treatment*Stages; 3.27 (See Appendix Table 5.4).

Table 5.5: The Kruskal-Wallis analysis of treatments based on the mean area under the chlorophyll content index progress curve and mean dry weight of dry bean (*Phaseolus vulgaris* (L.)

Treatment	Mean AUCCIPC Value	Mean AUCCIPC rank	Mean Dry Weight Value (g)	Mean Dry Weight rank
0% Nitrogen Control	297.33	54.5	0.51	28
100% Nitrogen Control	649.08	69	6.65	69.5
00040 I2	215.60	7	0.49	25.25
00040 I3-3	271.95	44	0.67	51.25
87051 I2-1	196.53	5	0.77	41
87051 I3-1	279.83	45.5	0.47	22.75
87091 T1-2	298.03	54.5	0.61	45.5
87091 C1-2	281.75	47	0.74	56.25
93015 T1-1	455.35	51	1.81	49.25
CCI3	211.75	13.25	0.45	16.5
CI1-G	280.88	50.5	0.53	31.5
CI2	217.18	19	0.45	19.5
CI3-2	266.63	37.5	0.52	29.5
CT1-6	232.40	19.5	0.46	18.25
CT2-2	238	20.75	0.41	8.5
G663 I1-1	248.15	28	0.60	35.75
G663 I3-1	297.85	50.5	0.54	32.25
Royes I1-2	214.90	12.5	0.67	51.75
Royes I3-3	259.53	31.5	0.58	43
S16	264.43	38.25	0.60	36.5
S18	263.38	35.75	0.78	33
S20B	243.78	25.25	0.61	40.25
S21	246.23	27.25	0.58	43.25
S26	244.65	25.75	0.46	18
S20A	269.85	41.75	0.58	32
S28A	263.73	34.5	0.77	51.5
S28B	263.03	37.5	0.47	21.5
S29	235.38	21	0.48	21.75
S30	219.63	8	0.57	35.75
S33A	242.20	25	0.44	16.75
S33B	287.88	51	0.48	22.5
SCI	298.20	55.5	1.05	60
Ukulinga I1-1	258.83	35.25	0.59	40
WB74	292.43	53.75	0.65	44.75
XS21	486.85	66.5	1.91	49.75
Sum of ranks		**1242.5**		**1242.5**
H Value		**47.12**		**33.72**
Degrees of freedom		**34**		**34**

Chi square at p <0.05 is 48.602

5.4 Discussion

Nitrogen is mobilized and relocated in plants using two mechanisms. Firstly, nitrogen is taken up as nitrate via the roots and secondly, remobilized and distributed throughout the plant (Daniel-Vedele, et al., 1998, Tsay, et al., 2007). Nitrogen relocation and storage is important for both annuals and perennials. Annuals require nitrogen for seed production because the nitrogen content determines the viability of seeds and seedling survival (Masclaux-Daubresse, et al., 2010). The chlorophyll content was measured at specific growth stages to establish at which growth stage the rhizobia began fixing nitrogen. Secondly, the total chlorophyll accumulated over the plants life cycle was measured together with the total dry weight (biomass). All the crops used were early maturing varieties and at the time of planting , the growth stages which were approximately a week apart, influencing the vegetative and reproductive growth stages for nutrient uptake, nitrogen fluxes and biomass (Buehring, et al., 2003, Kazemi, et al., 2005, Moosavi, et al., 2011). Analysis of variance (ANOVA) was used to analyse the chlorophyll content over the various growth stages. However, the initial ANOVA showed high coefficient of variance levels, which was due to the low number of replicates. Non-parametric Kruskal-Wallis analysis was used to analyse the mean total chlorophyll content (AUCCIPC) and mean dry weight as the data did not meet the criteria for ANOVA.

Soybean (*Glycine max*)

Figure 5.1 shows the fluctuations in chlorophyll content over the life cycle of soybean. The 0% Nitrogen Control only produced chlorophyll meter readings in the vegetative growth stages, with declining chlorophyll content from the V1-V4 growth stages. In contrast, the plants treated with the 100% Nitrogen Control showed a gradual increase in chlorophyll content between the V2 and R1 growth stages, with a peak chlorophyll content at the R3 growth stage. The plants treated with the rhizobial isolates shown in Figure 5.1 all showed decreases in chlorophyll content between the V1 and V3 growth stages. At the V3 Stage, the plants showed increases in chlorophyll content up until the R1 Stage. This increase was indicative of some of the isolates actively fixing atmospheric nitrogen resulting in increases in chlorophyll content. This trend was apparent as a result of some isolates such as 93015 T1-1,

as shown in Appendix Table 5.1, with some rhizobial isolates causing increases at the V2 Stage.

Table 5.2 shows the Kruskal-Wallis analysis of the AUCCIPC and dry weights of soybean plants treated with rhizobial isolates. The higher the ranking scores, the higher the mean total chlorophyll content and dry weight. The 0% Nitrogen Control had an AUCCIPC ranking of 18.5 whereas the 100% optimum Nitrogen Control had an AUCCIPC ranking of 29.25. The two commercial controls, WB74 and XS21, had rankings of 43.5 and 33, respectively, much higher than the 100% optimum Nitrogen Control. The freshly isolated isolate 93015 T1-1 had a ranking of 60.5 and Isolate 87051 I2-1 a ranking of 59, again much higher than the controls. These results indicated that some of the freshly isolated isolates fixed atmospheric nitrogen that translated into higher chlorophyll content more efficiently than the commercial controls.

The mean dry weight rankings showed that the 0% and 100% Nitrogen Control were ranked 7 and 48 respectively. In comparison, the rhizobial isolates 87051 I2-1 and 93015 T1-1 were ranked 51 and 49.5 respectively, slightly higher than the 100% Nitrogen Control. This indicated that the isolates may have effectively fixed nitrogen, as shown by the high chlorophyll content, but this was not effectively translated into high biomass. Based on the Chi square distribution at 34 degrees of freedom, the p value (< 0.05) is 48.602. The H value was lower than the Chi square value and therefore there was no significance between treatments (Table 5.2).

Cowpea (*Vigna unguiculata*)

Figure 5.2 shows the freshly isolated isolates 00040 I2, CI2 and Ukulinga I1-1 inoculated onto cowpea plants increased in chlorophyll content at the V2 Stage, with the peak chlorophyll readings at the R1 Stage. In comparison, the 0% Nitrogen Control declined in chlorophyll content over the four vegetative growth stages, whilst the 100% Nitrogen Control chlorophyll content fluctuated throughout the plant's life cycle. There were significant differences between the treatments and between the different growth stages. However, the coefficient of variance of 77 was high and this suggests a high degree of experimental error due to the low number of replicates.

The 0% Nitrogen Control mean AUCCIPC rank was 14.5 and for the 100% Nitrogen Control was 53. The two commercial controls, WB74 and XS21, scored 18 and 50, respectively. The

freshly isolated rhizobial isolates 00040 I2, 87051 I2-1, 87091 C1-2, CCI3, CI3-2, Royes I1-2 and Ukulinga I1-1 had a score of 64.5 which ranked them higher than the 100% Nitrogen Control (Table 5.3). The H value was 54.23, higher than the Chi square value of 48.602. This implies that the treatments were significantly different.

The mean dry weight score for the 0% Nitrogen Control was 14.75 and for the 100% Nitrogen Control was 52. The commercial isolates WB74 and XS21 had scores of 14.5 and 39.5, respectively, much lower than the 100% Nitrogen Control. A number of freshly isolated rhizobial isolates scored higher than the 100% Nitrogen Control. These included: 87051 I2-1, CI1-G CI2; G663 I3-1; Royes I1-2; Ukulinga I1-1 and CI3-2, with the highest score of 67.5. The H value of 52.73 was higher than the Chi square value of 48.602 and therefore the treatments were significantly different (Table 5.3).

Dolichos (*Lablab purpureus*)

Inoculation with Royes I1-2, Royes I3-3 and 87091 C1-2 resulted in increases in plant chlorophyll content at the V2 stage. Rhizobial isolates Royes I3-3 and 87091 C1-2 had caused peak chlorophyll content at the V4 stage, whilst Royes I1-2 had caused peak chlorophyll content at the R1 Stage. The 100% Nitrogen Control plants had a high chlorophyll content at the V1 and V4 stages. The treatments, stages and interaction of treatment and stages all showed significant differences. However, the CV% was high at 79.2 (Figure 5.3).

The mean AUCCIPC score for the 0% Nitrogen Control was 16 and for the 100% Nitrogen Control was 65.5. The plants treated with the commercial controls, WB74 and XS21, were scored 14 and 50, respectively. The only rhizobial isolates that scored as high as the 100% Nitrogen Control were CI2 and Royes I1-2. The H value was lower than the Chi square value of 48.602, so the differences were not significant (Table 5.4).

The mean dry weight score for 0% Nitrogen Control was 26.5 and 100% Nitrogen Control was 69.5 (Table 5.4). Plants treated with the commercial controls, XS21 and WB74, scored 54.5 and 17.5, respectively. Plants treated with CI2, Ukulinga I1-1 and Royes I1-2 scored above 60. The H value of 52.54 was higher than the Chi Square value therefore the differences were not significant.

Dry Bean (*Phaseolus vulgaris*)

Figure 5.4 showed the plants treated with both the 0% Nitrogen Control and 100% Nitrogen Control had high chlorophyll content at the V1 growth stage. The plants treated with the rhizobial isolates XS21 and 93015T1-1 had lower chlorophyll content at the V1 growth stage but showed exponential increases at the V3 growth stages. The treatments, stages and interactions between the treatments and growth stages were significantly different. However, the CV% of the experiment was high at 42.8.

The mean AUCCIPC score for 0% Nitrogen Control was 54.5 and for the 100% Nitrogen Control was 69. Only two rhizobial isolates SCI and 87091 T1-2 were scored equal or higher than the 0% Nitrogen Control. The commercial controls WB74 scored 53.75 and XS21 scored 66.5 (Table 5.5). The H value was lower than the Chi square value of 48.602, therefore the difference was not significant.

The 0% Nitrogen Control had a mean dry weight score of 28 and the 100% Nitrogen Control was 69.5. SCI was the only rhizobial isolate to score 60, which was higher than WB74 and XS21, which were ranked 44.75 and 49.75, respectively. The H value was 33.72, lower than the Chi square value of 48.602, therefore the difference was not significant.

Chlorophyll readings were high at the vegetative cotyledon stage (VC) due to the nitrogen that was available in the soil and the nitrogen available cotyledons. No additional nitrogen was added to the fertilizer mix except for the 100% Nitrogen Control for two reasons. Firstly, to ensure that the freshly isolated isolates had a high probability of infection and secondly to ensure that the chlorophyll readings were from the changes in chlorophyll content indirectly resulted in the effectiveness of the freshly isolated isolate at fixing atmospheric nitrogen. Plants assimilate, utilize, remobilized, store and translocate nitrogen, resulting in nitrogen fluxes during the plants' life cycle (Masclaux-Daubresse, et al., 2010). During senescence, photosynthetic proteins in leaves such as plastids are extensively degraded to create a source of nitrogen for growing parts such as new leaves, flowering, pod formation and seed development (Malagoli, et al., 2005, Diaz, et al., 2008, Lemaître, et al., 2008).

Nitrate and other mineral nutrients are required optimal growth and development. Nitrate uptake depends on the response of the plant to environmental conditions, stress conditions and variations during ontogeny (Imsande and Touraine, 1994). Chlorophyll content readings were high at the Vegetative Stage (V1) due to the residual nitrogen in the potting medium and from

the cotyledon during germination. Chlorophyll content decreased between the vegetative stages V2 and V4, with some isolates showing increases in chlorophyll content at the V2 and V3 growth stages. This was due to the lack of nitrogen in the potting medium, which stimulated some of the *Rhizobium* and *Bradyrhizobium* spp. isolates to begin nitrogen fixation. This was visible particularly at the V4 and R1 growth stages, which showed increases in chlorophyll content.

During the reproductive stages, the chlorophyll content in the leaves progressively declined, from growth stages R1 to R7. Crawford, et al. (1982) found the stalks and leaves of maize plants acted as nitrogen sources for the production of grain whilst the shank, husk and cob acted as nitrogen sinks during the reproductive stages. The greatest differences in chlorophyll content were noted between the R3 and R6 growth stages, in which the pod and seeds develop and the seed mature. During the R1 and R3 growth stages, flowers and pods were fragile and easily fell off the plant whilst chlorophyll readings were being taken. This decreased the number of flowers that formed pod and pods, which in turn, produced seed. Abiotic and biotic stresses, causes loss of leaf nitrogen as leaf litter, prior to pod filling, the vegetative structures (leaves, stems and roots) then cannot effectively mobilize nitrogen for reproductive structures, during the reproductive growth stages (Triboï-Blondel, 1988, Rossato, et al., 2001, Malagoli, et al., 2005). This inefficient nitrogen mobilization results in early senescence and pod development (Diepenbrock, 2000, Rossato, et al., 2001).

All the crops were able to fix nitrogen in varying degrees using isolates from cowpea, pigeon pea, soybean and groundnut. The plants treated with some of the freshly isolated isolates were capable of producing higher chlorophyll content in comparison to the 100% Nitrogen Control and the 0% Nitrogen Control with the potential for high dry weights.

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Chapter 6: Nodulation and leghaemoglobin production of four legumes inoculated with freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp.

Abstract

Nodulation is an integral part of nitrogen fixation. Nodules form by infection of the root hairs of the host plant by *Rhizobium* or *Bradyrhizobium* spp. The nodules have the capacity to fix atmospheric nitrogen. Nitrogen plays a vital role in the growth and development of leguminous plants. Leghaemoglobin is an oxygen-binding compound in rhizobial nodules which aids cytochromes and maintains oxygen levels in respiring bacteroids in the nodule and buffers the low free oxygen concentrations, preventing deactivation of the oxygen-labile nitrogenase enzyme. Soybean (*Glycine max* L), cowpea (*Vigna unguiculata* L), dolichos (*Lablab purpureus* L) and dry bean (*Phaseolus vulgaris* L) were treated with 17 freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp., two commercial *Rhizobium* spp. strains XS21 and WB74 and two controls: 0% Nitrogen Control and 100% Nitrogen Control. Each plant and isolate combination was planted in two rhizotrons and harvested 30 days post-inoculation. The aim of this study was to investigate firstly, the total number of nodules formed on the host and secondly, the number of nodules containing leghaemoglobin. The numbers of nodules and fraction of these containing leghaemoglobin varied amongst the different crops, as well as the location of nodules. Cowpea and dolichos showed a tendency to form nodules around the crown of the tap root and surrounding lateral roots, whilst dry bean, although capable of forming nodules at the crown of the tap root, formed more nodules on the lateral roots, which spanned the entire root system. In contrast, soybean did not form many nodules, due to poor drainage. Only four rhizobial isolates produced nodules on soybean and only one isolate, 93015T1-1, was capable of producing a single nodule containing leghaemoglobin. The lack of drainage reduced the oxygen levels in the soil, affected nodulation, and nitrogen fixation and induced nodule senescence, which affected overall plant growth and development. However, cowpea and dolichos showed that they were able to

nodulate with a number of the rhizobial isolates tested and fix nitrogen, with most nodules containing leghaemoglobin. Dry bean also nodulated well and fixed nitrogen, as reflected by the presence of leghaemoglobin. However, only eight of the nineteen isolates were compatible. Both controls for all the tested crops did not produce any nodules.

Keywords: nodulation; leghaemoglobin; cowpea; soybean; dolichos; dry bean; Rhizobium; Bradyrhizobium

6.1 Introduction

The symbiotic relationship between rhizobia and legumes are initiated by infection of legume root hairs by rhizobia, resulting in nodular growths (Malik, et al., 1984). Legumes infected with mutualistic rhizobial strains obtain extra nitrogen for plant growth and development, allowing legumes to grow in nitrogen-poor environments (Brockwell, 1982, Martínez-Romero, 2009). The nitrogen levels in the soil environment determine whether nodulation does or does not occur on legumes. In low nitrogen soil environments, legumes recognise rhizobia easily and infection occurs (Dixon and Wheeler, 1986).

Infection by most *Rhizobium* spp. occurs within five hours of inoculation in the presence of emergent root hairs, with little to no infection in mature, fully elongated root hairs (Calvert, et al., 1984). According to Dixon and Wheeler (1986), the first step in nodule formation is the recognition of the *Rhizobium* spp. in the legume root hair. Root hair curling seals the bacterium within the root hair, preventing enzymes required for root penetration from diffusing away. Morphological changes of the root hair begins with the presence of an infection sack, preceded by an infection thread in conjunction with the formation of the nodule meristem in the root cortex (Werner, et al., 1992). Differentiation of the host cell organelles, involves increases in the number of Golgi bodies, endoplasmic reticuli and microtubules. The infection thread enters the cortex of the root, and approximately 3-4 days after infection, a defined swelling or nodule develops. At 6 days after inoculation, the connection between the nodule meristem and the vascular system of the host plant is formed (Calvert, et al., 1984). Lastly, the plant establishes a supply and transport system to supply the nodule with carbon sources, for ammonia assimilation, nitrogenase activity and oxygen mechanisms (Werner, et al., 1992).

The term leghaemoglobin refers to the haemoglobins found in the nodules of legumes (Downie, 2005). Leghaemoglobin is a monomeric 16 kiloDalton protein with a high affinity for oxygen, which functions in a similar way to haemoglobin in mammals, binding reversibly to a singular oxygen molecule (Wittenberg, et al., 1972). The haem group is the binding site for the oxygen molecule with concentrations of leghaemoglobins reaching up to 700 µM in nodules (Bergersen, 1982). Leghaemoglobin is essential for symbiotic nitrogen fixation, with the purpose of transporting oxygen to the respiring bacteroids in the nodule and buffering the low free oxygen concentrations, preventing deactivation of the oxygen-labile nitrogenase enzyme. The leghaemoglobin is located in the cytosol of infected cells and is absent from the bacteroid and peribacteroid space (Appleby, 1984, O'Brian, et al., 1987, Santana, et al., 1998).

Nodules are classed in two categories, determinate and indeterminate nodules. Determinate or spherical nodules are formed by dividing cells congregating into a central mass. When cell division ceases, the size of the resulting nodule increases due to cell enlargement. Determinate nodules do not possess a persistent meristem, resulting in cell division discontinuing at the early stages (Patriarca, et al., 1996). Indeterminate or meristematic nodules are the result of the infection thread continually growing and branching from the meristem to the apex of the nodule, resulting in an elongated shape. Branched nodules are formed from the dividing meristem. The youngest section of the nodule is furthest from the apex of the nodule and the oldest is the initial site of infection. The active region of nitrogen fixation is at the core of the nodule, which contains mature cells encasing bacteroids with the capability of fixing nitrogen (Dixon and Wheeler, 1986).

Nodulation is affected by other factors including lectins and Nod factors, which are specific lipo-chitin oligosaccharides, that are synthesized by nodulation genes (*nod* genes) (Lerouge, et al., 1990). Over fifty different *nod* genes have been identified in legume nodulation. One of the characteristics of Nod factors is their host-specificity, brought about by structural variations in Nod factors that are generated by rhizobial strains having different host ranges, different intracellular and extracellular alkalinisation and membrane potential depolarization, induction of formation and deformation of root hairs, changes in ion concentrations and early nodulin gene expression and formation of the nodule environment (Broughton and Perret, 1999, D'Haeze and Holsters, 2002). Flavonoids are important because they activate the *nodD* transcriptional regulator gene, which in turn activates other bacterial nodulation genes, resulting in the synthesis of Nod factors (Geurts and Bisseling, 2002).

The purpose of this observational study was to investigate the ability of 17 freshly isolated bacterial isolates to infect and effectively nodulate the root system of four legumes, and to document the number of nodules formed after 30 days post inoculation and the number of nodules containing leghaemoglobin. Due to a limited number of rhizotrons only two replications were possible for this study.

6.2 Materials and Methods

Seventeen strains freshly isolated strains and two commercial strains of *Rhizobium* spp. and *Bradyrhizobium* spp. were grown on yeast mannitol agar (YMA): mannitol (uniVAR, USA) 10 $g.l^{-1}$; K_2HPO_4 (BDH Chem, England) 0.66 $g.l^{-1}$; $MgSO_4.7H_2O$ (Associated Chem Enterprises, South Africa) 0.2 $g.l^{-1}$; yeast extract (Merck, South Africa) 0.4 $g.l^{-1}$; NaCl (Merck, South Africa) 0.1 $g.l^{-1}$; $FeCl_3.6H_2O$ (Merck, South Africa) 0.6 $mg.l^{-1}$; bacteriological agar (Merck, South Africa) 15 $g.l^{-1}$ and 10 ml Congo red (Sigma, USA) solution and incubated at 28 °C (MRC Orbital Shaker incubator, Israel) for 1 week.

Table 6.1 the varieties of legumes used for experimentation. All crops used were of an early maturing variety.

Table 6.1: Shows the variety of seeds, scientific names and supplier of seed used in experimentation.

Common Name	Scientific Name	Supplier
Soybean	*Glycine max* (L.)Merrill	Link Seed, Greytown, South Africa
Cowpea	*Vigna unguiculata* (L.)Walp.	Agricol, Cato Ridge, KwaZulu-Natal
Dry bean	*Phaseolus vulgaris* var. Gadra (L.)	Proseeds, Pietermaritzburg, South Africa
Dolichos	*Lablab purpureus* (L.)Sweet	Agricol, Cato Ridge, KwaZulu-Natal

All seeds were sterilized using a 5% NaOCl solution (Unilever, South Africa) solution for one minute and rinsing thoroughly in sterile, distilled water, before using a 70% ethanol solution and thoroughly rinsing in sterile distilled water before drying on sterile filter paper in individual sterile Petri dishes. Two inoculated seeds per rhizotron were planted into autoclaved soil (30 minutes heating time). Two replicates per treatment per crop were used due to a limited number of rhizotrons. A completely randomised design was used. Two

controls were also planted, a 0% Nitrogen Control and a 100% optimum Nitrogen Control. *Rhizobium* spp. treatments were treated with a nitrogen free fertilizer solution (KSO_4 0.43 $g.l^{-1}$; $MgSO_4$ 0.11 $g.l^{-1}$; Ca_2SO_4 0.04 $g.l^{-1}$; Nutrifix 0.05/25 L; phosphorus 0.14 $g.l^{-1}$ and nitrogen (LAN) 0.43 $g.l^{-1}$ (100% Nitrogen Control). Temperatures were maintained in a glasshouse at approximately 28°C for a period of 30 days. Plants were treated with pesticides to control powdery mildew, red spider mite and mealy bug. The plants were removed at 30 days. The nodule number and the number of nodules containing leghaemoglobin were counted and averaged. All plants were, treated with pesticides to control powdery mildew (*Erysiphe polygoni*. D. C.); mealy bug (*Pseudococcus filamentosus* Guen.) and red spider mite (*Tetranychus urticae* Koch.).

6.3 Results

After a period of 30 days post-inoculation, no nodules were found on any of the control plants. Total number of nodules and number of leghaemoglobin containing nodules formed varied amongst the different crops (Figures 6.1; 6.3; 6.5; 6.7). The location of the nodules on treated plants varied. Cowpea plants developed nodules around the top third of the tap root system (Figure 6.4). Soybean and dry bean had nodulation around the lateral roots throughout the root system (Figures 6.2 and 6.8). Dolichos had nodulation started around the top third of the tap root and progressing to the lateral roots, developing on the entire root system (Figure 6.6). The majority of nodules formed were determinate in shape and varied in size. Cowpea and dolichos produced larger nodules than soybean and dry bean. The root system was distinctively smaller in soybean than on the other three legumes.

Powdery mildew, red spider mite and mealy bug infestations affected a number of plants. Due to poor drainage of the rhizotrons, some plants, particularly the soybean and dry beans, did not flourish as expected.

Soybean (*Glycine max*)

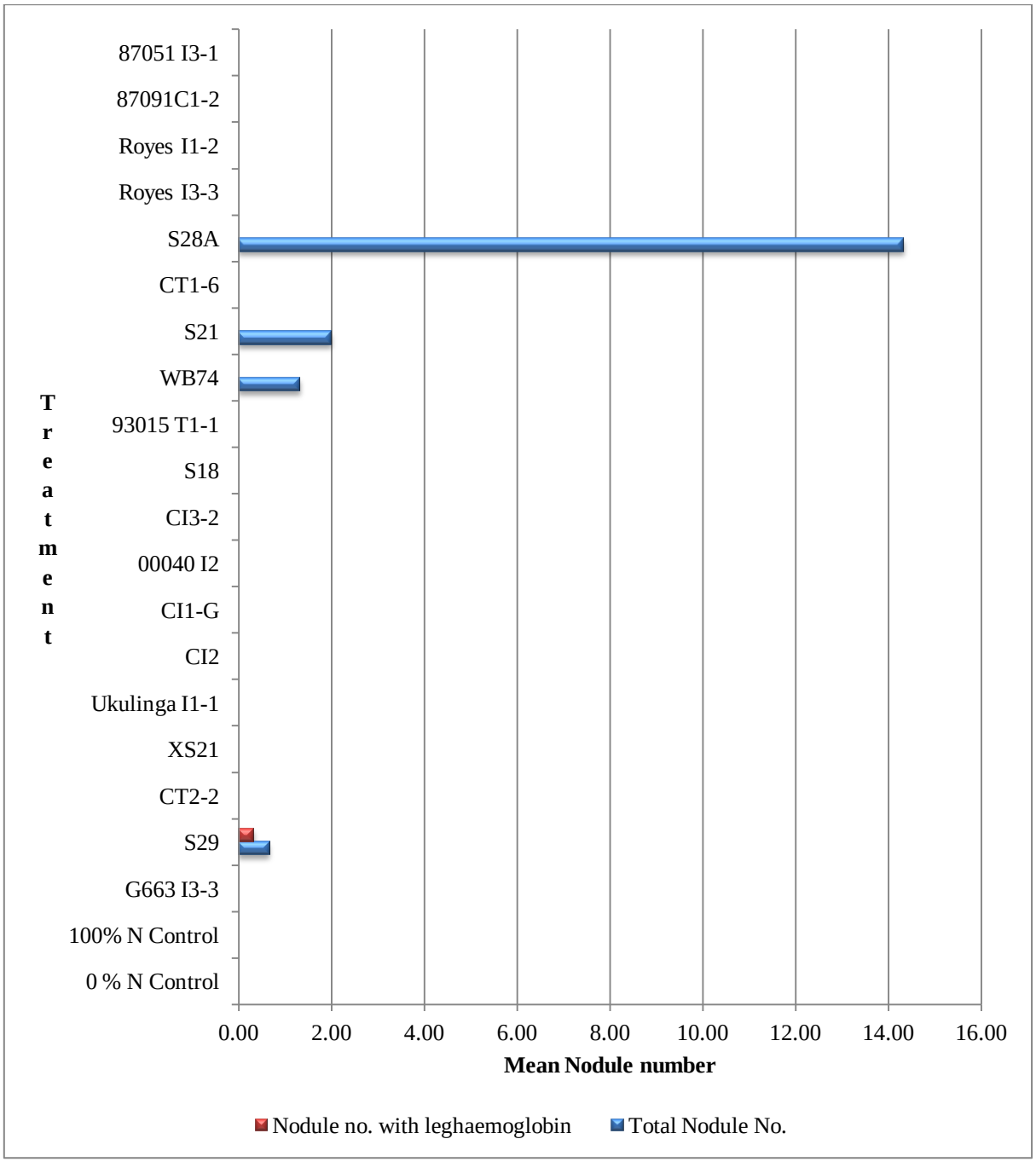

Figure 6.1: Soybean nodulation: number of nodules formed and number of nodules containing leghaemoglobin

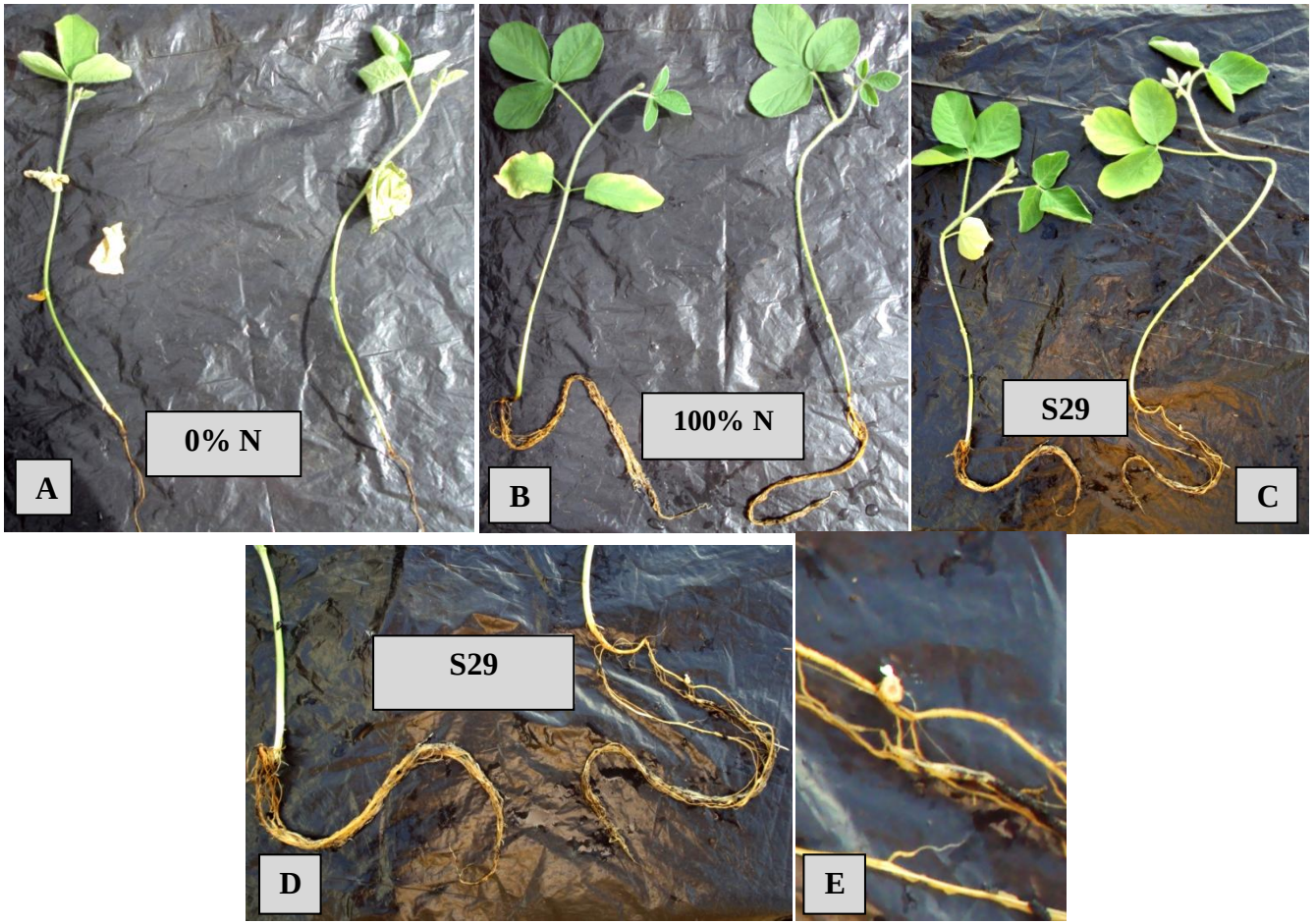

Figure 6.2 Examples of soybean nodulation: A: 0% Nitrogen Control plant showing pale green leaves, curling and drying with a minimal root structure and only main taproot visible. Figure 6.2B: 100% Nitrogen Control plant showing dark green leaves, lateral roots visible, unifoliate leaves yellow at the edges and drying. Figure 6.2C: A plant treated with Isolate S29 showing dark green leaves and some lateral roots. Figure 6.2D: An enhanced view of the root structure with nodulation by Isolate S29. Figure 6.2E: Close up of a single visible nodule with leghaemoglobin.

Cowpea (*Vigna unguiculata*)

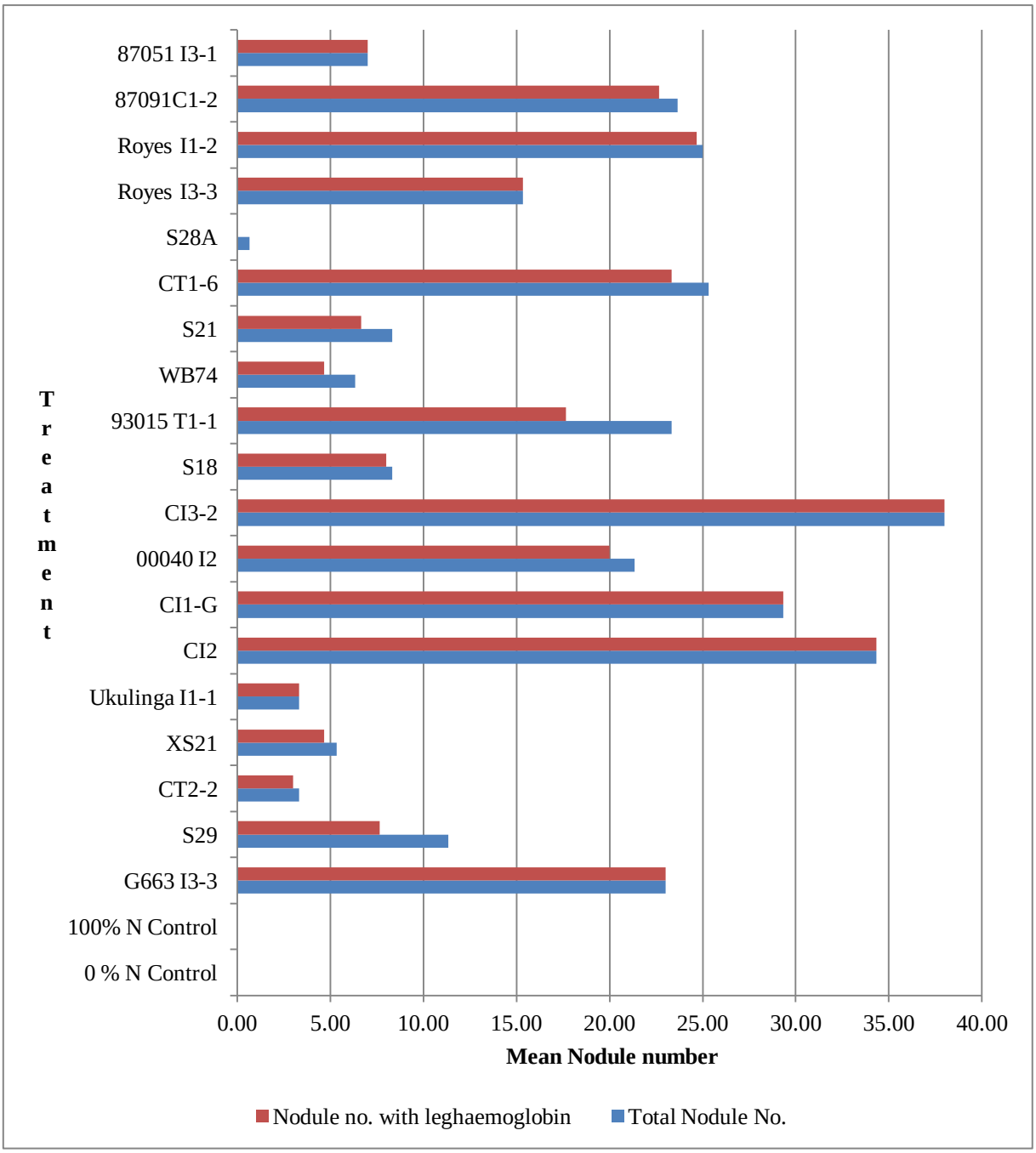

Figure 6.3 Cowpea nodule number and number of nodules containing leghaemoglobin. The untreated controls did not produce any nodules, with majority of the treatments producing nodules with an equally high leghaemoglobin producing nodules. Commercial strains WB74 and XS21 produced nodules but were not as many and effective as Isolates CI2, CI1-G, G663 I3-1, CI3-2, 87091 C1-2 and Royes I1-2.

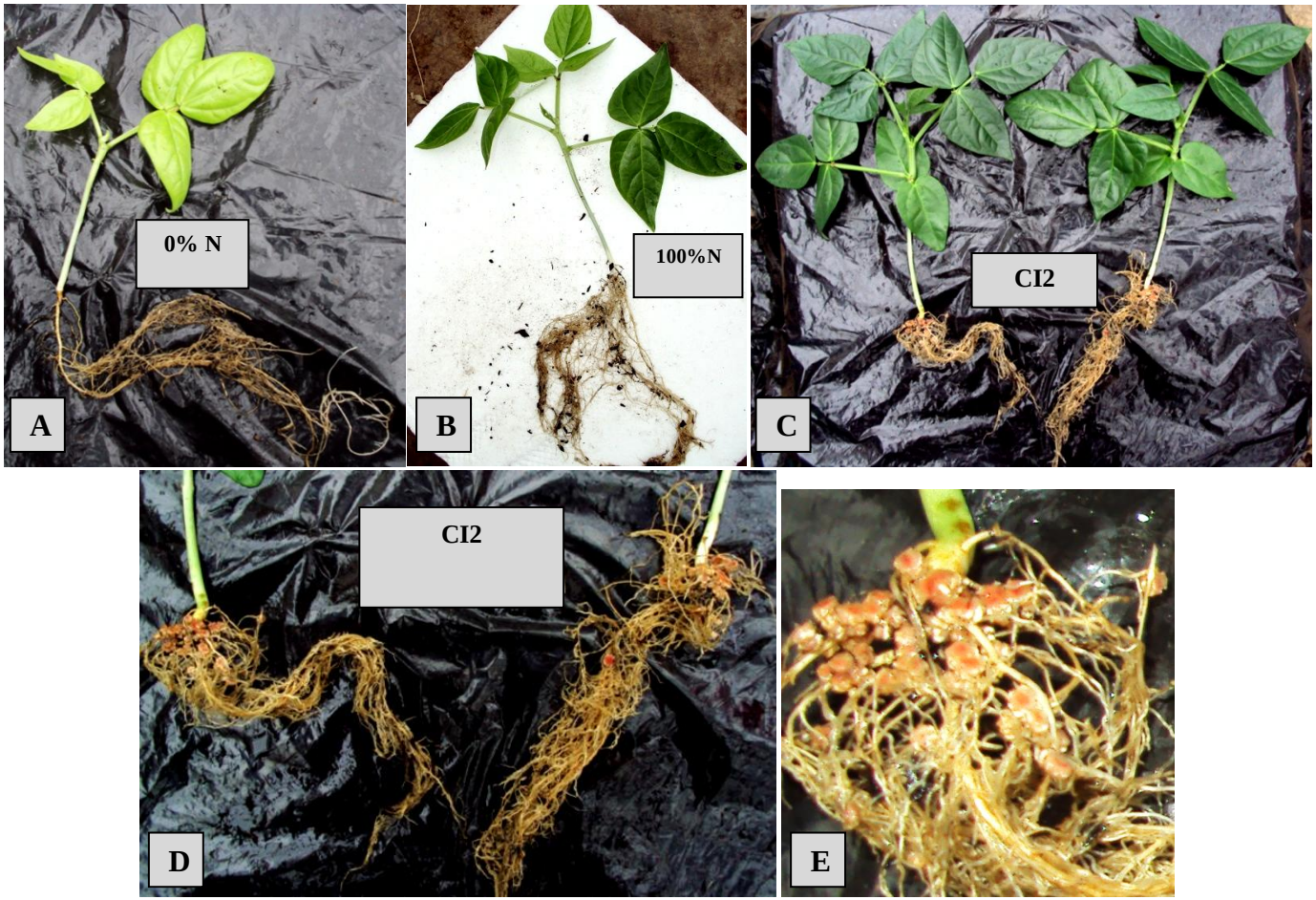

Figure 6.4 A: Examples of cowpea nodulation: 0% Nitrogen Control plant with light green to yellow leaves and no nodules. Figure 6.4B: 100% Nitrogen Control plant with dark green leaves and zero nodule formation. Figure 6.4C: Isolate CI2 plant showing dark green leaves and a number of red leghaemoglobin containing nodules, indicating nitrogen fixation. Figure 6.4D: Isolate CI2 showing a number of nodules containing leghaemoglobin mainly situated around the top third of the root structure and main tap root, with some nodules forming on the lateral roots.
Figure 6.4E: Close up of nodules with leghaemoglobin grouped at the root cap.

Dolichos (*Lablab purpureus*)

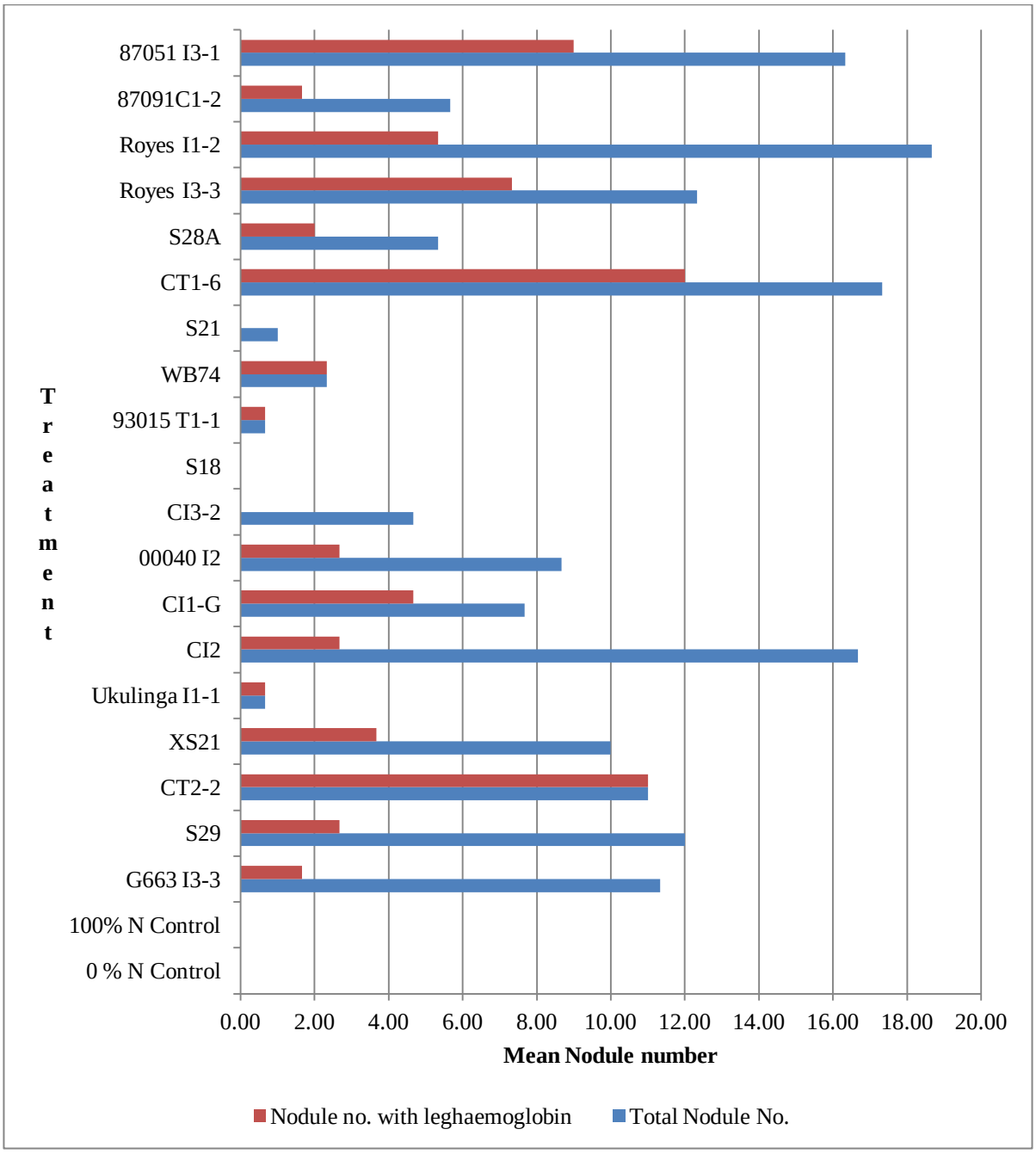

Figure 6.5 Dolichos number of nodules formed and number of nodules containing leghaemoglobin

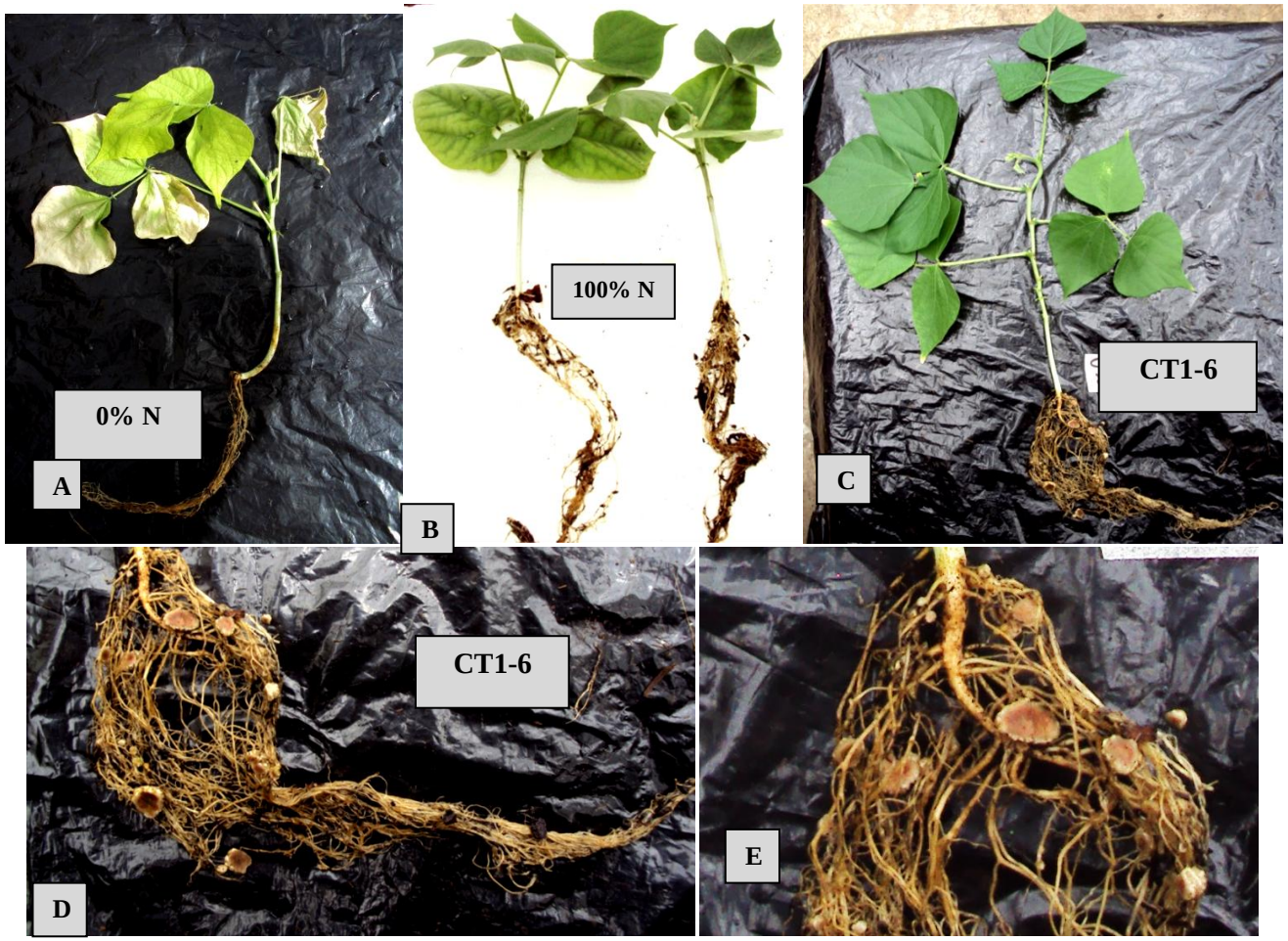

Figure 6.6A: Examples of dolichos nodulation: 0% Nitrogen Control plant showing chlorosis of the leaves, drying of leaves and poor root development. Figure 6.6B: 100% Nitrogen Control plant showing dark green leaves. The unifoliate leaves showed magnesium deficiency but the trifoliate leaves showed no signs of nutrient deficiency. Figure 6.6C: Treatment with Isolate CT1-6 causing dark green leaves and nodules on roots. Figure 6.6D: Enhanced view of the root system of dolichos treated with Isolate CT1-6, showing pale pink nodules containing leghaemoglobin formed on the main tap root and lateral roots.

Dry bean (*Phaseolus vulgaris*)

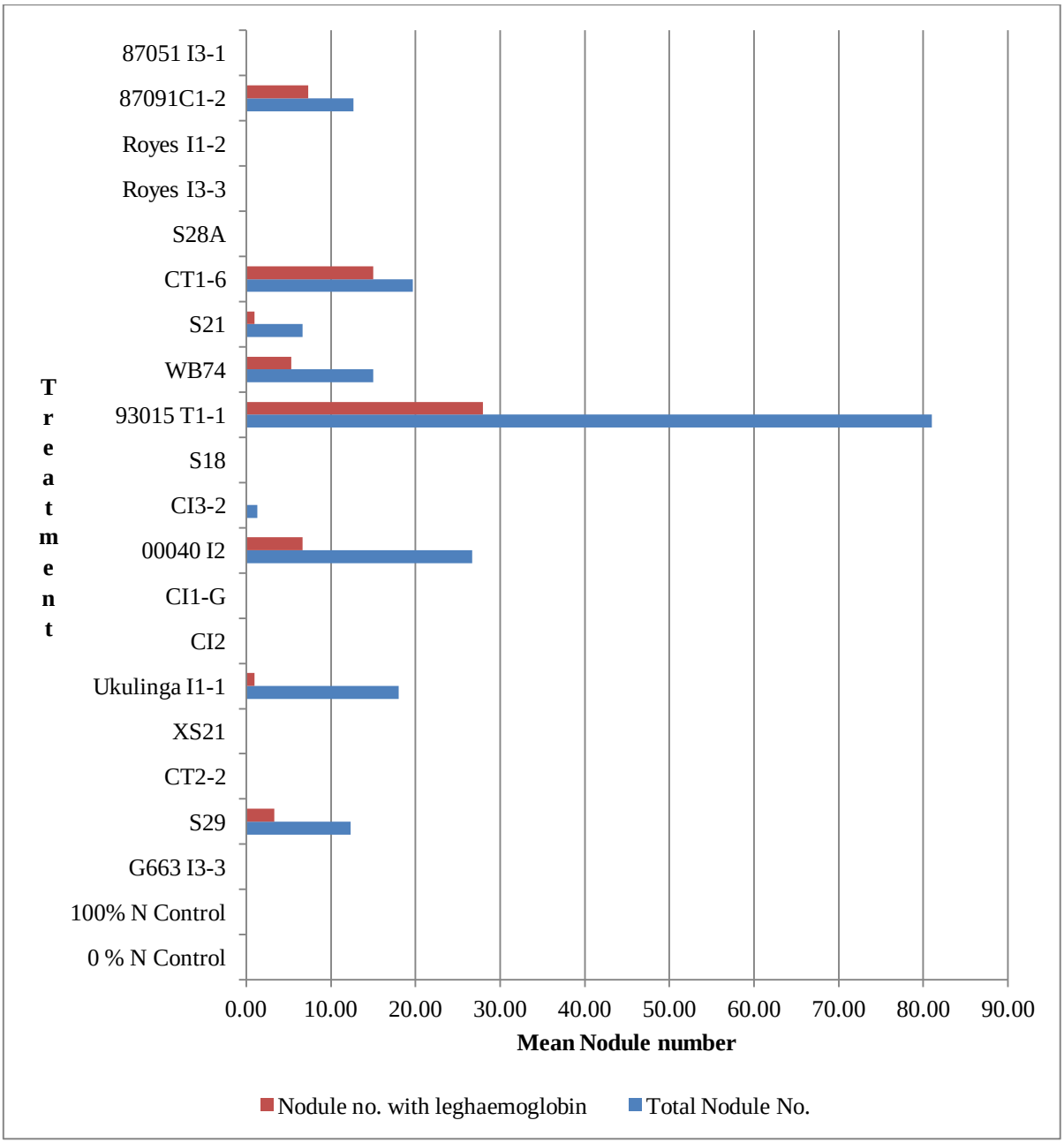

Figure 6.7: Dry bean nodulation: Number of nodules formed and number of nodules containing leghaemoglobin

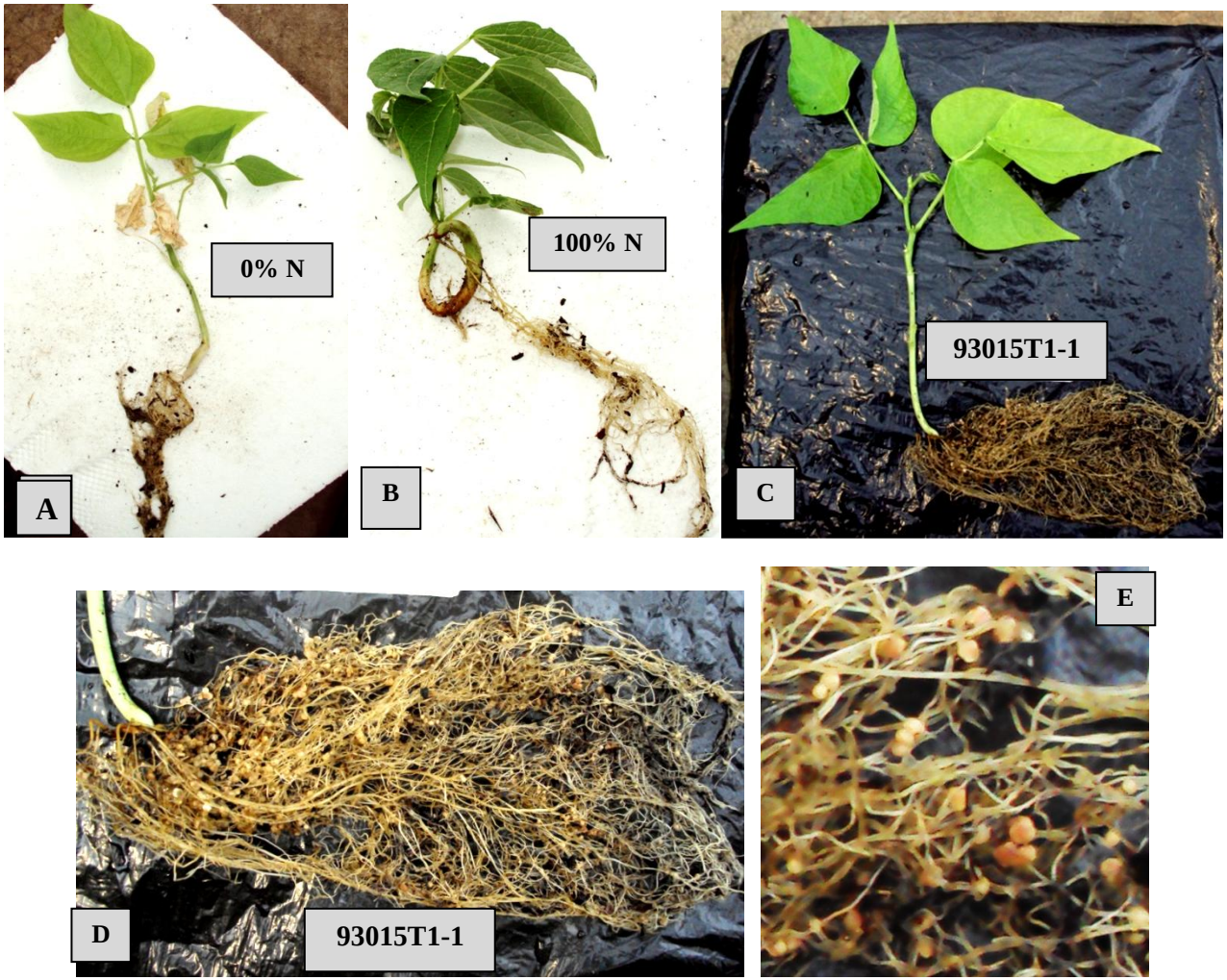

Figure 6.8A: dry bean nodulation: 0% Nitrogen Control showing light green leaves and dried unifoliate leaves and trifoliate leaves. Figure 6.8B 100% Nitrogen Control showing dark green leaves. Figure 6.8C: A plant treated with Isolate 93015T1-1 showing green leaves and nodules formed on root. Figure 6.8D: Enhanced view of roots showing a number of small nodules on the lateral roots ranging from a high concentration of nodules at the top third of the root system through to the bottom of the root system. Figure 6.8E: Nodules are small with pale pink to red centre.

6.4 Discussion

Initially nodules began with a white centre, but with the production of leghaemoglobin, a red centre began to form and spread across the entire inside of the nodule. This was seen in the younger nodules, particularly those found on cowpea, on which the majority of nodulation occurred around the crown of the main tap root and top third of the lateral root system situated closest to the base of the stem. Nodules formed clusters in most treatments varying in size. All were determinate in shape. The 0% and the 100% Nitrogen Control plants did not produce nodules, confirming that the soil was sterilized effectively. The 100% Nitrogen Control plants did not produce any nodules as the abundance of nitrogen available limited the plants ability to produce the sugars required to bind specifically with lectins. Various lectins show specificity with different sugars, allowing specific attachment of the *Rhizobium* spp. to the root of legumes via sugar-lectin interactions (Ridge, et al., 1998).

On soybean, only four isolates caused the production of nodules, and S28A produced the highest number of nodules. However, S29 was the only isolate to produce a leghaemoglobin-containing nodule (Figure 6.1). The soybean plants of the 0% Nitrogen Control developed pale green first trifoliate leaves, and yellow, dry unifoliate leaves (Figure 6.2A). The 100% Nitrogen Control showed the plant had only grown to produce the second trifoliate. Figure 6.2B shows the leaves are dark green. However, the root structure was a main tap root with lateral roots present. Isolate S29 produced only one leghaemoglobin containing nodule and two nodules in total (Figure 6.2 C, D and E). Matsunami, et al. (2007) found that even the supernodulating soybean cultivar Sakukei 4, the moderately nodulating Enrei cultivar and non-nodulating cultivar En1282 decreased their capacity for nodule formation if they suffered from poor drainage or waterlogging and this can be shown in the yield differences between control plants and waterlogged plants. Similar results were also reported by Sugimoto, et al. (2000), who showed that the vegetative growth of soybean was significantly reduced by waterlogging and flooding of the soil.

Ampomah, et al. (2008) found that cowpea was capable of nodulating faster and slow growing *Bradyrhizobium* spp. isolates than groundnut and soybean. Similar results were visible in Figure 6.3, with 18 of the 19 isolates producing leghaemoglobin containing nodules on cowpea. The most effective isolates were CI3-2, CI1-G, CT1-6 and CI2, which were

originally isolated from cowpea nodules. However, isolates that originated from other crops such as groundnut, soybean and a variety of pigeonpea cultivars were also able to nodulate cowpea roots. The two commercial soybean strains XS21 and WB74, produced less than 10 nodules in comparison to the other isolates tested, with even fewer leghaemoglobin containing nodules. Figure 6.4A shows the distinct yellowing from nitrogen deficiency along the leaves and leaf veins and the contrasting dark green appearance of the leaves in Figure 6.4 B. Figure 6.4 C shows the effect of nitrogen fixation by the dark green leaves and Figure 6.4D and 6.4E shows the bright red nodules containing leghaemoglobin.

Dolichos also responded to many of the rhizobial treatments, and formed active root nodules. Figure 6.5 shows that the isolates 87051 I3-1, Royes I1-2, CT1-6 and CI2 were able to form the highest total number of nodules. Approximately half of the nodules contained leghaemoglobin. The best isolates for the production of leghaemoglobin were 87051 I3-1, CT1-6 and CT2-2. In comparison the commercial strains XS21 produced leghaemoglobin in approximately half of the nodules, and WB74 with about a third of the nodules containing leghaemoglobin. Figure 6.6A (0% Nitrogen Control) shows symptoms of nitrogen deficiency with the outer edges of the leaves drying and becoming brittle, and a poor root system. Figure 6.6B shows dark green leaves for the 100% Nitrogen Control. However, the unifoliate leaves did show some magnesium deficiency. This could have been due to the poor drainage experienced with the use of the rhizotrons as none of the other plants showed any magnesium deficiency. Figure 6.6C shows the effect of nitrogen fixation with dark green leaves, visually almost equal to the leaves of the 100% Nitrogen Control. Notably more trifoliate leaves were produced during the same period and the shoots were noticeably longer than those of the 100% Nitrogen Control. Figure 6.6D and 6.6E show that nodule formation occurred around the crown of the main tap root and top third surrounding lateral roots, but that nodules were also found on the lateral roots towards the younger end of the root system. Nodules were determinate in shape and varied in size. They were not bright red but instead a pink to pale pink to light brown in colour.

Figure 6.7 shows only eight of the nodule-producing isolates on dry bean, with Isolate 93015 T1-1 producing the most nodules and nodules containing leghaemoglobin. Isolate CT1-6 produced the third most nodules but had the second most nodules with leghaemoglobin. The 0% Nitrogen Control developed pale green second trifoliate leaves and dried unifoliate and first trifoliate leaves (Figure 6.8A). The newly developed leaves were initially dark green but as the leaves developed in size, chlorosis took place. Figure 6.8B shows dark green leaves.

However, the root structure of the 100% Nitrogen Control was not as pronounced in size in comparison to the 0% Nitrogen Control. Isolate 93015T1-1 caused moderately green leaves, in comparison to the control plants. The root system of the treated plants were more abundant in comparison to the two controls (Figure 6.8D and E), suggesting that this isolate stimulated root development even though it may not have been a strong nitrogen fixer.

Cowpea and dolichos are both capable of nodulating with a variety of rhizobial isolates, forming nodules at the root crown and as the plant grows, nodules that form on the lateral roots and subsequently nodule formation occurs further down the root system. Examples of the cowpea and dolichos plants are in Figures 6.4A-E and 6.6A-E, illustrating that the location of nodule formation could also play a factor in effectively fixing and transporting nitrogen throughout the plant. Soybean and dry bean are also capable of nodule formation. However, external factors such as poor drainage affected the nodulation. Poor root formation by the soybean plants showed that this crop is particularly susceptible to waterlogging.

Nodule senescence coincides with the reproductive pod filling growth stage in the host plants (Lawn and Brun, 1974, Bethlenfalvay and Philips, 1977, Van de Velda, et al., 2006). Nodule senescence can be induced early due to various stresses (Gogorcena, et al., 1997, González, et al., 1998, Matamoros, et al., 1999, Van de Velda, et al., 2006). This results in the change in colour of nodules from nitrogen fixing red or pink to green, indicating the degradation of the heme group (Roponen, 1970). Nodule senescence is rapid and causes a wide variety of proteolytic activities resulting in protein degradation and oxidative, and plant cell death (Pladys and Vance, 1993, Puppo, et al., 2005, Van de Velda, et al., 2006). This explains why many of the nodules formed were not red in colour as the poor drainage affected the nodules, thus inducing early nodule senescence.

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Chapter 7: The effect of potassium silicate on chlorophyll content and dry weight of legumes inoculated with freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp. and, identification of the best performing strains.

Abstract

Silicon is not considered to be an essential nutrient but benefits the plant by increasing the overall strength of the plant. It increases the capacity for nutrient uptake and has been shown to be beneficial to *Rhizobium* and *Bradyrhizobium* spp. This study aimed firstly, investigate the effects of freshly isolated nitrogen fixing *Rhizobium* and *Bradyrhizobium* spp. plus potassium silicate (KSi) on four legumes by measuring the chlorophyll content. Secondly, it aimed to identify the best fourteen isolates using 16S rDNA sequencing. Soybean (*Glycine max* (L.) Merrill), cowpea (*Vigna unguiculata* (L.) Walp.), dry bean (*Phaseolus vulgaris* (L.) var. Gadra) and dolichos (*Lablab purpureus* (L.) Sweet) seeds were treated with 17 freshly isolated isolates of *Rhizobium* and *Bradyrhizobium* spp. and 2 commercial "multi-host" strains, XS21 and WB74 and watered with nitrogen-free fertilizer mixed with KSi. The controls plants were treated with: 0% nitrogen; 0% nitrogen with KSi; 100% nitrogen; and 100% nitrogen with KSi. The total chlorophyll content was calculated using area under the chlorophyll content index progress curve and plant dry weights were measured and ranked using the Kruskal-Wallis analysis. All controls treated with KSi had higher total chlorophyll contents and dry weights than the controls without. Results varied amongst the different crops. Twelve of the rhizobial isolates tested on soybean had a total chlorophyll content higher than the 0% N +KSi. The cowpea plants treated with 10 of the rhizobial isolates and KSi had higher total chlorophyll contents in comparison to the 100% optimum nitrogen with KSi. The dolichos plants did not enter the reproductive growth stage and the total chlorophyll content was based on the four vegetative growth stages. Seven rhizobial isolates tested on dry bean plants had higher total chlorophyll content that the 0% N +KSi. Dry weights varied amongst the different crops and amongst the rhizobial treatments. Fourteen rhizobial isolates selected based on total chlorophyll content and dry weight for identification using 16S rDNA

sequencing. Nine of the fourteen isolates identified as belonging to the *Bradyrhizobium* genus. The remaining five were identified as *Achromobacter xylosoxidans* Strain zc1, *Pseudomonas moraviensis* Strain IARI-HHS1-33 and *Pseudomonas* sp. LC182, *Pseudomonas chlororaphis* Strain UFB2 and *Bacillus acidiceler* Strain TSSAS2-2.

Keywords: *vegetative growth stages; reproductive growth stages*; *potassium silicate; total chlorophyll content; chlorophyll meter; soybean; cowpea; dolichos; dry bean; Rhizobium spp; Bradyrhizobium spp;. Pseudomonas; Achromobacter; Bacillus*

7.1 Introduction

Nitrogen is required for protein and enzyme synthesis in all organisms (Pellett, 1990). The invention of the Haber-Bosch synthesis of ammonia industrialized the fixation of nitrogen and today at least 40% of world agriculture is now dependent on this process (Smil, 1999). In contrast, legumes, working in symbiosis with *Rhizobium* spp., benefit the environment with their ability to fix atmospheric nitrogen, introducing fresh nitrogen into the cropping system. Biological nitrogen fixation (BNF) therefore reduces the need for nitrogen fertilizers in cropping systems (Power, 1987).

Silicon is not considered to be an essential, as a nutrient for plant growth, but its application has proven beneficiary to many plants (Mali and Aery, 2008). Benefits from the application of silicon fertilizers have been noted in silicon accumulating and non-silicon accumulating plants (Epstein, 1999). Silicon is absorbed from soil solutions and concentrations may vary from 1- 40 $mg.L^{-1}$ (Hallmark, et al., 1982). Plants take up silicon readily in the form of monosilicic acid/ orthosilicic acid (H_4SiO_4). Monosilicic acid can replace the anions of phosphate (HPO_4^{2}) and form bonds with calcium, magnesium, aluminium and iron, and thus making more phosphates available for plants (Daroub and Snyder, 2007). Silicon maybe deposited in the form of silica gel to strengthen the cell walls of epidermal cells of leaves, stems, and hulls by forming a silica double layer (Yoshida, 1965, Raven, 2003). Application of silicon positively affects the bacteroids and symbiosomes in nodules of nitrogen fixing cowpeas (Nelwamondo, et al., 2001). There are three types of plants: those that actively take up silicon from a water solution; those that passively take up silicon with water; and those that

reject silicon uptake and only take up water. This phenomena is completely crop dependent (Mittani and Ma, 2005).

The use of 16S rDNA has become the standard of bacterial phylogeny and taxonomy (Garrity and Holt, 2001, Menna, et al., 2006). This technology has ensured the increase in the number of rhizobial species from eight identified species before 1980, to 53 identified rhizobial species in 2006 (Frank, 1889, Cohn, 1942, Jordan, 1982, Chen, et al., 1988, Dreyfus, et al., 1988, Jarvis, et al., 1997, de Lajudie, et al., 1998, Willems, 2006). One of the problems with 16S rDNA for identification of *Bradyrhizobium* spp. is that there is a high degree of similarity between the gene sequences (Willems, 2006). The *Bradyrhizobium elkanii* 16S rDNA only has a 4% divergence in comparison to the other species in the genus *Bradyrhizobium* (Willems, et al., 2001, Willems, 2006).

The purpose of this study was firstly, to determine the total chlorophyll content, and final dry weights of four legume crops inoculated with freshly isolated strains of *Rhizobium* spp. and *Bradyrhizobium* spp. supplemented with silicon (KSi) as a drench; and secondly, to identify the 14 freshly isolated *Rhizobium* spp. and *Bradyrhizobium* spp. isolates that performed best, using 16SrRNA sequencing, PCR and BLAST.

7.2 Materials and Methods

Table 7.1 the varieties of legumes used for experimentation. All crops used were of early maturing cultivars.

Table 7.1: Variety of seeds, scientific names and supplier of seed used in experimentation

Common Name	Scientific Name	Supplier
Soybean	*Glycine max* (L.) Merrill	Link Seed, Greytown, South Africa
Cowpea	*Vigna unguiculata* (L.) Walp.	Agricol, Cato Ridge, KwaZulu-Natal
Dolichos	*Lablab purpureus* (L.) Sweet	Agricol, Cato Ridge, KwaZulu-Natal
Dry bean	*Phaseolus vulgaris* var. Gadra (L.)	Proseeds, Pietermaritzburg, South Africa

Nineteen freshly isolated strains of *Rhizobium* spp. and *Bradyrhizobium* spp., and two commercial strains, XS21 and WB74, were grown on yeast mannitol agar (YMA): mannitol

(Saarchem, South Africa) 10 $g.L^{-1}$; K_2HPO_4 (Saarchem, South Africa) 0.66 $g.L^{-1}$; $MgSO_4.7H_2O$ 0.2 $g.L^{-1}$ (Saarchem, South Africa); yeast extract 0.4 $g.L^{-1}$; NaCl (Saarchem, South Africa) 0.1 $g.L^{-1}$; $FeCl_3.6H_2O$ 0.6 $mg.L^{-1}$; bacteriological agar 15 $g.L^{-1}$; and 10 ml Congo red (Sigma-Aldrich, South Africa), and incubated at 28 °C (MRC Orbital Shaker Incubator; Israel) for 1 week. All the seeds were sterilized using a 5% NaOCl solution (Unilever, South Africa) solution for one minute and rinsed thoroughly in sterile distilled water, before using a 70% ethanol solution and thoroughly rinsing in sterile distilled water before drying on sterile filter paper in individual sterile Petri dishes. Each rhizobial isolate was scraped off inoculated YMA plates, mixed with a 2% gum guar sticker, and mixed with sterilized seed of each crop and left to dry. Four controls were, used in this trial: a 0% Nitrogen Control (0%N); 0% Nitrogen Control with potassium silicate (0%N + KSi); 100% (optimum) Nitrogen Control (100%N); and 100% (optimum) nitrogen with potassium silicate (100%N + KSi) Control. Four seeds per pot were planted into steam autoclaved soil (30 minutes heating time), with three pots per replicate for each treatment using a completely randomized design. The soil pH was 6.09. Plants treated with a nitrogen-free fertilizer solution (K_2SO_4 0.42 $g.L^{-1}$; $MgSO_4$ 0.11 $g.L^{-1}$; $CaSO_4$ 0.05 $g.L^{-1}$; Nutrifix® 0.05/25 L; phosphorus 0.14 $g.L^{-1}$). Limestone ammonium nitrate (LAN) (Blackwood's, South Africa) (0.44 $g.L^{-1}$) was added to the nitrogen-free fertilizer solution for the 100% optimum nitrogen and 100% optimum nitrogen with potassium silicate Controls. Potassium silicate (Agsil, South Africa) 1.07 ml/25 L was added to the nitrogen-free fertilizer solution to treat all the rhizobial treatments and, the 0%N and 100% optimum nitrogen with potassium silicate Controls.

Glasshouse temperatures were maintained at approximately 28 °C for a period of 90 days until the Reproductive Stage 7 was completed. The final chlorophyll content was measured using a chlorophyll meter (Opti-Sciences CCM 200 Plus, USA). Chlorophyll content was measured over four vegetative stages and reproductive stages, namely: first true leaves (V1); first trifoliate stage (V2); second trifoliate stage (V3) and third trifoliate stage (V4); first open flower (R1); initial pod development stage (R3); mature pod and fully viable seed stage (R6) and the initial senescent stage (R7). The plants harvested when pods and seed were mature. The plant material was oven dried, overnight at 40 °C and thereafter weighed. Dry weight of total biomass, was measured for each plant for all four crops and results were analysed using one-way ANOVA (Genstat 14). The area under the chlorophyll content index progress curve (AUCCIPC) was to determine the total chlorophyll content and this result was analysed using

one-way ANOVA (Genstat 14). Kruskal-Wallis analysis of rankings the area under the chlorophyll content index progress curve (AUCCIPC) and dry weights (Genstat 14). All plants were, treated with pesticides to control powdery mildew (*Erysiphe polygoni* D.C.); mealy bug (*Pseudococcus filamentosus* Guen.) and red spider mite (*Tetranychus urticae* Koch.).

One constraint encountered was that a complete factorial of treatments was not possible because of a limited amount of available glasshouse space. The rhizobial isolates that were to be inoculated without the aid of KSi were left out of the trial. A second problem was that the dolichos plants did not enter the reproductive growth phase.

Identification of the freshly isolated rhizobial isolates was performed on fourteen of the nineteen-rhizobial isolates that performed best in the nodulation and potassium silicate trials. The fourteen rhizobial isolates namely: 93015 T1-1; S29; CI3-2; CI2; CI1-G; Ukulinga I1-1; Royes I1-2; Royes I3-3; S21; G663 I3-1; CT1-6; 87091C1-2; 87051I3-1 and 00040 I2, were grown on YMA plates and sent to Inqaba Biotech (South Africa) for DNA extraction, PCR and sequencing using 16S rDNA and identified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

7.3 Results

Increases in leaf size, plant height and stem thickness were observed in cowpea, dolichos, dry bean and some soybean plants treated with KSi. Due to the height of the plants, many of the plants fell over, even with supporting structures in place, resulting in loss of emerging flowers and pods. Fourteen of the best performing isolates based on nodule number formed (results not shown), and total chlorophyll content and dry weight were identified (Table 7.6). Due to the lack of glasshouse space, a full factorial trial was not possible. The significance of the results are based on the H-value obtained using the Kruskal-Wallis analysis in comparison to the Chi square value obtained from the Chi square distribution table. The rankings listed in the tables below are based on the three replicates and due to the heterogeneity of the trial, each replicate was ranked and their mean ranking was used for the analysis in the tables below (Tables 7.2-7.5).

Soybean (*Glycine max*)

Table 7.2 ANOVA and Kruskal-Wallis analysis of treatments based on the area under the chlorophyll content index progress curve and dry weight of soybean (*Glycine max* L. Merrill)

Treatment	Mean Total Chlorophyll (AUCCIPC)	Mean AUCCIPC Rank	Mean Dry Weight (g)	Mean Dry Weight Rank
0% N Control+ KSi	313.95	34	0.2167	21.67
0% N Control	231.90	13.33	0.17	19.5
100% N Control + KSi	534.49	63.67	2.3544	68
100% N Control	407.36	38.67	0.3744	43
0004I2 + KSi	285.25	26.33	0.1778	21.33
87051I3-1 + KSi	267.56	23.67	0.1667	17
87091C1-2 + KSi	378.72	35	0.2911	40.83
93015T1-1 + KSi	327.29	39	0.2844	49
CI1-G + KSi	366.92	44.33	0.2611	34.83
CI2 + KSi	258.53	22.67	0.3933	53.67
CI3-2 + KSi	341.90	37.67	0.2744	44.83
CT2-2 + KSi	391.04	49.67	0.1822	21.67
CTI-6 + KSi	236.29	21	0.1589	19
G663 I3-1 + KSi	196.63	9.33	0.1622	16.33
Royes I1-2 + KSi	190.97	9	0.1778	22.17
Royes I3-3 + KSi	423.86	54	0.6378	55.5
S18 + KSi	342.63	39.67	0.2422	33.5
S21 + KSi	346.02	40	0.4433	44.5
S28A + KSi	358.74	39.67	0.37	43.33
S29 + KSi	263.91	23	0.1344	18.25
Ukulinga I1-1 + KSi	355.02	43.67	0.2356	35
WB74 + KSi	388.69	43.33	0.3267	39.67
XS21 + KSi	432.72	54.33	0.2533	41.67
Sum of ranks		**805.01**		**823.75**
H Value		**34.36**		**34.53**
Degrees of freedom		**22**		**22**

ANOVA Table	Mean Total Chlorophyll	Mean Dry Weight
F Value	1.54	14.87
P Value	0.107	<.001
cv%	34.8	55.2
LSD	189.95	0.3257

Chi square at $p<0.05$ is 33.924.

Cowpea (*Vigna unguiculata*)

Table 7.3 ANOVA and Kruskal-Wallis analysis of treatments based on the area under the chlorophyll content index progress curve and dry weight of cowpea (*Vigna unguiculata* L. Walp.)

Treatment	Mean Total Chlorophyll (AUCCIPC)	Mean AUCCIPC Rank	Mean Dry Weight (g)	Mean Dry Weight Rank
0% N Control+ KSi	418.48	6.67	0.217	10.33
0% N Control	337.89	5.33	0.17	4
100% N Control + KSi	1472.19	37.33	2.354	56.33
100% N Control	1162.50	30.67	0.374	55.83
0004I2 + KSi	1651.25	46.33	4.33	43.33
87051I3-1 + KSi	2041.06	62.33	3.567	36.33
87091C1-2 + KSi	1769.25	52	4.287	44.33
93015T1-1 + KSi	1163.82	29.33	3.619	38.67
CI1-G + KSi	2007.48	59	2.792	28.33
CI2 + KSi	1943.95	56.33	5.384	51.67
CI3-2 + KSi	1909.55	56	3.686	36
CT2-2 + KSi	983.55	23.67	3.532	34.33
CTI-6 + KSi	921.6	21.67	3.506	34.83
G663 I3-1 + KSi	1871.38	56	4.026	29
Royes I1-2 + KSi	1082.88	27.67	4.588	47
Royes I3-3 + KSi	1748.98	52.33	4.718	53.33
S18 + KSi	1466.54	39.67	1.796	17
S21 + KSi	973.25	21.67	3.706	38.67
S28A + KSi	489.65	9.33	0.841	9
S29 + KSi	332.03	5	0.562	18
Ukulinga I1-1 + KSi	1168.78	30.33	4.091	40.67
WB74 + KSi	1805.71	50.67	4.636	38.67
XS21 + KSi	1045.87	25.67	4.21	44
Sum of ranks		**805**		**813.65**
H Value		**56.57**		**38.19**
Degrees of freedom		**22**		**22**

ANOVA Table	Mean Total Chlorophyll	Mean Dry Weight
F Value	10.86	3.95
P Value	<.001	<.001
cv%	22.6	45
LSD	481.45	2.284

Chi square at $p<0.05$ is 33.924

Dolichos (*Lablab purpureus*)

Table 7.4 ANOVA and Kruskal-Wallis analysis of treatments based on the area under the chlorophyll content index progress curve and dry weight of dolichos (*Lablab purpureus* L. Sweet)

Treatment	Mean Total Chlorophyll (AUCCIPC)	Mean AUCCIPC Rank	Mean Dry Weight (g)	Mean Dry Weight Rank
0% N Control+ KSi	173.65	22.67	0.615	9.33
0% N Control	120.80	4.33	0.329	7.67
100% N Control + KSi	360.69	67	12.018	56.67
100% N Control	280.29	35.33	11.408	55.67
0004I2 + KSi	195.28	30.33	3.878	30.67
87051I3-1 + KSi	326.41	65.67	7.83	54.67
87091C1-2 + KSi	242.95	51.33	8.046	56.67
93015T1-1 + KSi	174.19	23	5.342	37
CI1-G + KSi	214.03	40.33	5.169	37.67
CI2 + KSi	210.54	38	10.184	55.33
CI3-2 + KSi	178.56	26	6.497	44.83
CT2-2 + KSi	152.95	12.67	2.72	11
CTI-6 + KSi	176.33	23	2.689	22
G663 I3-1 + KSi	248.79	54.33	7.384	51
Royes I1-2 + KSi	218.96	43.33	6.083	44.83
Royes I3-3 + KSi	226.41	44.33	6.336	45.67
S18 + KSi	240.37	50	3.662	28.67
S21 + KSi	204.80	33.67	5.089	38.33
S28A + KSi	165.75	19.67	0.476	8.33
S29 + KSi	207.50	35.33	0.668	8.5
Ukulinga I1-1 + KSi	206.51	36	5.177	16
WB74 + KSi	151.59	14.67	3.597	38.33
XS21 + KSi	201.23	34	4.048	31.67
Sum of ranks		**804.99**		**818.51**
H Value		**42.18**		**47.20**
Degrees of freedom		**22**		**22**

ANOVA Table	Mean Total Chlorophyll	Mean Dry Weight
F Value	4.55	2.58
P Value	<.001	0.003
cv%	21.1	67.7
LSD	73.42	5.767

Chi square at $p<0.05$ is 33.924

Dry bean (*Phaseolus vulgaris*)

Table 7.5 ANOVA and Kruskal-Wallis analysis of treatments based on the area under the chlorophyll content index progress curve (AUCCIPC) and, dry weight of dry bean var. Gadra (*Phaseolus vulgaris* L.)

Treatment	Mean Total Chlorophyll (AUCCIPC)	Mean AUCCIPC Rank	Mean Dry Weight (g)	Mean Dry Weight Rank
0% N Control+ KSi	291.58	34.33	0.575	24.17
0% N Control	245.13	26.67	0.571	18
100% N Control + KSi	972.80	67	6.529	56.33
100% N Control	910.35	66	15.081	67.33
0004I2 + KSi	225.96	23	0.688	20.33
87051I3-1 + KSi	163.75	20	0.839	29.83
87091C1-2 + KSi	355.04	39.5	0.982	32.33
93015T1-1 + KSi	502.2	48.67	6.962	61.33
CI1-G + KSi	320.26	36.33	0.659	32
CI2 + KSi	354.53	42.83	2.118	38.33
CI3-2 + KSi	210.65	25.67	0.793	25.33
CT2-2 + KSi	172.11	22.33	0.506	20.67
CTI-6 + KSi	465.57	48.67	3.024	51.17
G663 I3-1 + KSi	305.43	30	1.572	38.67
Royes I1-2 + KSi	189.37	23.33	0.319	20.67
Royes I3-3 + KSi	427.26	46.67	4.699	49
S18 + KSi	196.92	25.33	0.07	9.33
S21 + KSi	258.42	31	1.782	39.67
S28A + KSi	135.55	16.67	0.173	14.83
S29 + KSi	258.35	29	2.208	52
Ukulinga I1-1 + KSi	225.87	26	1.309	36.33
WB74 + KSi	272.23	30.67	1.087	37.33
XS21 + KSi	377.97	45.33	1.944	45.33
Sum of ranks		**805**		**826.13**
H Value		**30.69**		**41.13**
Degrees of freedom		**22**		**22**

ANOVA Table	Mean Total Chlorophyll	Mean Dry Weight
F Value	3.60	6.41
P Value	<.001	<.001
cv%	57.0	95.3
LSD	319.03	3.69

Chi square at $p<0.05$ is 33.924

Table 7.6. Probable identification of plant growth promoting rhizobia based on partial sequencing of 16S rDNA genome

Isolate ID	16S RNA closest relative	Accession No.	Similarity
00040I2	Uncultured *Bradyrhizobium* sp. Clone G16-9-D12	FJ193476.1	99%
93015T1-1	*Achromobacter xylosoxidans* strain zc1	KR136349.1	97%
CI2	*Bradyrhizobium pachyrhizi* strain B2RC3	JQ689186.1	99%
CI1-G	*Bradyrhizobium elkanii* strain CCBAU 53142	EF394150.1	98%
CI3-2	*Bradyrhizobium elkanii* strain CCBAU 53142	EF394150.1	97%
CT1-6	*Bradyrhizobium paxllaeri* strain LMTR 13	NR133707.1	98%
G663I3-1	*Bradyrhizobium japonicum* strain N2-225	KF995119.1	98%
Royes I1-2	*Pseudomonas moraviensis* strain IARI-HHS1-33	KF054775.1	99%
Royes I3-3	*Pseudomonas* sp. LC182	KJ534492.1	99%
S21	*Bradyrhizobium japonicum* strain SCAUs36	KP219176.1	98%
87091 C1-2	*Bradyrhizobium japonicum* strain CCBAU 33175	FJ418696.1	98%
87051 I3-1	*Bradyrhizobium japonicum* strain NA 6090	AB070565.1	97%
S29	*Bacillus acidiceler* strain TSS AS2-2	GQ284498.1	99%
Ukulinga I1-1	*Pseudomonas chlororaphis* strain UFB2	CP011020.1	99%

7.4 Discussion

Masclaux, et al. (2001) divided nitrogen fluxes in two categories. The first was the vegetative stage allowed for roots and leaves to act as sink organs allowing the assimilation of minerals to form amino acids and synthesize proteins. The second category is the reproductive stages, which remobilized the accumulated nutrients to other parts of the plant to allow for the production of flowers, pods and eventually seeds. Nitrogen affects the leaf area, maintenance, photosynthetic activity and dry matter partitioning to the reproductive parts of the plant (Muchow, 1988, Guitman, et al., 1991, Vouillot and Devienne-Barret, 1999, Prystupa, et al., 2004, Arduini, et al., 2006). The nutrient status of the plant and environment plays a vital role in the plants ability to defend itself against insect infestations (Meyer and Keeping, 2005, Keeping, et al., 2014, Miles, et al., 2014). The 100% Nitrogen Control had a high dry weights than the plants treated with the rhizobia and silicon. The silicon in the form of KSi can

increase the plants defence against insects (Meyer and Keeping, 2005, Keeping, et al., 2014) . The 100% Nitrogen + KSi Control produced the highest dry weights compared to the other treatments in spite of the infestations of red spider mite, powdery mildew and mealy bug, which affected majority of the crops.

Soybean (*Glycine max*)

Table 7.2 showed that there were significant differences between the treatments for the total chlorophyll content of soybean. The plants treated with 0% N + KSi and 100% optimum N + KSi Controls both produced higher total chlorophyll contents than the 0% N and 100% N Controls without potassium silicate. Soybean plants treated with rhizobial isolates XS21 + KSi and Royes I3-3 + KSi had higher total chlorophyll contents than the 100% N without KSi Control. The plants treated with twelve of the nineteen-rhizobial isolates had higher total chlorophyll contents than the 0% + KSi. The plants treated with seventeen of the nineteen isolates had higher total chlorophyll contents than the 0% N Control.

There were significant differences between the treatments for total dry weight of soybean. The plants treated with 100% N + KSi Control had a higher dry weight than the other treatments. The plants treated with CI2 +KSi and Royes I3-3 + KSi had higher dry weights than the control plants treated with 100% N. Plants treated with 0% + KSi had a higher dry weight than the 0% N Control. The plants treated with twelve of the nineteen-rhizobial isolates had higher dry weights than the 0% N + KSi Control. Fifteen of the nineteen-rhizobial isolates tested on soybean showed higher dry weights than the 0% N Control (Table 7.2).

Cowpea (*Vigna unguiculata*)

There were significant differences between treatments for total chlorophyll content on cowpea (Table 7.3). The total chlorophyll content for the plants treated with 0% N+ KSi Control was higher than the 0% N Control. Likewise, the plants treated with 100 % N + KSi Control had higher chlorophyll content than the 100% N Control. The cowpea plants inoculated with eighteen of the nineteen-rhizobial isolates had higher total chlorophyll contents than the 0% N and 0% N +KSi Controls. In comparison, the plants inoculated with twelve of the nineteen-rhizobial isolates had higher chlorophyll contents than the 100% N Control, with nine rhizobial isolates producing higher chlorophyll contents than the 100% N + KSi Control.

According to Nelwamondo and Dakora (1999), silicon fertilization causes increased nodule numbers and the amounts of nitrogen fixed by *Bradyrhizobium* spp. infected cowpea.

In Table 7.3 plants treated with the 0% N +KSi Control had higher dry weights than the plants treated with 0% N Control only. There were significant differences in the dry weights of cowpea between the different treatments, particularly between the 100% N and 100% N +KSi Controls. All the plants inoculated with the rhizobial treatments had higher dry weights than the 0% N, 0% N +KSi and 100% N Controls (Table 7.3). Table 7.3 also shows that the plants treated with sixteen of the nineteen-rhizobial isolates had higher dry weights than the 100% N + KSi. Dakora and Nelwamondo (2003) showed with *Bradyrhizobium* spp. inoculated cowpea in addition with metasilicic acid, there were significant increases in the weight of the roots but not for shoots.

Dolichos (*Lablab purpureus*)

There were significant differences in the total chlorophyll content between the treatments (Table 7.4). The plants treated with 0% N + KSi Control had higher total chlorophyll content than the plants treated with the 0% N Control. Likewise, the 100% + KSi Control had higher total chlorophyll content than the 100% N Control. The plants treated with rhizobial isolates all had higher total chlorophyll contents than the 0% N Control. The plants treated with sixteen of the nineteen-rhizobial isolates had higher total chlorophyll contents than the 0% N + KSi. Only the plants treated with 87051 I3-1 + KSi had higher total chlorophyll content than the 100% N Control. Nitrogen fluxes were reported by Gastal and Lemaire (2002) in the form of fluctuations in nitrogen uptake and distribution in crops were evident in fluctuating chlorophyll content of plants as a results of each of the isolates during the crop's vegetative stages. The chlorophyll content measured only during the first four vegetative growth stages, possibly due to vernalization, which lowered the expected total chlorophyll content.

There were significant differences in the total dry weight of dolichos between the various treatments (Table 7.4). The plants treated with the 0% N + KSi Control had a higher dry weight than the 0% N Control. Likewise, with the 100% N +KSi Control had a higher dry weight than the 100% N Control. All of the plants treated with nineteen-rhizobial isolates had higher dry weights than the 0% N Control. The plants treated with eighteen of the nineteen-rhizobial isolates had higher dry weights than the 0% N + KSi Control. However, none of the dolichos plants treated with rhizobial isolates produced dry weights that were higher than the 100% N and 100% +KSi Controls.

Dry bean (*Phaseolus vulgaris*)

Dry bean is generally thought to be a poor nitrogen fixing legume (Akter, et al., 2014). Table 7.5 showed significant differences in the total chlorophyll content amongst the treatments. The plants treated with the 0% N + KSi Control had higher dry weights than the 0% N Control. Likewise, the plants treated with the 100% N +KSi Control produced higher dry weights than the 100% N Control. Plants treated with eleven of the nineteen-rhizobial isolates had higher total chlorophyll contents compared to the 0% N Control. Plants treated with eight of the nineteen-rhizobial isolates had higher total chlorophyll contents than the 0% N +KSi Control. None of the rhizobial isolates tested was, capable of producing total chlorophyll contents close to the 100% N and 100% N +KSi Controls.

There were significant differences between the treatments for the total dry weights of dry bean (Table 7.5). The plants treated with the 0% N +KSi had slightly higher dry weight than the 0% N Control. The plants treated with the 100% N Control had a higher total dry weight than the 100% N +KSi Control. This is due to the weight of the plants treated with 100% N +KSi and grown in 12cm diameter pots falling over during the reproductive stages, which resulted in flowers and pods that were forming to prematurely fall off. Plants treated with thirteen of the nineteen-rhizobial isolates had higher dry weights than both the 0% N and 0% N +KSi Controls.

A full factorial trial would have allowed comparative analysis of the KSi treated plants against the plants without KSi treatment and the possible synergistic effect of KSi with rhizobial isolates in terms of changes in chlorophyll content and dry biomass. Silicon in conjunction with potassium had shown increases in nitrogen fixation capacity in chickpeas (*Cicer arietinum*) under water stressed conditions (Kurdali, et al., 2013). Nelwamondo and Dakora (1999) showed a synergistic effect between *Bradyrhizobium* spp. and silicon, which promoted significant increases in plant dry biomass. The flower stem thickness of hydroponically grown Gerbera plants increased with the addition of a silicon treatment (Savvas, et al., 2002). It also enhances chlorophyll content levels of rice and melon and, reduces transpiration levels in *Cucumis melo* L. (Lu and Cao, 2001, Isa, et al., 2010). Ma (2004) found that silicon plays numerous roles in helping plants to resist abiotic and biotic stresses. Whilst Mattson and Leatherwood (2010) showed that weekly drenches with silicon increased not only the leaf silicon content of several varieties of flowers, but also the height, fresh weight, diameter, dry weight, flower diameter and leaf thickness. All the plants showed increases in chlorophyll content with the silicon treatments and some of the rhizobial isolates

with KSi had high dry weights namely: 93015 T1-1; S29; CI3-2; CI2; CI1-G; Ukulinga I1-1; Royes I1-2; Royes I3-3; S21; G663 I3-1; CT1-6; 87091C1-2; 87051I3-1 and 00040 I2.

The *Bradyrhizobium* genus is considered to be diverse due to its large host range (Sprent, 2001). Nine of the fourteen freshly isolated isolates identified using 16S rDNA sequencing as belonging to the genus *Bradyrhizobium*. It is thought that *Bradyrhizobium japonicum* is the main rhizobial species to infect soybean roots (Mabood and Smith, 2005). The isolate S21 was isolated from soybean nodules. However, G663-I3-1 was isolated from groundnut and Isolates 87091C1-2 and 87051I3-1 were isolated from pigeonpea. Dhar, et al. (2013) found *Bradyrhizobium* spp. strains from pigeon pea (*Cajanus cajan* L.), which aligned to *Bradyrhizobium japonicum* using 16S rDNA sequencing. Likewise Yang, et al. (2005) found *Bradyrhizobium* spp. isolated from peanut (*Arachis hypogaea* L.) is phylogenically related to *Bradyrhizobium japonicum*. The *Bradyrhizobium pachyrhizi* strain is considered to infect tuberous root- producing legumes such as yam (Rodriguez-Navarro, et al., 2004, Ramirez-Bahena, et al., 2009, Delamuta, et al., 2015). The rhizobial isolate CI2 from cowpea nodules showed a high degree of similarity to *Bradyrhizobium pachyrhizus* strain B2RC3 based on 16S rDNA sequencing. Both the Isolates CI1-G and CI3-2 identified with a high degree of similarity to *Bradyrhizobium elkanii* Strain CCBAU 53142. Zhang, et al. (2008) showed that cowpea is capable of forming synergistic relationships with a number of *Bradyrhizobium* spp. including *Bradyrhizobium elkanii*. Isolate 00040I2 identified with 99% similarity to an uncultured *Bradyrhizobium* sp. Clone G16-9-D12.

The endophytic bacteria *Achromobacter xylosoxidans*, as described by , is able to produce indole-3-acetic acid, solubilize phosphate and fix nitrogen in wheat varieties. A study by Benata, et al. (2008) shows that *A. xylosoxidans* is capable of forming root nodules on *Prosopis juliflora* (Schwartz) D. C. However, studies carried out by Wedhastri, et al. (2013) and Guimarães, et al. (2012) show that *A. xylosoxidans* can infect legume roots and fix nitrogen of soybean and cowpea respectively. The isolate 93015T1-1 was initially isolated from pigeon pea (*Cajanus cajan* L.) and identified with 99% degree similarity related to *Achromobacter xylosoxidans* Strain zc1.

Some strains of the rhizosphere bacterial genus *Pseudomonas* enhance plant growth by making nutrients available to the plant but also protects the plant by secreting antibiotics and hormones, and by producing siderophores (Glick, 1995, Botelho and Mendonça-Hagler, 2006, Vyas, et al., 2009). The Isolate Ukulinga I1-1 identified with a 99% similarity to

Pseudomonas chlororaphis Strain UFB2. *Pseudomonas chlororaphis* is a non-pathogenic soil bacteria, with the capacity to produce rhamnolipids (Gunther IV, et al., 2005). Rhamnolipids are used as natural emulsifiers and biosurfactants and have a high capacity for antimicrobial and antifungal activity (Guerra-Santos, et al., 1986, Desai and Banat, 1997, Tjeerd van Rij, et al., 2005). The Isolate Royes I3-3 was identified as *Pseudomonas* sp. LC182. The isolate Royes I1-2 was initially isolated from pigeonpea and identified with a 99% similarity to *Pseudomonas moraviensis* IARI-HHS1-33 strain. It was first identified from soil in the Czech Republic (Tvrzová, et al., 2006). A study by Sura-de Jong, et al. (2015) showed that some *P. moraviensis* strains are capable of phosphate solubilization, indole-3-acetic acid production and are moderate selenate hyperaccumulators and nitrite reducers.

The Isolate S29 was initially isolated from soybean root nodules and tested Gram negative (results not shown). Its identification, based on 16S rDNA, showed a 99% similarity to *Bacillus acidiceler* Strain TSSAS2-2. The *Bacillus acidiceler* is a Gram positive to Gram variable strain that can grow well under acidic conditions (Peak, et al., 2007). According to Burdon (1946) and Vincent, et al. (1962), rhizobia have the capacity to be sudanophilic, which results in Gram variability.

Five of the fourteen isolates were not of the genera *Bradyrhizobium* or *Rhizobium*. However, each of the five bacterial strains has the capacity for plant growth promotion but this does not necessarily include the infection and formation of root nodules in legumes except in the case of *Achromobacter xylosoxidans*.

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An Overview of the Major Research Findings and their Implications

Introduction

The cost of nitrogenous fertilizers has driven research for more sustainable solutions such as biological nitrogen fixation. The search for new rhizobial strains with not only the potential to form nodules but also to effectively fix atmospheric nitrogen in a range of legumes is of importance, firstly, because it will reduce the use of nitrogenous fertilizers; secondly, it will reduce the use of many biological nitrogen-fixing products to a single product; and thirdly, rhizobial strains applied as seed treatments reduce labour and related costs throughout the growing season. The purpose of the present study was to isolate freshly isolated *Bradyrhizobium* spp. and *Rhizobium* spp. from nodules of various legumes and to test them for multi-host plant growth promotion. The achieve this, the objectives of this study were focused on: 1) the isolation of freshly isolated rhizobial isolates from nodules of soybean (*Glycine max* L.); cowpea (*Vigna unguiculata* L.); groundnut (*Arachis hypogaea* L.) and pigeon pea (*Cajanus cajan* L.); 2) The development of a visual growth scale based on the vegetative and reproductive growth stages for soybean; cowpea; dolichos (*Lablab purpureus* L.) and dry bean (*Phaseolus vulgaris* L.); 3) The measurement of total chlorophyll and total leaf nitrogen content based on the uptake of various levels of nitrogen during specific vegetative and reproductive growth stages; 4) The *in vitro* testing of freshly isolated rhizobia for plant growth promotion properties such as phosphate solubilization and indole-3-acetic acid production, and biological control by producing of siderophores; 5) The multi-host testing of freshly isolated rhizobia *in vivo* using soybean, cowpea, dolichos and dry bean as test crops; 6) The nodulation and presence of leghaemoglobin of test crops inoculated with the 20 best rhizobial isolates; 7) The presence of a synergistic effect of rhizobia with the use of silicon in the form of potassium silicate (KSi) on total chlorophyll content and dry biomass; and the identification of the 14 best performing rhizobial isolates using 16S rDNA sequencing. This overview is a summary of the major findings and their implications.

Keywords: Rhizobia; nodulation; KSi; nitrogen fixation; plant growth stages; chlorophyll content; leaf nitrogen content

Major findings and their implications

- Thirty-one rhizobial isolates in total were isolated from soybean, cowpea, pigeonpea and groundnut nodules. These isolates were subjected to Gram staining, and the colony morphology of each culture grown on YMA was noted. Two commercial strains selected for exclusive use on soybean, WB74 and XS21, were used as commercial controls for subsequent trials. The implication of this is that nodulation of multiple crops allows for a wider variety of rhizobia to be isolated with the potential for multi-host plant growth promoting properties.
- The visual scales of the plants growth stages were based on the first four vegetative growth stages and the initial flowering, podding, mature pod and senescent reproductive growth stages.
- Increases in nitrogen fertilizer content linearly increased the total chlorophyll content and total leaf nitrogen content of each of the test crops. The use of a visual growth scale in conjunction with a nitrogen uptake trial helps evaluate: 1) how much of nitrogen taken up by the plant at the various growth stages based on increases in chlorophyll content and leaf nitrogen content; 2) at which growth stages did nitrogen fluxes occur; 3) to track the total chlorophyll content and leaf nitrogen content over the growth cycle of the four crops; 4) the differences between the vegetative and reproductive growth stages and the effect of the uptake of nutrients on the reproductive cycle and yield.
- Twelve of the 31 strains of rhizobia tested, including the commercial strains, were positive for IAA production; 15 strains tested positive for phosphate solubilization; 18 strains tested positive for siderophore production. Seven strains tested positive for all three *in vitro* tests whilst only two isolates, Ukulinga I1-1 and 93015 T1-1, were strongly positive for all three tests. Positive results for production of siderophores and indole-3-acetic acid, and phosphate solubilization, in addition to nitrogen-fixing capability, allows for greater uptake of nutrients and enhanced plant growth.

- Thirty-one freshly isolated rhizobium strains and two commercial strains were ranked for nitrogen fixation on multiple crops based on the total chlorophyll and dry biomass. Chlorophyll content profiles were based on the chlorophyll content at specific growth stages to determine nitrogen fluxes. Many of the rhizobial isolates began nitrogen fixing during the V2 and V3 growth stages. Peak readings were during the V4 and R1 growth stages, and then declined almost exponentially during the R3, R6 and R7 growth stages. Chlorophyll content and dry biomass differed among the test crops. Testing for multi-host infection by the rhizobial strains using multiple crops impact nitrogen fixation in the following ways:
    - The testing of rhizobial strains on crops that have a specific host range and those with a wide host range. This allows for a more detailed analysis of the host range of rhizobia;
    - The effect of each strain on the different crops had varying results and therefore, strains need to be assessed based on their performance across a range of crops and also individually;
    - The determination of the growth stage in which infection and nitrogen fixation take place, based on either chlorophyll content or leaf nitrogen content;
    - To determine the effect of infection at the V2 and V3 growth stages;
    - To determine the effect on the overall chlorophyll content and yield in comparison to optimum fertilizer applications and with no fertilizer.
- Based on the different test crops, cowpea and dolichos plants were the most nodulated crops followed by dry bean and soybean. Many of the rhizobial isolates that infected cowpea plants and effectively formed nitrogen fixing nodules were also leghaemoglobin-containing nodules. In dolichos plants infected with rhizobia almost half of the nodules contained leghaemoglobin. Nine rhizobial isolates tested on dry bean plants produced nodules, of which eight contained leghaemoglobin. Determining how long it takes for nitrogen fixing nodules to form is crucial in the impact on nitrogen uptake, plant growth and yield. Nodulation is vital for nitrogen fixation and therefore understanding nodule formation and the factors which impact nodulation are important:
    - Nodulation testing should be done in pots rather than the current rhizotron because of drainage issues and the need for space for plant growth, and root and nodule formation;
    - More research is needed in the area of the time required for infection of legume roots, formation of nodules and production of leghaemoglobin in nodules;

- The number of nodules and location of nodules play a vital role in the affectivity of nitrogen fixation, which requires more research;
- The factors, that affect nodulation such as leghaemoglobin degradation, nodule age and water logging, affect nitrogen fixation need to be quantified;
- The physical characteristics of nodules, such as size, influence the nitrogen fixation of legumes.

- The synergistic effect of rhizobia with KSi showed not only in total chlorophyll content but also in dry weights, particularly in the case of treated cowpea plants. Soybean, dolichos and dry bean plants treated with rhizobia and KSi had higher total chlorophyll contents than the 0% N+KSi and 0% N Controls. Of the fourteen rhizobial isolates selected for identification, nine were identified as *Bradyrhizobium* spp. The remaining five isolates identified as *Achromobacter xylosoxidans*, *Bacillus acidiceler* and three strains of the genus *Pseudomonas*. The effect of silicon fertilization, combined with rhizobia could enhance biological nitrogen fixation. Further research is proposed:
  - A repeat of a full factorial trial is required for comparable statistics;
  - Silicon aids in the uptake of nutrients and therefore is beneficial when used in conjunction with rhizobia for increased uptake of nitrogen in comparison to ammonia and nitrate fertilizers;
  - Silicon provides a level of insect protection and plant turgidity. However, this is dependent on the nutrient status of the plant;
  - More research needs to be done in the area of when rhizobia infect nodules and how the delay in infection at the V2 or V3 growth stages affects overall plant growth;
  - Evaluate the impact on the effect of silicon in nitrogen fixation and yield.
- It is important to identify rhizobial strains that work individually or in combination with other strains for increased effectiveness in nitrogen fixation and yield.

Appendices from Chapter 5

5.1 Soybean (*Glycine max* (L.) Merrill)

Appendix 5.1: Chlorophyll readings at four vegetative and reproductive stages of soybean (*Glycine max* (L.) Merrill) inoculated with 31 freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp., two commercial strains XS21 and WB74, and two fertilizer controls, 0% Nitrogen Control and 100% Nitrogen Control.

Treatment	Significant Difference	VC	V1	V2	V3	R 1	R 3	R 6	R 7
0 Control	a	1.7	1.4	0.7	0.3	0.0	0.0	0	0
100 Control	abc	2.3	1.1	1.5	2.3	2.8	4.1	1.3	0.5
00040I3-3	abc	3.0	3.2	1.9	2.0	2.5	1.9	1.3	0.6
00040 I2	bc	11.6	8.6	4.6	4.4	8.6	6.2	4.6	1.4
87051 I2-1	bc	12.9	8.5	5.8	8.9	9.1	5.2	2.2	1.3
87051 I3-1	abc	6.3	5.7	3.1	1.1	2.1	1.3	0.8	0.5
87091 T1-2	abc	4.2	2.6	2	1.2	1.8	1.4	0.6	0.6
87091C1-2	abc	6.5	5	3.3	3.4	3.7	2.9	1.7	0.7
93015 T1-1	c	11.6	12.4	8.2	8.5	6.8	4.1	3.4	1.6
CCI3	abc	13.9	7.4	5.6	4.3	4.5	3.3	1.8	1.1
CI1-G	abc	2.2	2.8	1.7	1.1	2.6	2.2	1.6	0.3
CI2	abc	8.0	6.8	4	6.0	7.7	4.7	3	0.9
CI3-2	ab	2.7	1.6	1.4	2.0	1.8	1.2	0.9	0.4
CT1-6	abc	11.9	9.1	2.7	2.5	1.6	1.1	0.8	0.5
CT2-2	abc	4.0	3.8	2.1	4.9	3.7	2.3	1.4	0.6
G663 I1-1	ab	3.4	4.3	1.3	0.7	1.4	0.7	0.6	0.2
G663 I3-1	a	0.0	0	0	0.0	0.0	0.0	0	0
Royes I1-2	abc	4.5	4.4	2.6	2.9	6.1	2.9	2.2	1.1
Royes I3-3	abc	3.3	3.6	0.8	0.5	3.2	2.8	1.1	0.4
S16	ab	1.0	2	2	1.4	2.9	1.9	1.1	0.5
S18	abc	2.3	2.1	1.1	1.0	3.6	2.6	1.3	0.5
S20 B	abc	1.2	2.7	1.9	1.2	2.6	2.1	1.2	0.7
S21	bc	14.7	10	5.3	4.4	9.2	6.7	3.7	0.9
S26	abc	6.8	6.7	4.3	7.5	7.2	5.4	3.1	0.8
S20A	a	0.0	0	0	0.0	0.0	0.0	0	0
S28A	abc	5.3	2.1	2	1.6	3.2	2.5	1.5	1.4
S28B	a	0.0	0	0	0.0	0.0	0.0	0	0
S29	abc	8.1	5.8	2.8	4.1	9.8	6.3	3.2	1.2
S30	abc	2.8	1.9	2.8	4.7	2.2	2.1	1	0.4
S33A	abc	5.7	4.2	5.7	6.4	7.6	5.0	3.7	1
S33B	abc	3.5	2.7	1.6	0.8	2.8	2.5	1.5	0.3
SCI	abc	2.3	2.6	3.8	4.5	3.5	2.8	2.1	0.8
Ukulinga I1-1	bc	9.1	6.3	5.3	8.0	8.1	7.5	4.1	1.4
WB74	abc	4.7	4.1	2.9	2.4	5.5	4.0	2.1	0.9
XS21	abc	4.9	4.8	1.7	1.1	2.4	2.1	1.4	0.7

	F Value	P Value	cv %	LSD/DMRT
Treatment	<.001	4.5	125	2.7
Stages	<.001	10.2		1.3
Treatment*Stages	1	0.3		7.6

5.2 Cowpea (*Vigna unguiculata* (L.)Walp.)

Appendix 5.2: Chlorophyll readings at four vegetative and reproductive stages of cowpea (*Vigna unguiculata* (L.) Walp.) inoculated with 31 freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp., two commercial strains XS21 and WB74, and two controls 0% Nitrogen Control and 100% Nitrogen Control.

Treatment	Significant Difference	VC	V1	V2	V3	R 1	R 3	R 6	R 7
0 Control	ab	10.3	6.3	3.6	0	0	0	0	0
100 Control	ghi	25.5	24.3	15.9	19.2	19.2	23.3	6.4	2.1
00040I3-3	ab	14.2	6.6	5.5	0	0	0	0	0
00040 I2	i	8.5	5.7	21.8	39.5	45.6	31.5	4.8	1.6
87051 I2-1	ab	11	7.6	1.6	0	0	0	0	0
87051 I3-1	efghi	7.4	5.5	14.7	24.2	31.9	20.7	6.9	1.5
87091 T1-2	a	9.5	4.6	1.2	0	0	0	0	0
87091C1-2	hi	12.9	8.3	39.5	27.3	32.2	25.2	6.4	2.2
93015 T1-1	abcd	15.6	4.7	11.8	6.8	8.3	6.7	1.2	0.6
CCI3	ghi	10.4	6.9	37.8	25.3	26.2	19.8	7.7	1.5
CI1-G	efgh	9.1	9	19.9	23.3	23.9	15.1	7.1	1.9
CI2	efgh	7	6.5	39.4	35.5	8.7	7.3	5.1	1.7
CI3-2	hi	8.6	6.1	37	38.3	31.7	18.1	7.3	1.5
CT1-6	def	13.3	4.6	17.9	22	14.2	11.4	4	1.2
CT2-2	bcde	8.9	5.1	7	19	19.1	5.5	2.1	0.8
G663 I1-1	a	7.1	2.7	2.8	5.8	0	0	0	0
G663 I3-1	fghi	11.4	6.7	25	26.2	24.7	16.5	7.9	1.3
Royes I1-2	hi	11.5	5.8	28.5	27.8	41.5	29.2	7.2	1.5
Royes I3-3	def	6	4.3	12.5	26.1	16.3	15.6	3.3	2.1
S16	ab	14.8	4.1	1	0.9	0	0	0	0
S18	cdef	19.8	7.5	6.3	6.3	20.8	16.5	4.4	0.8
S20 B	ab	7.9	4.8	4.3	2	2.5	0.7	0	0
S21	abcd	9.1	5	8.9	4.8	13.1	10.2	4.1	0.8
S26	a	6.9	3.9	0	0	0	0	0	0
S20A	ab	7.1	4.7	4.4	4	4.1	3.5	2.3	0.4
S28A	abc	8.3	8.2	5.6	6.7	4.6	3.2	1.2	0.2
S28B	defg	11.1	5.9	16.4	19.1	19	11.7	5.7	1.7
S29	defg	18.1	6.9	10.3	18.8	20.2	14.2	2.5	1.2
S30	ab	11.6	5.9	5.9	0	0	0	0	0
S33A	a	9.1	5.5	2	0	0	0	0	0
S33B	abc	9.6	5.3	4.3	4.9	7.5	5	0	0
SCI	abc	6.8	4	7.5	7.7	7.3	4.2	1.9	0.4
Ukulinga I1-1	hi	6.8	5.7	16.5	26.1	51.2	33.4	4.8	2.2
WB74	ab	11.9	5.3	3.9	2.1	0	0	0	0
XS21	efgh	10.4	6.5	17.3	26.2	27.1	19	3.3	1

	F Value	P Value	cv %	LSD/DMRT
Treatment	<.001	12.4	77.6	4.97
Stages	<.001	37.62		2.38
Treatment*Stages	<.001	1.89		14.05

5.3 Dolichos (*Lablab purpureus* (L.) Sweet)

Appendix 5.3: Chlorophyll readings at four vegetative and reproductive stages dolichos (*Lablab purpureus* (L.) Sweet) inoculated with 31 freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp., two commercial strains XS21 and WB74, and two controls 0% Nitrogen Control and 100% Nitrogen Control.

Treatment	Significant Difference	VC	V1	V2	V3	R 1	R 3	R 6	R 7
0 Control	abc	11.7	3.9	3.3	0.6	0	0	0	0
100 Control	m	30	17.2	16.7	20.7	15	8	3.2	1.2
00040I3-3	a	9.5	4	1.9	0	0	0	0	0
00040 I2	abcdefghi	9.4	4.1	3.2	3.5	8.8	3.2	2.6	1
87051 I2-1	ab	9.1	2.8	3.3	1.6	0	0	0	0
87051 I3-1	hijk	14.1	3.3	5.6	15	9.9	5.2	2.7	1.1
87091 T1-2	cdefghij	11.5	3.2	5.8	4.9	11.9	7.2	3	1.1
87091C1-2	jkl	14.5	6.7	8	18.6	14.3	6.5	4	1.3
93015 T1-1	a	10.2	3.3	2.1	0	0	0	0	0
CCI3	abcd	15.3	3.9	3.4	1.8	0	0	0	0
CI1-G	dfghijk	12.4	5.2	4.9	13	9.8	5.9	1.4	0.6
CI2	kl	11	4.6	9.2	10.8	23.9	13.1	6.7	1.2
CI3-2	fghijk	6.6	6.6	7.8	16.6	9	4.2	1.7	0.6
CT1-6	fghijk	9.9	4.6	4.5	10.7	11.3	7.5	3.8	1.2
CT2-2	ghijk	8.2	3.8	4.1	12.1	14.7	8	2.7	1
G663 I1-1	abcdefghi	11.3	3.5	3.9	8.9	7.5	4.5	2.1	0.8
G663 I3-1	abcdefghi	11.7	7.6	5	5.1	7.3	3.7	1.7	0.4
Royes I1-2	lm	13.4	5.9	8.5	17.7	24.5	14.1	5	1.1
Royes I3-3	kl	18.5	5.8	4.8	17.5	17.1	9.6	4.3	1.2
S16	ab	11.5	3.1	2.8	1.2	0	0	0	0
S18	abc	12.9	3.5	5.1	1.4	0	0	0	0
S20 B	abcdef	10.3	5	5.1	2.6	1.2	0.8	0.4	0.3
S21	abcdefghi	11.9	3.1	3.9	10.1	3.1	3	1.7	0.3
S26	abcdefgh	12	3.9	4	4.7	4.5	0.9	0	0
S20A	abcde	18.9	3.4	2	0	0	0	0	0
S28A	bcdefghij	13.7	4.9	4.1	10	6.5	4.8	1.6	0.4
S28B	abcdefgh	21.9	5.5	1.6	0	0	0	0	0
S29	abcdef	17	4.7	2.7	0.6	0	0	0	0
S30	abc	12.4	3.6	3	1.3	0	0	0	0
S33A	ijk	11	7.5	5.2	13	12.1	8.9	3.2	1.2
S33B	abc	15.4	5.3	2.3	0	0	0	0	0
SCI	abcdefg	6.6	4.4	4.6	4.3	3.4	2.5	1.3	0.4
Ukulinga I1-1	ijk	9.6	5.3	5.6	12.4	16.2	7.5	3.4	1
WB74	abc	15.4	2.8	2.9	0	0	0	0	0
XS21	ijk	15.1	6.7	5.4	6.4	14.3	7.3	4.7	1.1

	F Value	P Value	cv %	LSD/DMRT
Treatment	<.001	7.94	79.2	2.96
Stages	<.001	56.91		1.41
Treatment*Stages	0.038	1.25		8.36

5.4 Dry bean (*Phaseolus vulgaris* (L.)

Appendix 5.4: Chlorophyll readings at four vegetative and reproductive stages dry bean (*Phaseolus vulgaris* (L.) inoculated with 31 freshly isolated strains of *Rhizobium* and *Bradyrhizobium* spp., two commercial strains XS21 and WB74, and two controls, 0% Nitrogen Control and 100% Nitrogen Control

Treatment	Significant Difference	VC	V1	V2	V3	R 1	R 3	R 6	R 7
0 Control	cd	11.7	7.5	3.8	2.5	4.9	3.4	2.3	1.7
100 Control	f	14.8	13.4	12.5	10.1	10.8	7.9	4	2.4
00040I3-3	bc	11.4	3	3.7	4.3	4.8	3.5	1.8	1.5
00040 I2	ab	3.9	3.3	3.2	3.1	4.9	2.5	1.5	1.4
87051 I2-1	a	2.6	3	2.8	4	5.6	3.2	0	0
87051 I3-1	abc	4.5	3.9	4.8	4.4	5.4	4	2.1	1.3
87091 T1-2	abc	4.8	4.8	5.5	3.8	5.2	4	2.6	1.6
87091C1-2	abc	4.3	4	3.6	3.9	5.1	4.1	2.7	1.9
93015 T1-1	de	4.6	4.6	4.6	9.3	10	8.6	2.3	1.5
CCI3	ab	3.2	3.8	3.8	3.8	3.2	2.5	1.7	1.3
CI1-G	bc	6.8	8.2	3.2	3.8	4.5	3.4	1.9	1.3
CI2	ab	4.4	3.4	3.4	3.8	4.4	3	1.1	0.8
CI3-2	abc	3.2	3.1	4.1	4.1	6.4	4.7	2.5	2.1
CT1-6	ab	3.4	2.7	2.9	3.5	4.7	3.7	1.9	1.4
CT2-2	abc	9.7	2.5	3.2	2.7	3.6	3.2	2.2	1.9
G663 I1-1	abc	4.3	4	4.1	3.5	4.1	3.5	2.3	1.1
G663 I3-1	cd	10.3	9.9	2.8	4.5	4.3	2.9	1.8	1.4
Royes I1-2	ab	3.9	3.5	2.9	3.5	3.6	3	1.7	1.3
Royes I3-3	abc	6	5.2	4.1	3.8	4.6	2.7	2.1	1.5
S16	abc	3.2	3.4	4.6	5.9	4.2	3.5	1.9	1.3
S18	abc	3	3.3	3.2	6.9	5.3	3.2	1.5	1.1
S20 B	abc	4.9	4.1	5	4.3	4.1	2.7	1.6	1.3
S21	abc	4.3	3.6	4.2	3.3	5.3	3.8	1.4	1.2
S26	ab	3.7	3.4	3.9	3.4	4.8	3.6	1.9	1.5
S20A	abc	4.9	3.5	3.5	3.6	6	4	2.2	1.3
S28A	abc	4.4	3.8	4.2	6.5	4.3	3.4	1.4	1.1
S28B	abc	4.2	4.1	3.5	4.3	5.1	3.6	1.9	1.5
S29	ab	4.1	3.7	4	3.5	3.9	3.1	2.2	1.3
S30	ab	3.9	3.8	4.5	3.6	3.2	2.4	1.9	1.4
S33A	abc	4.2	4.1	4.3	3.7	4	3.3	1.8	1.4
S33B	abc	5	3.8	3.2	5	5.8	4.6	1.9	1.2
SCI	abc	4.2	3.5	3.5	5.8	4.6	4.2	2.9	2
Ukulinga I1-1	abc	4.5	3.5	4.4	4	5.7	3.1	1.8	1.4
WB74	bc	6.6	6.4	4	4.3	5.2	3.7	2	1.4
XS21	e	4.6	5.1	5.1	9.6	10.8	9.8	2.1	1.5

	F Value	P Value	cv %	LSD/DMRT
Treatment	<.001	8.52	42.8	1.16
Stages	<.001	52.75		0.55
Treatment*Stages	0.004	1.39		3.27